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*Original Article*

# Expression of CYP1B1 and B7-H3 significantly correlates with poor prognosis in colorectal cancer patients

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**Abstract:** Cytochrome P450 1B1 (CYP1B1) is a phase I xenobiotic-metabolizing enzyme (XME) that is overexpressed in colorectal cancer (CRC) tissue, but the prognosis value of CYP1B1 in CRC remains elusive. Additionally, B7-H3 has a key role in tumor cell immune evasion by inhibiting functions of T cells. Thus, in this study, we aimed to identify a new marker for predicting the prognosis of CRC and to study the relationship between tumor metabolism and tumor immunity. We analyzed CYP1B1 and B7-H3 expression in 231 patients with CRC using immunohistochemistry, and we investigated the relationship between CYP1B1 and B7-H3 expression using real-time PCR and Western blot analysis in two human CRC cell lines. Kaplan-Meier survival analysis was used to calculate overall survival (OS) rates, and Cox proportional regression model was performed for multivariate analysis. We found that both CYP1B1 and B7-H3 expression were aberrantly increased in CRC tissues compared to normal colorectal tissues. Moreover, high expression of CYP1B1 was significantly correlated with poor OS, and multivariate analysis indicated that CYP1B1 was a valuable independent prognostic biomarker. Furthermore, CYP1B1 expression was significantly correlated with B7-H3 expression in CRC tissues samples and two human CRC cell lines. In conclusion, these results indicate that CYP1B1 may be a novel predictive biomarker for prognosis of CRC patients, and there is a strong correlation between CYP1B1 and B7-H3 *in vivo* and *in vitro*.

**Keywords:** Colorectal cancer, CYP1B1, B7-H3, immunohistochemistry, survival analysis

## Introduction

Colorectal cancer (CRC) is one of the most common forms of cancer, and it is the second leading cause of cancer-related deaths globally [1]. Although there has been progress in the diagnosis and therapeutic strategies of CRC, the 5-year survival rate is poor. Thus, it is imperative to explore new treatment strategies for CRC and identify novel markers to improve the prognosis.

The etiology of CRC has not been fully identified. However, previous studies have indicated a strong association between high levels of heterocyclic aromatic amines and polycyclic aromatic hydrocarbons (PAHs) and an increase in the risk of developing CRC [2]. Cytochrome P450 1B1 (CYP1B1) can activate carcinogenic compounds by metabolizing PAHs, heterocyclic aromatic amines, and other procarcinogens [3, 4]. CYP1B1 also participates in the metabolism of fatty acids and sex hormones, which are both important to CRC development [5]. CYP1B1 is a phase I xenobiotic-metabolizing enzyme (XME), and it has been reported to participate in lipid metabolism [6]. Overexpression of CYP1B1 protein has been demonstrated in different tumors, including those found in the brain, colon, esophagus, and skin [7]. Overexpression of CYP1B1 in CRC tissues in comparison with normal colorectal tissues supports development of CYP1B1-targeted anticancer therapies [8]. Additionally, increased expression of CYP1B1 also was detected in lymph node metastasis [9]. Moreover, CYP1B1 polymorphisms have been implicated as risk factors of CRC [10], and carcinogenesis mediated by CYP1B1 may rely

