RESEARCH ARTICLE

The prognostic impact of serum bilirubin in stage IV colorectal cancer patients

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Background: Bilirubin played a great role in antioxidation and anticancer and has been considered as a promising prognostic factor of non-liver disease-related death in various cancers. The aim of this study was to assess the prognostic value of pre-treatment serum bilirubin in stage IV CRC patients.

Methods: Serum bilirubin including TBIL, DBIL, and IBI which were tested at pre-treatment were investigated in 154 stage IV CRC patients in Zhongda Hospital, Nanjing, China, from July 2005 to July 2011. X-tile program was used to determine the optimal cut-off values of these three biomarkers. Kaplan-Meier analysis, univariate, and multivariate cox regression as well as time-dependent ROC curve analysis were performed to evaluate the relations between serum bilirubin and survival outcomes.

Results: We got the results that the optimal cut-off points of serum TBIL, DBIL, and IBI levels were 12.9, 6.1, and 4.8 μmol/L, respectively. Univariate analysis showed that elevated TBIL, DBIL, and CEA were significantly associated with poor 5-year OS in stage IV CRC patients. Multivariate cox analysis indicated that the high DBIL (HR=1.603, 95%CI=1.053-2.442, $P<.028$) and CEA (HR=1.785, 95%CI=1.123-2.837, $P=.014$) could be identified as independent factors for poor OS. Furthermore, time-dependent ROC curves demonstrated that high DBIL had similar prognostic efficacy as elevated CEA for poor OS (AUC=0.63 and 0.61, respectively).

Conclusions: Pre-treatment elevated TBIL and DBIL levels were associated with poor OS in stage IV CRC patients. Moreover, DBIL could be considered as an independent prognostic biomarker for OS. Furthermore, DBIL had similar prognostic efficacy as CEA for OS.

KEYWORDS
bilirubin, colorectal cancer, overall survival, prognosis

1 | INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide and it also has been supposed as the fourth most commonly leading cause of cancer death in male and the third in female.[1] With limitation of useful early diagnostic biomarkers and invasiveness of colonoscopy, it was reported approximately 25% CRC patients present with metastases at initial diagnosis,[2] which was the

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Abbreviations: CRC, colorectal cancer; HR, hazard ratio; 95%CI, 95% confidential interval; TBIL, total bilirubin; DBIL, direct bilirubin; IBI, indirect bilirubin; OS, overall survival; LFTs, Liver function tests; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; UGT1A1, uridine diphosphate glucuronosyltransferase 1A1; ROC, receiver operating characteristic; NSCLC, non-small-cell lung cancer.

Lin Yang and Lu-Yao Ge are contributed equally to this work.

key contributor to poor 5-year overall survival outcome reported for CRC, though obvious improvements of treatment regimens had been implement recently. As we knew, the primary treatment for metastatic CRC was surgery or systemic chemo-radiotherapy. However, it has become increasingly difficult to identify the best way to integrate systemic chemotherapy with curative surgery for metastatic CRC patients.[3] Thus, finding optimal markers to identify optional therapeutic options for certain subgroups of CRC patients would have enormous clinical benefits. Compared with traditional prognostic markers, such as tumor size, tumor TNM stage and differential grade, blood-based biomarkers became more and more popular and posed greater potential to predict prognosis and guide treatment for CRC patients because it is easily accessible and minimally invasive.

Liver function tests (LFTs), a group of blood tests, were frequently included as baseline tests for inpatients and usually used for reflecting the liver function, such as the integrity and functionality of hepatocytes, and conditions of biliary tract. Bilirubin, the end point product of heme catabolism, was a member of LFTs. It was historically considered to have no physiological function.[4] Interestingly, nowadays bilirubin has been demonstrated by many experimental and clinical researches to play important protective roles in antioxidant and anticancer, giving a rise in the poor ability of patients with cancer scavenging plasma free radical.[5-7] Moreover, an inverse relationship between serum bilirubin level and cancer risk also has been reported in CRC.[4,8-10] At same time, serum bilirubin was also widely investigated as a useful prognostic indicator for non-liver disease-related mortality in various cancers, including CRC.[11-15] However, as for Zhang's study, they only reported stage II-III CRC patients.[15] And Cao's study conducted in northern Chinese population, only reported patients with rectal cancer, the cut-off value was evaluated by ROC curve, and only stage I-III patients were included.[12] In a word, the relationship between bilirubin level and survival of stage IV CRC has not been clearly delineated.

