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Clinical significance of the combined measurement of serum B7-H1 and interleukin-10 in colorectal cancer patients

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Abstract

Colorectal cancer (CRC) patients have been shown to express a cytokine signature that is detectable in serum and contributes to cancer pathogenesis. The objective of this study was to evaluate the potential clinical significance of preoperative circulating cytokine levels in CRC patients.

The expression of serum B7-H1 and IL-10 was assessed by ELISA in 89 patients and 64 health volunteers. As a control marker, CEA serum levels were measured by electrochemical luminescence detection. The receiver operating characteristic (ROC) curve was used to analysis to demonstrate the potential diagnostic value of these biomarkers.

The expression of serum B7-H1 was significantly increased in CRC patients ($P = .001$) and associated with the progression of TNM stage and a positive association with serum IL-10 levels was also evident. Furthermore, serum B7-H1 and IL-10 expression was not influenced by age, gender, tumor location, or mass, whereas a relationship existed with tumor metastasis and TNM stage. The serum levels of B7-H1 and IL-10 on the 7th postoperative day were significantly decreased compared with that of preoperative serum levels ($P = .001$, $P = .003$ respectively). The area under the ROC curves (AUC) for B7-H1 and IL-10 were 0.7063 and 0.5706, respectively. The optimal sensitivity and specificity of B7-H1 for discriminating between colon cancer patients and healthy controls were 85.21% and 56.43%, respectively, using a cut-off value of 3.46 ng/mL. However, the combined ROC analysis using B7-H1 and IL-10 revealed an AUC of 0.8791, with a sensitivity of 90.63% and a specificity of 75.18%.

The outcomes of the present study demonstrate the clinical significance of serum B7-H1 and IL-10 concentrations. Combined detection of B7-H1 plus IL-10 showed significantly increased sensitivity and specificity for discriminating between colorectal cancer patients and healthy controls compared these markers detection individual. The measurement of B7-H1 or IL-10 in sera following surgery may provide an additional tool for assessing the curative effects of surgery in CRC patients.

Abbreviations: AUC = the area under the ROC curves, CEA = carcinoembryonic antigen, CRC = colorectal cancer, IL = interleukin, ROC = receiver operating characteristic.

Keywords: B7-H1, colorectal cancer, diagnostic marker, IL-10

1. Introduction

Colorectal cancer (CRC) remains a major health burden with increasing morbidity and mortality rates each year despite advances in diagnosis and treatment.[1] Accounting for nearly 8.5% of total cancer related deaths annually,[2,3] it is one of the most frequently diagnosed cancers among high-income and most middle-income countries.[4] CRC is known to be a biologically heterogeneous disease characterized by the activation of several immunologic and oncogenic pathways.[5]

Evidence from preclinical models indicates a pivotal role for the programmed cell death ligand 1 (PD-1 or CD274) T cell co-receptor and its ligand, B7-H1/PD-L1, in maintaining an immunosuppressive tumor microenvironment.[6–8] B7-H1 was identified as a member of the B7 family of molecules based on a homology search of the EST database.[9] B7 family members of negative costimulatory molecules, including B7-H1, have been shown to be overexpressed in some cancers, including ovarian, lung, and breast cancer.[10–12] We previously demonstrated that B7-H1 exhibits a high prognostic significance, promotes tumor tolerance, and may contribute to regulatory T cell (Treg) development in the CRC tolerogenic milieu.[13] Higher serum B7-H1 levels have also been reported in CRC patients and are closely correlated with clinicopathological parameters, further

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supporting its potential value as a marker for early diagnosis and prognosis.

Ligation of T cells by B7-H1 moderately stimulates growth and preferential secretion of IL-10 and interferon-γ in vitro, and promotes antibody and helper T cell responses in vivo.[14] Although the inducible expression of B7-H1 in the tumor microenvironment is complex, it is clear that the costimulatory molecule IL-10, in a subset of T cells, plays an important role.[15] IL-10 is primarily secreted by Th2 cells, mononuclear macrophages, B lymphocytes, and keratinocytes under normal conditions, however, an unusual increase in its expression occurs during tumorigenesis. In the body of a cancer patient, the Th2 cell subset, which is the primary source of IL-10, increases abnormally. Furthermore, regulatory T cells, another source of IL-10, secrete high levels of IL-10 and low levels of IL-2. Additionally, tumors by themselves may generate IL-10, leading to increased expression of IL-10.[16]

The potential value of serum IL-10 and B7-H1 as diagnostic and prognostic markers have been reported in several studies.[17–19] However, the sensitivity and specificity of the B7-H1 or IL-10 blood tests for CRC patients still requires improvement. It is also necessary to further clarify the clinical significance of the PD-1 signaling pathway in vivo by measuring serum expression of B7-H1 and IL-10. Therefore, this study investigates the relationship between the serum expression levels of B7-H1 and IL10 relative to several clinicopathological parameters in CRC patients. We also explored the diagnostic sensitivity of B7-H1 and IL-10 and the predictive value of patient immune status relative to the expression of pathway members.