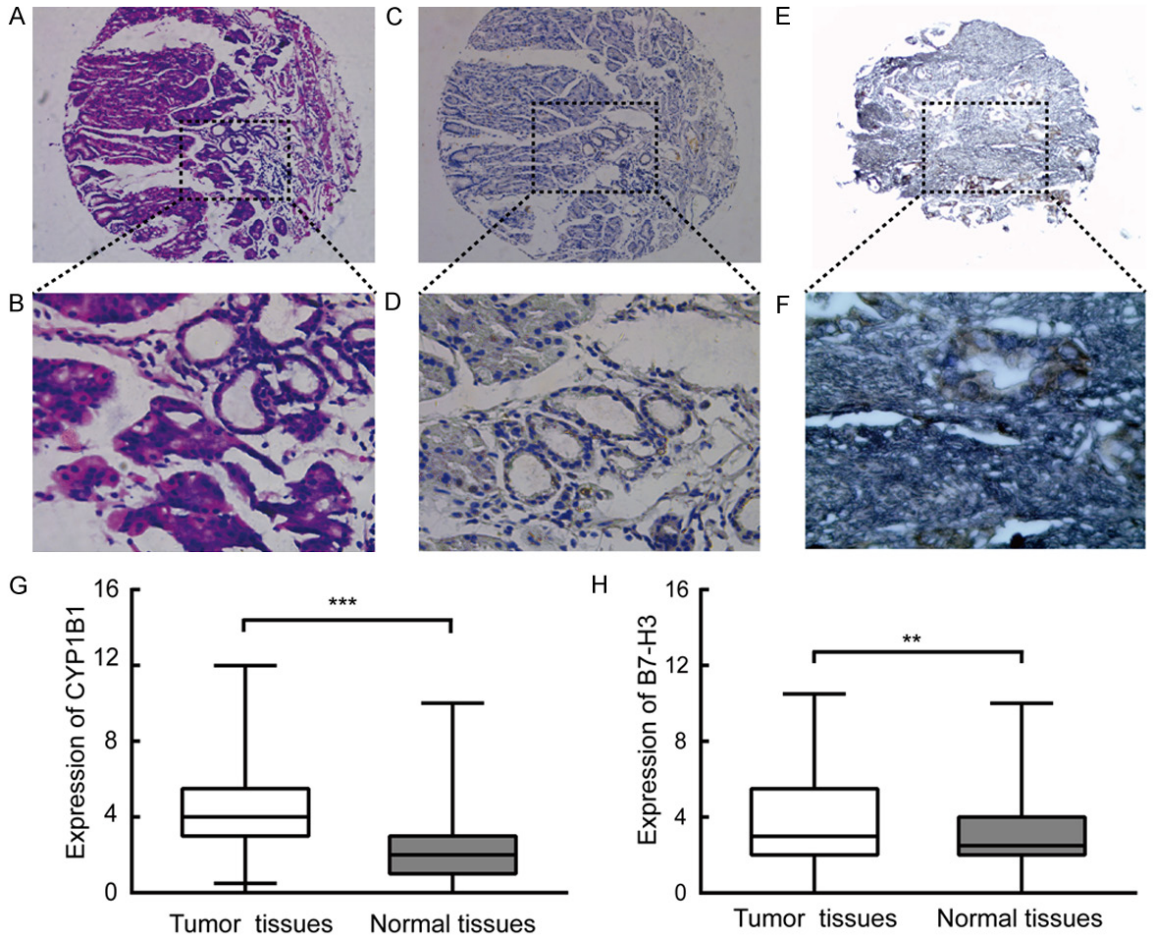


**Figure 1.** Representative immunohistochemical results of CYP1B1 and B7-H3 expression in colorectal cancer (CRC) patients. (A and B) show typical immunohistochemical staining pattern of colorectal adenocarcinoma (hematoxylin-eosin). (C and D) were typical representative of images, which showed that CYP1B1 expression was elevated in the tumor tissue. (E and F) show typical representative of images, which showed that B7-H3 expression was elevated in the tumor tissue. The photomicrographs were at 50 × and 200 × magnification. (G, H) Statistical analysis shows that the expression of both CYP1B1 and B7-H3 is significantly increased in CRC tissues compared with normal colorectal tissues. $^{**}P < 0.01$, $^{***}P < 0.001$.

on CYP1B1 enzymatic activity. According to these findings, CYP1B1 might be a key mediator in CRC development. Thus, there is potential importance in exploring the prognostic value of CYP1B1 in CRC. However, there are currently few reports on the relationship between CYP1B1 expression and the prognosis of CRC [9], and there have been no further studies to confirm the results.

B7-H3 contributes to tumor cell immune evasion [11] and is aberrantly expressed in CRC [12]. Several studies have been conducted to illustrate the roles of B7-H3 in CRC. Previous research from our lab demonstrated that B7-H3 not only gives the promotion of cell invasion and migration, but it also augments anti-apoptosis and the epithelial-mesenchymal transition (EMT) in CRC cells [13-15]. Moreover, recent studies have shown that B7-H3 can regulate lipid metabolism in lung cancer [16] and reprograms glucose metabolism in breast cancer [17]. However, the relationship between tumor metabolism and B7-H3 in CRC patients remains unclear.

Reprogramming of energy metabolism and evading immune destruction are considered new hallmarks of cancer [18]. Thus, investigating the relationship between tumor metabolism and tumor immunity is of potential significance. In the current study, we evaluated the associa-

tion between patient outcomes and the expression of CYP1B1 and B7-H3. In addition, we studied the relationship between the two genes.

## Materials and methods

### *Patients and samples*

This study was conducted from samples of 231 patients with CRC who underwent an initial surgical resection between June 2006 and November 2011 at the Affiliated Hospital of Jiangnan University (Wuxi, China) and with the permission of the Research Ethics Committee. We obtained formalin-fixed paraffin-embedded tissues from 231 CRC specimens from the Department of Pathology at this hospital. All 231 patients received follow up care by telephone or letter until October 2017. Clinicopathological parameters that were collected included gender, age, tumor location, depth of tumor, lymph metastasis, TNM stage, vascular invasion, neural invasion and differentiation.