Hence, this retrospective study was conducted in eastern Chinese population to investigate the prognostic impacts of serum bilirubin levels including total bilirubin (TBIL), direct bilirubin (DBIL) and indirect bilirubin (IBI) in 154 stage IV CRC patients. To our knowledge, this is the first study to assess serum bilirubin in stage IV CRC patients.

2 | MATERIALS AND METHODS

2.1 | Study population

Between July 2005 and July 2011, 154 patients with stage IV CRC who were confirmed by histology or cytology in Zhongda Hospital, Jiangsu, China, were enrolled in this retrospective analysis. The inclusion criteria were as followed: (1) age ≥18 years; (2) TBIL, DBIL and IBI were determined within 2 weeks before initial medical therapy. The exclusion criteria were as followed: (1) previous diagnosis of malignancy; (2) mingled with other cancers; (3) underlying known hepatobiliary and pancreatic diseases; (4) elevated parameters in liver function tests (AST>40 U/L; ALT>50 U/L); (5) with any reasons resulting in jaundice; and (6) with clinical parameters and laboratory results loss. At last, 154 patients were enrolled in this study and informed consents were obtained from all eligible patients. This study was approved by the ethics committee of Southeast University.

2.2 | Clinical parameters and laboratory results

The following clinical parameters were evaluated: age, gender, tumor site, treatment type and metastatic organ, which were retrieved from medical records. At the same time, laboratory results including routine tumor markers for CRC, such as carcinoembryonic antigen (CEA) (upper physiological value: 5 ng/mL) and carbohydrate antigen 19-9 (CA19-9) (upper physiological value: 37 U/mL) measured using electrochemiluminescence by ELECSYS 2010 (Roche, Basel, Switzerland) and biochemical data (TBIL, DBIL and IBI) detected by Olympus AU5421 automatic analytical system (Olympus Co. Ltd., Tokyo, Japan) were also collected from medical records. All enrolled patients' blood samples were obtained at 6-8 clock in the morning before initial medical therapy.

2.3 | Follow-up

Patients were followed up regularly until death or dating up to September 1, 2016 according to 7th edition of the TNM-UICC/AJCC classification for CRC (every 3-6 months for first 2 years, every 6 months for the third to fifth years). The overall survival (OS) was defined as the time from the date of diagnosis to last follow-up or death. Follow-up data for patients were computed from medical records, physical examinations, laboratory examinations, or telephone follow-up.