### *Tissue microarray (TMA) construction, immunohistochemistry, antibodies, and reagents*

Typical cylindrical tissue cores from paraffin-embedded colorectal tumors and adjacent normal colorectal tissue were selected by two proficient colorectal pathologists. Two tumor tissue cores and adjacent normal colorectal tissue cores with diameter of 1.0 mm were selected from each specimen to construct the TMA. The expression level of CYP1B1 and B7-H3 was determined by a standard immunohistochemistry (IHC) approach (**Figure 1**). The TMA sections were deparaffinized in xylene and then rehydrated in descending dilutions of ethanol. The sections underwent antigen retrieval and endogenous peroxidase blocking. Subsequently, sections were incubated overnight at 4°C with a primary antibody of either anti-CYP1B1 antibody (1:200, Abcam, Hong Kong, China) or anti-B7-H3 antibody (1:150, Abcam, Hong Kong, China). Sections were incubated with polymerase and stained with 3,3'-diaminobenzidine (DAB, reagent A, B, and C, GTVisionTM III Kit supply, Shanghai, China). Finally, hematoxylin was used to counter stain the sections. Sections were incubated with PBS only and without primary antibodies as negative controls. CYP1B1 and B7-H3 expression were scored according to two indicators: the percent area of positive stain and the intensity of staining. The density of staining was assigned to one of the five categories: 0 (≤ 5%); 1 (6%-25%); 2 (26%-50%); 3 (51%-75%); and 4 (76%-100%) according to the proportion of positively stained areas relative to the entire block [19]. The intensity of staining was described as: 0 = no staining, 1 = weak staining, 2 = moderate staining, or 3 = strong staining. The two scores were then multiplied to calculate the final score that ranges from 0 to 12. Expression was scored by two pathologists without knowledge of the patients' basic information or the outcomes. If there were discrepancies, a final score was adopted to reassess the staining using a multihead microscope.

### *Cell lines and culture*

SW480 and CaCo-2 cells are human CRC cell lines maintained in our laboratory that have differing expression of B7-H3. CaCo-2 cells were stably transfected with B7-H3 siRNA (CaCo-2-shB7-H3) to reduce expression of B7-H3. SW480-B7-H3-EGFP was constructed from SW480 cells to express high levels of B7-H3. CaCo-2-NC and SW480-NC cells transfected with a mock vector were used as negative controls. All medium (HyClone GE Healthcare Life Sciences, USA) were supplemented with 10% fetal bovine serum (Invitrogen, USA). The cells were incubated in a humidified atmosphere with 5% $CO_2$ at 37°C.

### *RNA isolation and relative real-time PCR*

TRIzol reagent (Invitrogen, USA) was used to isolate total RNA from $1.5 \times 10^6$ cells. Total RNA was then quantified using NanoDrop 2000 (Thermo Scientific, USA). First strand cDNA was synthesized from 1 μg RNA using AMV reverse transcriptase. mRNA expression levels of CYP1B1 and B7-H3 were determined using QuantiNova SYBR Green PCR kit (QIAGEN, German). β-actin was used as an internal control. The polymerase chain reaction (PCR) reactions were performed using a Prism 7300 real-time PCR instrument (Applied Biosystems Inc, USA). The primers for CYP1B1 were F: TTGACTCTGGAGTGGGAGTG, R: TCGGTGAGTGGCGTCAATTC. The primers for B7-H3 were F: AGCACTGTGGTTCTGCCTCACA, R: CACCAGCTGTTTGGTATCTGTCAG. The primers for β-actin were F: AGCGAGCATCCCCCAAAGTT, R: GGGCACGAAGGCTCATCATT. Specific expression of double-stranded DNA was evaluated by the compara-

**Table 1.** Correlation between clinicopathological parameters and expression of CYP1B1, B7-H3 in 231 cases of CRC patients

| Parameters | Total (N = 231) | CYP1B1 expression | | *P* | B7H3 expression | | *P* |
|---|---|---|---|---|---|---|---|
| | | High | Low | | High | Low | |
| Gender | | | | 0.026 | | | 0.858 |
| Male | 130 | 60 | 70 | | 77 | 53 | |
| Female | 101 | 32 | 69 | | 61 | 40 | |
| Age | | | | 0.414 | | | 0.221 |
| < 60 | 88 | 38 | 50 | | 57 | 31 | |
| ≥ 60 | 143 | 54 | 89 | | 81 | 62 | |
| Tumor location | | | | 0.448 | | | 0.920 |
| Colon | 91 | 39 | 52 | | 54 | 37 | |
| Rectum | 140 | 53 | 87 | | 84 | 56 | |
| Vascular invasion | | | | 0.643 | | | 0.488 |
| Negative | 194 | 76 | 118 | | 114 | 80 | |
| Positive | 37 | 16 | 21 | | 24 | 13 | |
| Neural invasion | | | | 0.251 | | | 0.247 |
| Negative | 196 | 75 | 121 | | 114 | 82 | |
| Positive | 35 | 17 | 18 | | 24 | 11 | |
| Depth of tumor | | | | 0.275 | | | 0.371 |
| T1/T2 | 67 | 23 | 44 | | 37 | 30 | |
| T3/T4 | 164 | 69 | 95 | | 101 | 63 | |
| Lymph metastasis | | | | 0.916 | | | 0.034 |
| N0 | 112 | 45 | 67 | | 59 | 53 | |
| N1/N2 | 119 | 47 | 72 | | 79 | 40 | |
| Differentiation | | | | 0.896 | | | 0.428 |
| Well | 79 | 31 | 48 | | 50 | 29 | |
| Poor/Moderate | 152 | 61 | 91 | | 88 | 64 | |
| TNM stage | | | | 0.706 | | | 0.405 |
| I-II | 114 | 44 | 70 | | 65 | 49 | |
| III-IV | 117 | 48 | 69 | | 73 | 44 | |

*P-value* < *0.05* was considered statistically significant.

tive Ct method using $2^{-\Delta\Delta Ct}$. Experiments were performed in triplicate.

*Western blot analysis*

Total cell protein content was measured using a BCA protein assay kit (Cwbio, China). Proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Germany). After blocking with 5% non-fat milk, the membranes were incubated with CYP1B1 (1:1000, Abcam, Hong Kong, China), B7-H3 (1:1000, Abcam, Hong Kong, China), or β-actin (1:2000, ThermoFisher, USA) at 4°C overnight. The blots were incubated with a secondary antibody for 1 h and were analyzed using an ECL system (Beyotime, China).

*Statistical analysis*

Statistically significant differences between groups were determined by Student's t test. The results are expressed as the mean ± SD. The most appropriate cutoff value for CYP1B1 and B7-H3 score was obtained by generating receiver operating characteristics (ROC) curves. Chi-square analysis and Fisher's exact test were performed to analyze the association between clinicopathological parameters and the expression of CYP1B1 and B7-H3. The Kaplan-Meier method was used to plot OS curve and the log-rank test was used to determine statistical significance. Cox proportional hazard regression method was used to perform multivariate survival analysis. The SPSS ver-

**Table 2.** Correlation analysis of CYP1B1 and B7-H3 expression in 231 cases of CRC patients

| B7H3 expression | CYP1B1 expression | |
|---|---|---|
| | High | Low |
| High | 63 | 75 |
| Low | 29 | 64 |
| *P*-value | 0.028 | |

*P-value < 0.05* was considered statistically significant.

sion 20.0 software (IBM, USA) was used to carry out statistical calculations. P < 0.05 was considered statistically significant.

## Results

*Clinicopathological parameters of patients*

Clinicopathological parameters are presented in **Table 1**. Our study included 130 males (56.3%) and 101 females (43.7%). The median age of the patients was 61.3 and ranged from 27 to 87 years old. There were 79 (34.2%) cases with well-differentiated tumors and 152 (65.8%) cases with poor or moderate tumor differentiation. There were 114 (49.4%) cases of stage I-II disease and 117 (50.6%) cases of stage III-IV disease. We classified the disease stages according to the 2010 The Union for International Cancer Control (UICC) CRC TNM staging system.

*Expression of CYP1B1 and B7-H3 in colorectal tissue*

The typical immunohistochemical staining pattern of colorectal cancer can be seen in **Figure 1A** and **1B**. Positive expression of CYP1B1 was found in the majority of CRC tissues and was predominantly localized to the cytoplasm of tumor cells (**Figure 1C** and **1D**). Positive expression of B7-H3 was found in the majority of CRC tissues and was primarily localized to the cytoplasm and membrane of tumor cells (**Figure 1E** and **1F**). However, we found weak or no expression of CYP1B1 and B7-H3 in adjacent normal colorectal tissues. Data analysis indicated that the expression of both CYP1B1 and B7-H3 were increased in CRC tissues when compared to normal colorectal tissues (P < 0.05, **Figure 1G** and **1H**).

*Relationship between clinicopathological parameters and the expression of CYP1B1 and B7-H3*

We explored the relationship between clinicopathological parameters and the expression of CYP1B1 and B7-H3 in this study. In order to evaluate CYP1B1 expression, we used an immunohistochemical (ICH) semi-quantitative score. When the IHC score was equal to 4.75, the Youden index of the survival outcome was predicted by CYP1B1 expression. Thus, patients were defined as either high expression of CYP1B1 with IHC scores ≥ 5.0 or low expression of CYP1B1 with IHC scores < 5.0. We found that 92 cases had high expression of CYP1B1 (92/231, 39.8%), and we found a significant difference in IHC scores between male and female patients (P = 0.026, **Table 1**). However, expression of CYP1B1 was not significantly associated with tumor location, vascular invasion, neural invasion, differentiation, or TNM stage.

Using the same method, when the IHC score was equal to 2.75, the Youden index of the survival outcome was predicted by B7-H3 expression levels. Thus, patients were defined as either high expression of B7-H3 with IHC scores ≥ 3.0 or low expression of B7-H3 with IHC scores < 3.0. We found that 138 cases had high expression of B7-H3 (138/231, 59.7%), and we found that B7-H3 expression was significantly associated with lymph metastasis (P = 0.034, **Table 1**). Moreover, we assessed expression of CYP1B1 in tumor samples with high expression of B7-H3 (n = 138). Of these 138 samples, 63 cases also had CYP1B1 high expression. Data analysis indicated that CYP1B1 expression is positively correlated with B7-H3 expression in CRC tissues (P = 0.028, **Table 2**).