2.4 | Statistical analyses

IBM SPSS Statistical 20.0 (SPSS Inc. Chicago, IL, USA) and R 3.3.0 software (Institute for Statistics and Mathematics, Vienna, Austria) were used for statistical analysis. Kolmogorov-Smirnow test was selected to assess the normality of calculated parameters. Student's t test was used for normal distributed parameters, otherwise Mann-Whitney U test was performed. Chi-square test was used to compare categorical variables. For clinical practice, the continuous variables were changed to categorical variables, and the cut-off values of TBIL, DBIL and IBI were determined by X-tile 3.6.1 (Yale University, New Haven, CT, USA).[16] OS curves were established according to the Kaplan-Meier method and the differences were analyzed by the log-rank test. To identify the independent factors, multivariate Cox regression analyses were performed. Age, gender, and other variables with a P value <.10 in the univariate cox regression analysis were entered into the multivariate cox regression model using backward conditional method. Time-dependent receiver operating characteristic (ROC) curve analysis was used to further compare the performance of significant variables in multivariate analysis in predicting survival outcome. Two-tailed P values of <.05 were considered to be statistically significant.
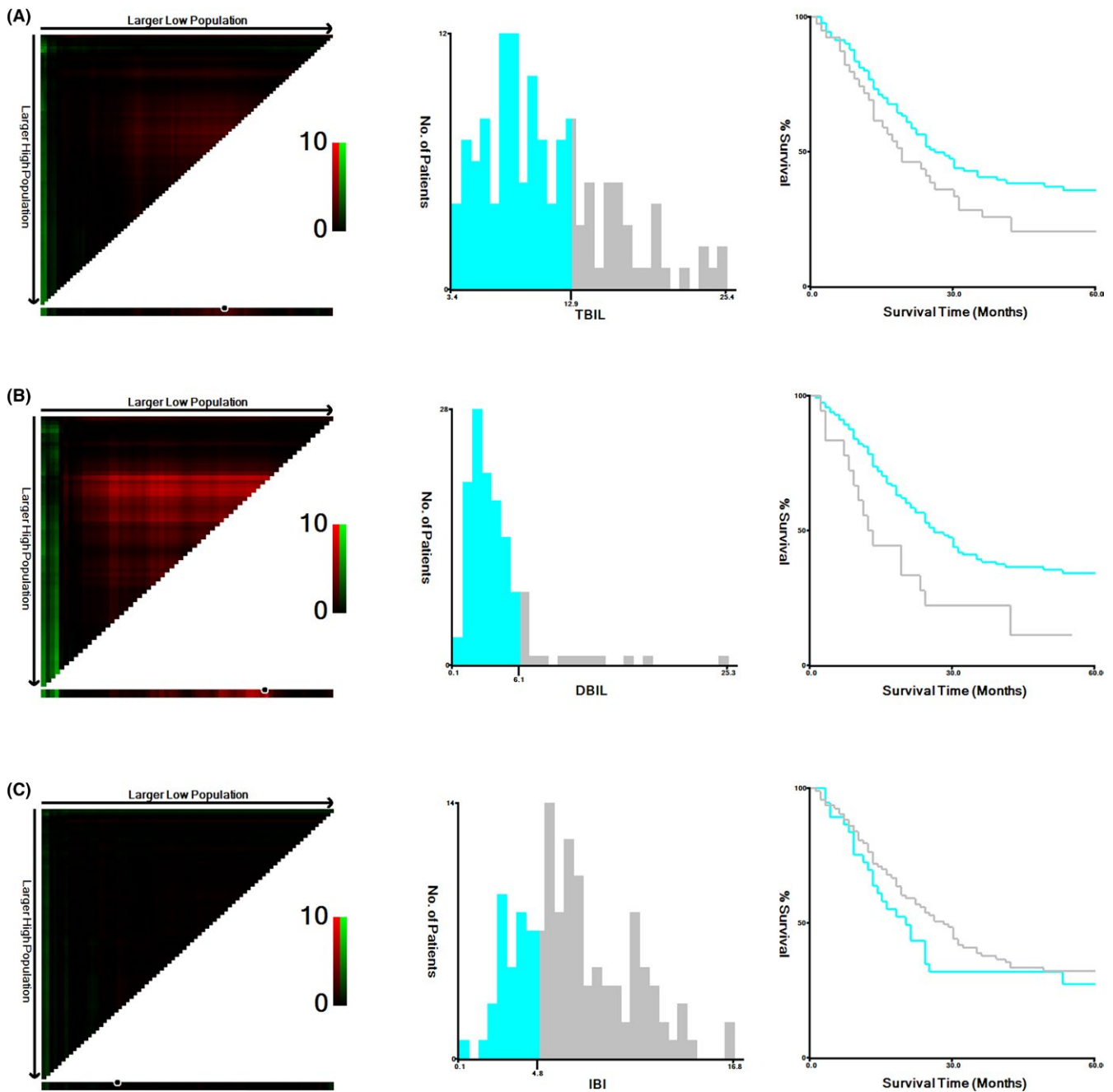
FIGURE 1 X-tile analyses for 5-year overall survival. The sample of CRC patients was equally divided into training and validation sets. X-tile plots of training sets were shown in the left panels, with plots of matched validation sets shown in the smaller inset. The optimal cut-off values highlighted by the black circles in left rectangular panels were also shown in histograms of the entire cohort (middle panels), and Kaplan-Meier plots were displayed in right panels. P values were determined by using the cut-off values defined in training sets and applying them to validation sets. The optimal cut-off values of TBIL, DBIL, and IBI for OS were 12.9 µmol/L, 6.1 µmol/L, and 4.8 µmol/L, respectively. (A) TBIL. (B) DBIL. (C) IBI.CRC, colorectal cancer; TBIL, total bilirubin; DBIL, direct bilirubin; IBI, indirect bilirubin; OS, overall survival

3 | RESULTS

3.1 | The optimal cut-off values for TBIL, DBIL, and IBI

The optimal cut-off values calculated by X-tile were 12.9 µmol/L for TBIL, 6.1 µmol/L for DBIL and 4.8 µmol/L for IBI (Figure 1). Patients were subsequently divided into high group and low group according to the optimal cut-off levels. The cut-off values for CEA and CA19-9 were

5 ng/mL and 37 U/mL, respectively, which was according to the reference range reported by Clinical Laboratory Department in Zhongda Hospital.

3.2 | Baseline characteristics of patients based on TBIL, DBIL, and IBI

Clinical baseline characteristics were summarized in Table 1. A total of 154 patients had a median age of 64 years (range 25-86) and were

TABLE 1 Baseline characteristics of stage IV patients with colorectal cancer based on TBIL, DBIL, and IBI

Characteristics	Total patients (N=154)	TBIL (N=154) (μmol/L)			DBIL (N=154) (μmol/L)			IBI (N=154) (μmol/L)		
		≦12.9 (N=98)	>12.9 (N=56)	P*	≦6.1 (N=119)	>6.1 (N=35)	P*	≦4.8 (N=42)	>4.8 (N=112)	P*
Age (y)										
≦64	83 (53.9%)	49 (31.8%)	34 (22.1%)	.199	62 (40.3%)	21 (13.6%)	.410	18 (11.7%)	65 (42.2%)	.092
>64	71 (46.1%)	49 (31.8%)	22 (14.3%)		57 (37.0%)	14 (9.1%)		24 (15.6%)	47 (30.5%)	
Gender										
Male	101 (65.6%)	61 (39.6%)	29 (26.0%)	.249	72 (46.8%)	29 (18.8%)	**.014**	29 (18.8%)	72 (46.8%)	.580
Female	53 (34.4%)	101 (24.0%)	8 (10.4%)		47 (30.5%)	6 (3.9%)		13 (8.4%)	40 (26.0%)	
Tumor site										
Rectal	59 (38.3%)	37 (24.0%)	22 (14.3%)	.851	47 (30.5%)	12 (7.8%)	.577	15 (9.7%)	44 (28.6%)	.685
Colon	95 (61.7%)	61 (39.6%)	34 (22.1%)		72 (46.8%)	23 (14.9%)		27 (17.5%)	68 (44.2%)	
Operation										
No	55 (35.7%)	31 (20.1%)	24 (15.6%)	.162	40 (26.0%)	15 (9.7%)	.316	12 (7.8%)	43 (27.9%)	.257
Yes	99 (64.3%)	67 (43.5%)	2 (20.8%)		79 (51.3%)	20 (13.0%)		30 (19.5%)	69 (44.8%)	
Chemo-radiotherapy										
No	39 (25.3%)	24 (15.6%)	15 (9.7%)	.753	31 (20.1%)	8 (5.2%)	.703	11 (7.1%)	28 (18.2%)	.880
Yes	115 (74.7%)	74 (48.1%)	41 (26.6%)		88 (57.1%)	27 (17.5%)		31 (20.1%)	84 (54.5%)	
CEA										
<5 ng/mL	45 (29.2%)	36 (23.4%)	9 (5.8%)	**.007**	42 (27.3%)	3 (1.9%)	**.002**	15 (9.7%)	30 (19.5%)	.278
≥5 ng/mL	109 (70.8%)	114 (40.3%)	12 (30.5%)		77 (50.0%)	32 (20.8%)		27 (17.5%)	43 (53.2%)	
CA 19-9										
<37 U/mL	74 (48.1%)	49 (31.8%)	25 (16.2%)	.522	62 (40.3%)	12 (7.8%)	.064	20 (13.0%)	54 (35.1%)	.948
≥37 U/mL	80 (51.9%)	49 (31.8%)	31 (20.1%)		57 (37.0%)	23 (14.9%)		22 (14.3%)	58 (37.7%)	