*Relationship between CYP1B1 B7-H3 expression and patient overall survival*

Overall survival (OS) was calculated from the date of surgery until the patient's death. The 5-year survival rate was 61.9% (143/231). Patients with high expression of CYP1B1 had significantly poor OS relative to patients with low expression of CYP1B1 (P = 0.009, **Figure 2A**). Additionally, high expression of B7-H3 was

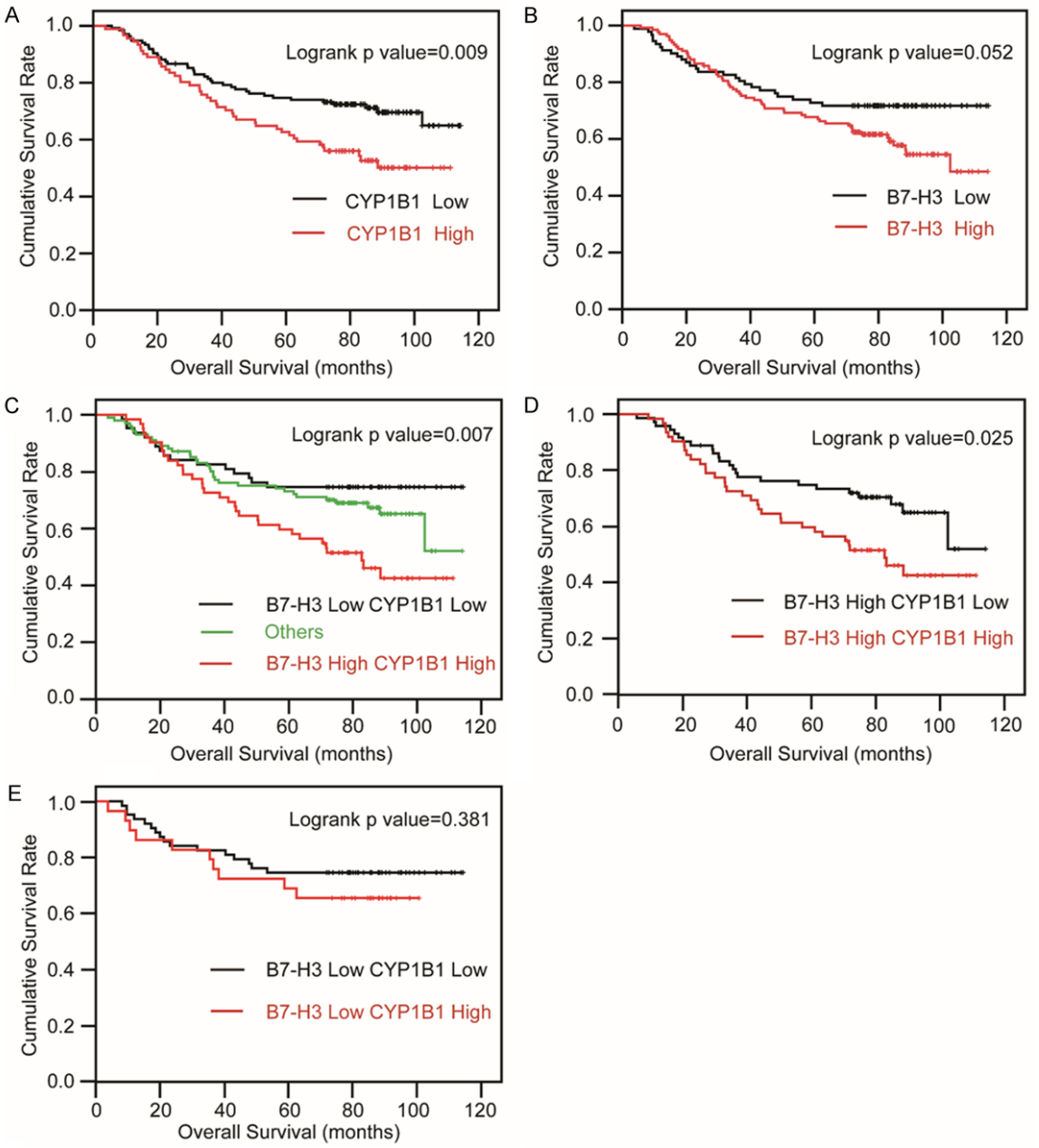


**Figure 2.** Relationship between CYP1B1, B7-H3 expression and patient overall survival (OS). Kaplan-Meier survival analysis was used to calculate OS rates. *P* values were calculated using the log-rank test. A. High expression of CYP1B1 was associated with poor OS of CRC patients. B. High expression of B7-H3 was not associated with poor OS of CRC patients. C. Patients with high expression of both CYP1B1 and B7-H3 had the poorest OS compared to patients with low expression of both CYP1B1 and B7-H3 and other patients, including patients that were either CYP1B1-high and B7-H3-low or CYP1B1-low and B7-H3-high. D. Patients were divided into two groups in subgroup analysis: B7-H3 High and B7-H3 Low. High expression of CYP1B1 was significantly correlated with poor OS in the B7-H3 high expression group. E. High expression of CYP1B1 was not correlated with poor OS in the B7-H3 low expression group. *P-value < 0.05* was considered statistically significant.