TBIL, total bilirubin; DBIL, direct bilirubin; IBI, indirect bilirubin; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

The bold represent that *P* value was statistically significant.

*Difference between groups was tested by Chi-square test.

mostly males (65.6%). Colon was the most common location site of the primary tumor (61.7%). A total of 99 patients (64.3%) underwent operation for CRC and up to 115 (74.7%) CRC patients underwent chemo-radiotherapy. The majority of CRC patients had high CEA level (70.8%). During the deadline of follow-up, 44 (28.6%) were still alive and 110 (71.4%) patients were dead. The median OS was 22.5 months (range 1-60). To study the correlation of TBIL, DBIL, and IBI with baseline characteristics of patients, comparison between the high and low groups for TBIL, DBIL and IBI was carried out. Our results revealed that TBIL and DBIL were significantly associated with CEA (P_{all}<.05). Moreover, DBIL was also closely associated with gender (P=.014).

3.3 | The association between baseline characteristics and clinical prognosis

The association between baseline characteristics and OS in stage IV CRC patients were listed in Table 2. Our results showed that TBIL (>12.9 μmol/L), DBIL (>6.1 μmol/L), tumor site (colon), chemo-radiotherapy (yes), CEA (≥5 ng/mL), and CA19-9 (≥37 U/mL) were significantly associated with decreased OS. Clinical baseline characteristics for the prediction of clinical prognosis were further investigated by univariate and multivariate analysis with Cox regression model. The significant characteristics in univariate analysis above were entered into the multivariate cox regression model to further determine the influence on OS (Table 2). Our results indicated that location of colon (HR=1.657, 95%CI=1.102-2.490, P=.015), elevated CEA (HR=1.785, 95%CI=1.123-2.837, P=.014), and high level of DBIL (HR=1.603, 95%CI=1.053-2.442, P=.028) identified by multivariate Cox regression could be considered to be independent markers for poor OS of CRC patients. Moreover, in univariate analysis, the OS was better in patients with high IBI than in those with low IBI (HR=0.794), but there was no statistical significance between them (P=.284). Time-dependent ROC curves demonstrated that high DBIL had similar prognostic efficacy as elevated CEA for reduced 5-year OS (AUC=0.63 and 0.61, respectively).

4 | DISCUSSION

In this retrospective study of 154 stage IV CRC patients, we indicated that pre-treatment elevated TBIL and DBIL levels were associated