not correlated with poor OS (P = 0.052, **Figure 2B**). Moreover, we evaluated the prognostic value of combined CYP1B1 and B7-H3 expression. Patients were divided into three groups: high expression of both CYP1B1 and B7-H3 (n = 63), low expression of both CYP1B1 and B7-H3 (n = 64) and other patients, including patients that were either CYP1B1-high and B7-H3-low or

**Table 3.** Univariate Kaplan-Meier survival analysis in 231 cases of CRC patients

| Parameters | Survival, Median (Range) | Log-Rank Chi square | *P*-value |
|---|---|---|---|
| Depth of tumor | | | 0.003 |
| T1/T2 | 98.5 (90.9-106.1) | 8.865 | |
| T3/T4 | 79.1 (72.7-85.7) | | |
| Lymph metastasis | | | < 0.001 |
| N0 | 96.3 (89.9-102.8) | 18.967 | |
| N1/N2 | 73.5 (65.7-81.3) | | |
| Neural invasion | | | < 0.001 |
| Negative | 88.8 (83.3-94.2) | 13.959 | |
| Positive | 60.2 (46.4-74.1) | | |
| Vascular invasion | | | 0.001 |
| Negative | 88.5 (82.9-94.0) | 11.788 | |
| Positive | 65.2 (51.6-78.7) | | |
| TNM stage | | | < 0.001 |
| I-II | 98.4 (92.3-104.4) | 27.000 | |
| III-IV | 71.0 (63.1-78.9) | | |
| CYP1B1 expression | | | 0.009 |
| Low | 89.8 (83.2-96.4) | 6.761 | |
| High | 75.9 (67.8-84.1) | | |

*P-value < 0.05* was considered statistically significant.

CYP1B1-low and B7-H3-high (n = 104). Statistical analysis revealed that low expression of both CYP1B1 and B7-H3 group had the most favorable OS while high expression of both CYP1B1 and B7-H3 group had the poorest OS (P = 0.007, **Figure 2C**).

We further conducted a subgroup analysis. Patients were divided into two groups: B7-H3 High (n = 138) and B7-H3 Low (n = 93). High expression of CYP1B1 was significantly correlated with poor OS in the B7-H3 high expression group (P = 0.025, **Figure 2D**). However, high expression of CYP1B1 was not correlated with poor OS in the B7-H3 low expression group (P = 0.381, **Figure 2E**).

Furthermore, we used univariate and multivariate analyses to evaluate the factors related to the prognosis of CRC patients. The univariate analysis indicated that neural invasion, vascular invasion, depth of tumor, lymph metastasis, TNM stage, and CYP1B1 expression were significantly associated with long-term mortality risk (P < 0.05, **Table 3**). In addition, multivariate Cox regression analysis shown in **Table 4** indicated that TNM stages III and IV were associated with a relative risk of death of 7.772 (95% CI, 3.796-15.910; P < 0.01) compared with stages I and II. Poor and moderately differentiated tumors were associated with a relative risk of death of 0.496 (95% CI, 0.249-0.988; P = 0.041). In addition, high expression of CYP1B1 was associated with a relative risk of death of 1.084 (95% CI, 0.563-2.085; P = 0.044). Multivariate Cox regression analysis indicated that high CYP1B1 expression might be a biomarker for predicting poor OS.

Combining both CYP1B1 and B7-H3 high expression as a variable, the multivariate Cox regression analysis indicated that TNM stages III and IV were associated with a relative risk of death of 14.665 (95% CI, 6.379-33.715; P < 0.01) compared to stages I and II. High expression of both CYP1B1 and B7-H3 was associated with a relative risk of death of 3.606 (95% CI, 1.690-7.694; P = 0.043).

*Relationship between CYP1B1 and B7-H3 expression in CRC cell lines*

Our results demonstrate a significant positive correlation between CYP1B1 expression and B7-H3 expression in CRC tissues samples. To further study the relationship between CYP1B1 and B7-H3 expression, we measured expression in two human CRC cell lines (SW480 and CaCo-2). Upregulation of B7-H3 expression resulted in the upregulation of CYP1B1 at both the mRNA and protein level (**Figure 3A-C**). Additionally, downregulation of B7-H3 expression resulted in the downregulation of CYP1B1 (**Figure 3D-F**). Overall, these results indicate that expression of CYP1B1 and B7-H3 are significantly correlated in CRC cell lines.

## Discussion

In current study, the prognostic role of CYP1B1 in CRC was investigated. High expression of CYP1B1 was associated with poor OS. Thus, CYP1B1 detection might be an effective measure to predict the prognosis of CRC patients. In addition, CYP1B1 expression was significantly correlated with B7-H3 expression in CRC tissues samples and two human CRC cells.