TABLE 2 Univariate/multivariate analysis for overall survival

Characteristics	Total patients (N=154)	Number of death (N=110)	Log-rank P value	Univariate analysis HR (95%CI)	P value	Multivariate analysis HR (95%CI)	P value
Age (y)							
≤ 64	83	55 (66.27%)	.152	1	.195		
>64	71	55 (77.46%)		1.309 (0.900-1.903)			
Gender							
Male	101	73 (72.28%)	.778	1	.781		
Female	53	37 (69.81%)		0.945 (0.636-1.404)			
Location							
Rectal	59	35 (59.32%)	**.005**	1	**.006**	1	**.015**
Colon	95	75 (78.95%)		**1.759 (1.176-2.630)**		**1.657 (1.102-2.490)**	
Operation							
No	55	42 (76.36%)	.193	1	.200		
Yes	99	68 (68.69%)		0.778 (0.529-1.143)			
Chemo-radiotherapy							
No	39	19 (48.72%)	**.012**	1	**.014**	1	.082
Yes	115	91 (79.13%)		**1.864 (1.132-3.067)**		1.566 (0.945-2.597)	
CEA							
<5 ng/mL	45	24 (53.33%)	**.002**	1	**.003**	1	**.014**
≥5 ng/mL	109	86 (78.90%)		**1.995 (1.267-3.142)**		**1.785 (1.123-2.837)**	
_CA19-9_							
<37 U/mL	74	45 (60.81%)	**.015**	1	**.017**	1	.555
≥37 U/mL	80	65 (81.25%)		**1.590 (1.086-2.328)**		1.139 (0.740-1.754)	
TBIL							
≤12.9 µmol/L	98	63 (64.29%)	**.022**	1	**.025**	1	.859
>12.9 µmol/L	56	47 (83.93%)		**1.540 (1.054-2.248)**		1.053 (0.593-1.870)	
DBIL							
≤6.1 µmol/L	119	78 (65.55%)	**.002**	1	**.003**	1	**.028**
>6.1 µmol/L	35	32 (91.43%)		**1.875 (1.241-2.834)**		**1.603 (1.053-2.442)**	
IBI							
≤4.8 µmol/L	42	30 (71.43%)	.276	1	.284		
>4.8 µmol/L	112	80 (71.43%)		0.794 (0.521-1.210)			

TBIL, total bilirubin; DBIL, direct bilirubin; IBI, indirect bilirubin; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; HR, hazard ratio; 95%CI, 95% confidential interval.
The bold represent that P value was statistically significant.

with poor OS, and DBIL was an independent prognostic factor for OS. To the best of our knowledge, our study was the first report on the prognostic role of DBIL in stage IV CRC patients.

X-tile program, a robust graphical tool verified by Yale University was used to determine the optimum cut-off values for TBIL, DBIL, and IBI, which were 12.9, 6.1, and 4.8 µmol/L, respectively.[16] However, these cut-off values varied from previous studies (date were listed in Table 3).[11-15] With the aspect to CRC, Gao used the threshold 2.6 µmol/L as cut-off value to classified the DBIL for stage I-IV rectal cancer,[12] and Zhang chose 3.6 µmol/L for stage II-III CRC,[15] which

TABLE 3 Published studies on the association between serum bilirubin level and survival of cancer patients

Study	Year	Cancer	Number	Method	TNM stage	Country	Ethnicity	Survival	Factors	Cut-off value	Significant in univariate analysis	Significant in multivariate analysis
Deng et al.[11]	2015	NPC	1327	ROC	I-IV	Guangzhou	Asian	OS	IBI	9.7 µmol/L	Yes	No
Deng et al.[11]	2015	NPC	1327	ROC	I-IV	Guangzhou	Asian	DMFS	IBI	9.7 µmol/L	Yes	Yes
Li et al.[13]	2015	NSCLC	1617	ROC	I-III	Guangdong	Asian	OS,DFS, LRFS, DMFS	TBIL	9.50 µmol/L	Yes	No
Li et al.[13]	2015	NSCLC	1617	ROC	I-III	Guangdong	Asian	OS, DFS, LRFS, DMFS	DBIL	3.45 µmol/L	Yes	No
Li et al.[13]	2015	NSCLC	1617	ROC	I-III	Guangdong	Asian	OS, DFS, LRFS, DMFS	IBI	6.95 µmol/L	Yes	Yes
Liu et al.[14]	2015	Breast cancer	2425	Spline curves	I-III	Houston	Caucasian	OS	TBIL	0.2 mg/dl	Yes	Yes
Cao et al.[12]	2016	Rectal cancer	469	ROC	I-IV	Beijing	Asian	OS	DBIL	2.6 µmol/L	Yes	Yes
Zhang et al.[15]	2016	CRC	986	X-tile	II-III	Shanghai	Asian	OS, DFS	DBIL	3.6 µmol/L	Yes	Yes
This study	2016	CRC	154	X-tile	IV	Nanjing	Asian	OS	TBIL	12.9 µmol/L	Yes	No
This study	2016	CRC	154	X-tile	IV	Nanjing	Asian	OS	DBIL	6.1 µmol/L	Yes	Yes
This study	2016	CRC	154	X-tile	IV	Nanjing	Asian	OS	IBI	4.8 µmol/L	No	No

ROC, receiver operating characteristic; NSCLC, non-small-cell lung cancer; NPC, nasopharyngeal carcinoma; CRC, colorectal cancer; TBIL, total bilirubin; DBIL, direct bilirubin; IBI, indirect bilirubin; OS, overall survival; LRFS, locoregional relapse-free survival; DMFS, distant metastasis-free survival; DFS, disease-free survival; RFS, relapse-free survival.

were slightly lower than our cut-off value of DBIL. Number of patients from different tumor stages and different geographic regions may be could explain this phenomenon. Furthermore, different reference ranges of routine biochemistry tests in different medical centers could also contribute to this difference.

Our study found that the OS was better in patients with high IBI than in those with low IBI, but there was no statistical significance between them, which indicated that elevated IBI level might be a protective factor for OS. At the same time, a previous study on naso-pharyngeal carcinoma (NPC) showed that IBI could inhibit NPC metastasis by inhibiting reactive oxygen species (ROS) production, and acted as a favorable prognostic factor.[11] What's more, IBI was also reported to be an independent prognostic factor in NSCLC.[13] On the other hand, it was reported that IBI could induce cell apoptosis in colon cancer cell lines by triggering mitochondrial depolarization,[17] suggesting that elevated IBI level could predict good outcome. As for the prognostic role of IBI in stage IV CRC, it should be confirmed in further study.

The study revealed some interesting associations between the variates and tumor prognosis. First, we found that the location of tumor was significantly more likely to be colon in patients with poor outcome. It might be due to that colon was the main place of converting unconjugated bilirubin to form urobilinogens by the function of bacterium in enterohepatic recirculation. Second, our study demonstrated that higher DBIL correlated with poor survival in stage IV CRC patients, and could be treated as an independent prognostic biomarker, which was also found in stage I-IV rectal cancer, stage II-III CRC, and other malignancies such as NSCLC previously. In addition, this study also showed elevated TBIL level was associated with poor OS, which was consistent with Li's study in NSCLC.[13] As we knew, bilirubin was mainly cleared by liver. In hepatocytes, unconjugated bilirubin (or IBI) could be converted into conjugated bilirubin (or DBIL) through conjugation with two molecules of glucuronic acid by the action of UGT1A1 enzyme, which indicated that UGT1A1 enzyme could play essential roles in the level of bilirubin.[7] It was reported congenital under-expression of UGT1A1 resulted into mild, chronic, fluctuating unconjugated hyperbilirubinemia (Gilbert's syndrome), which was due to an extra TA in the $(TA)_6$ locus of the promoter region of UGT1A1, leading to low expression of the enzyme.[7] Additionally, recent studies also reported that gene polymorphism of UGT1A1 enzyme was commonly seen in CRC,[8] and UGT1A1 gene polymorphism was related to prognosis of irinotecan-based chemotherapy in patients with advanced CRC,[18] thus in advanced CRC patients, the process of IBI converting to DBIL would be influenced, and then poor outcome occurred. Hence, taking the inconvenience and expensiveness of UGT1A1 into consideration, our results proved our hypothesis and indicated that serum bilirubin which was tested routinely could be an available prognostic biomarker for CRC.

On the other hand, our study had some limitations. First, only 154 stage IV patients could not be representative of all stage IV CRC patients in general. Second, only OS which were not able to exclude the influence of cancer unrelated to death was accessed in this study. The last but not least, the lack of an external validation cohort requires further studies.

In conclusion, pre-treatment elevated TBIL and DBIL levels were associated with poor OS in stage IV CRC patients. Moreover, DBIL could be considered as an independent prognostic biomarker for OS. Furthermore, high DBIL had similar prognostic efficacy as elevated CEA for reduced 5-year OS.

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