Previous studies have showed that CYP1B1 may play a significant role in tumor development and progression [20]. Hence, identifica-

**Table 4.** Multivariate Cox proportional hazard model

| Parameters | Multivariate | | | |
|---|---|---|---|---|
| | HR (*95% CI*) | *P*-value | HR (*95% CI*) | *P*-value |
| Gender (Female/Male) | 0.972 (0.511-1.851) | 0.953 | 0.938 (0.489-1.800) | 0.820 |
| Age (≥ 60/< 60) | 1.728 (0.890-3.355) | 0.108 | 1.657 (0.841-3.263) | 0.091 |
| Tumor location (Rectum/Colon) | 0.617 (0.326-1.168) | 0.160 | 0.643 (0.334-1.237) | 0.151 |
| Differentiation (Poor, Moderate/Well) | 0.496 (0.249-0.988) | 0.041 | 0.458 (0.228-0.922) | 0.053 |
| TNM stage (III-IV/I-II) | 7.772 (3.796-15.910) | < 0.01 | 14.665 (6.379-33.715) | < 0.01 |
| B7H3 (High/Low) | 1.772 (0.919-3.415) | 0.749 | - | - |
| CYP1B1 (High/Low) | 1.084 (0.563-2.085) | 0.044 | - | - |
| CYP1B1, B7-H3 (Both High/others) | - | - | 3.606 (1.690-7.694) | 0.043 |

Both high meant high expression of both CYP1B1 and B7-H3, others included CYP1B1-high and B7-H3-low, CYP1B1-low and B7-H3-high, CYP1B1-low and B7-H3-low. *P-value < 0.05* was considered statistically significant.

tion of the relationship between CYP1B1 expression and prognosis may contribute to better diagnosis and treatment of the disease. In the present study, we discovered that CYP1B1 immunoreactivity was increased in CRC tissues, but few or no detectable proteins were found in adjacent normal colorectal tissue. These results agree with previous studies [9]. Our results did not reveal a significant correlation between the expression of CYP1B1 and depth of tumor or lymph metastasis, suggesting that expression of CYP1B1 is independent of TNM status.

Previously, there have only been a few studies that have investigated the relationship between CYP1B1 expression and patient prognosis using immunohistochemistry for CRC [9]. Thus, we analyzed the relationship between CYP1B1 expression and CRC prognosis using this method. Patients with high expression of CYP1B1 had significantly poor OS compared to patients with low CYP1B1 expression. Additionally, a multivariate Cox regression analysis also indicated that the expression level of CYP1B1 was an independent predictor of OS. This suggests that CYP1B1 may be a valuable, independent prognostic biomarker. To date, this is the first report on the independent prognostic value of CYP1B1 in CRC patients.

We also found that B7-H3 expression was increased in CRC tissues and that B7-H3 expression was significantly correlated with lymph metastasis. These results indicate that CRC patients with high expression of B7-H3 in their interstitial lymphocytes are more likely to experience lymph metastasis. High expression of B7-H3 demonstrated no correlation with poor OS. Previous studies have indicated that the overexpression of B7-H3 was significantly linked with low OS in CRC patients [21, 22]. The discrepancy between the current study and previous studies is limited by the relatively small sample size in our study. Recent reports demonstrate that B7-H3 has a key role in tumor cell immune evasion, and it also affects chemosensitivity in non-immunological systems [23, 24]. Our pre-experiment found that there might be a relationship between CYP1B1 and B7-H3. The current study shows that CYP1B1 expression is positively correlated with B7-H3 expression in CRC tissues samples and two human CRC cells. To the best of our knowledge, there are no other reports to identify a connection between the two genes. However, the regulatory mechanism of CYP1B1 and B7-H3 is not clear, and further studies are needed.

The results indicate that CYP1B1 is a useful independent prognostic indicator. Although B7-H3 was not indicated as an independent prognostic biomarker in this study, we did find that the combination of CYP1B1 and B7-H3 might be useful in predicting OS. Additionally, patients with high expression of both CYP1B1 and B7-H3 had worse OS compared with all other combinations of expression levels. Further studies in CRC patients are required to confirm the validity of both the single detection of CYP1B1 and the combined detection of CYP1B1 and B7-H3.

While our study has novel findings, it does have some limitations. First, we did not analyze CYP1B1 and B7-H3 expression in metastatic lymph nodes and metastases. A second limitation is that our study has a limited and relatively

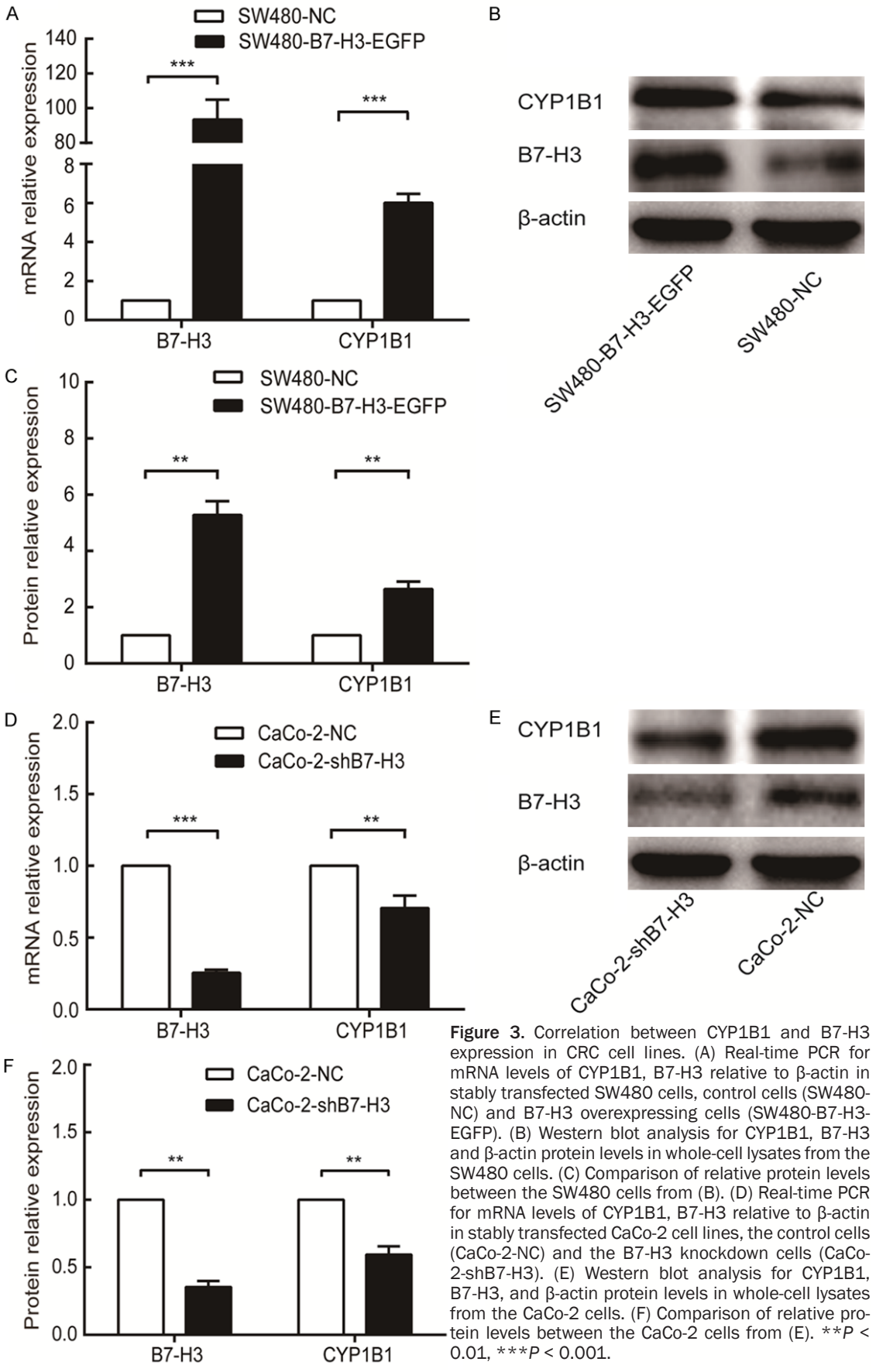


**Figure 3.** Correlation between CYP1B1 and B7-H3 expression in CRC cell lines. (A) Real-time PCR for mRNA levels of CYP1B1, B7-H3 relative to β-actin in stably transfected SW480 cells, control cells (SW480-NC) and B7-H3 overexpressing cells (SW480-B7-H3-EGFP). (B) Western blot analysis for CYP1B1, B7-H3 and β-actin protein levels in whole-cell lysates from the SW480 cells. (C) Comparison of relative protein levels between the SW480 cells from (B). (D) Real-time PCR for mRNA levels of CYP1B1, B7-H3 relative to β-actin in stably transfected CaCo-2 cell lines, the control cells (CaCo-2-NC) and the B7-H3 knockdown cells (CaCo-2-shB7-H3). (E) Western blot analysis for CYP1B1, B7-H3, and β-actin protein levels in whole-cell lysates from the CaCo-2 cells. (F) Comparison of relative protein levels between the CaCo-2 cells from (E). $^{**}P < 0.01$, $^{***}P < 0.001$.

small sample size. Moreover, the immunohistochemical test method is semi-quantitative, and the reported results depend on the quality of the antibody and pathologist [25]. This was a retrospective study. Therefore, large-scale prospective investigation and more accurate evaluating approaches are warranted.

In conclusion, our study demonstrates that high expression of CYP1B1 is significantly associated with poor OS in CRC patients, and CYP1B1 is an independent marker for determining the severity of the disease. Our study has revealed a strong correlation between CYP1B1 and B7-H3 both *in vivo* and *in vitro*, but the biological significance of CYP1B1 and the regulating mechanism between CYP1B1 and B7-H3 warrant further study.

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## Disclosure of conflict of interest

None.

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