2. Materials and methods

2.1. Patients and serum samples

Patients in the present study were enrolled from the Second Affiliated Hospital of Beihua University and the People's Hospital of Jilin City, China. The study was approved by the Research Ethics Committee of the hospital and written informed consent was obtained from all of the patients according to the committee's regulations. All samples were handled and anonymized according to ethical and legal standards. Eighty-nine paraffin-embedded CRC samples and matched serum samples were provided by the Second Affiliated Hospital of Beihua University and the People's Hospital of Jilin City for patients with complete histopathology and follow-up information from 2015 to 2016. With respect to the criteria, uncontrollable ascites or pleural effusion and serious comorbidities, such as interstitial pneumonia or uncontrollable diabetes mellitus, severe heart disease, other active malignancy, active inflammation, or other serious medical conditions were excluded. None of the patients received preoperative chemotherapy or radiotherapy. The clinicopathological characteristics of the patients are detailed in Table 1.

Sixty-four healthy age- and sex-matched controls were recruited from people who came and received health examina-

Table 1

The association between serum B7-H1, IL-10, CEA level and CRC clinicopathological parameters.

		B7-H1 (ng/ml)		IL-10 (pg/ml)		CEA (ng/ml)	
Patient Characteristics	**n**	**251658240$\bar{x} \pm S$**	***P***	**251658240$\bar{x} \pm S$**	***P***	**251658240$\bar{x} \pm S$**	***P***
Age							
<60	47	4.238 ± 3.402	.401	4.026 ± 3.154	.391	60.581 ± 31.350	.604
≥60	42	3.899 ± 3.025		4.566 ± 3.291		68.879 ± 56.703	
Gender							
Male	61	4.119 ± 3.415	.387	4.026 ± 3.154	.298	76.859 ± 35.017	.368
Female	28	3.987 ± 3.114		4.566 ± 3.291		77.481 ± 44.502	
location of tumor							
Colon	57	3.987 ± 3.021	.104	4.198 ± 3.321	.368	67.115 ± 41.693	.285
Rectum	32	4.321 ± 3.391		3.998 ± 3.112		73.750 ± 51.402	
Depth of infiltration							
T1+T2	19	3.789 ± 3.001	.375	4.047 ± 2.628	.440	63.899 ± 37.241	.341
T3+T4	60	4.214 ± 3.452		3.021 ± 1.069		64.645 ± 43.553	
Tumor metastasis							
metastasized	63	5.118 ± 4.421	.0001	5.225 ± 4.982	.002	67.001 ± 43.752	.076
Non-Metastasized	26	3.229 ± 1.844		3.341 ± 1.254		77.358 ± 37.496	
lymph nodes metastasis							
<4	33	3.367 ± 1.625	.0001	2.588 ± 1.232	.003	55.236 ± 47.351	.112
≥4	56	5.351 ± 1.024		4.125 ± 2.507		62.457 ± 37.169	
TNM stage							
I/II	29	2.475 ± 1.336	.0001	2.601 ± 1.546	.004	57.128 ± 39.160	.089
III/IV	60	5.541 ± 1.014		4.927 ± 2.331		63.685 ± 35.097	
Differentiation							
High	18	4.022 ± 3.370	.442	4.336 ± 3.292	.412	60.565 ± 47.117	.105
Middle	57	4.673 ± 2.148		3.689 ± 1.273		62.120 ± 33.095	
Low	14	4.101 ± 3.318		4.014 ± 1.215		65.153 ± 42.506	
Histology							
Squamous	2	4.846 ± 1.312	.406	3.845 ± 1.106	.863	65.726 ± 34.502	.418
glandular	78	4.120 ± 1.498		3.903 ± 1.037		64.580 ± 31.794	
Mucoid	9	4.625 ± 1.501		4.729 ± 1.068		62.425 ± 43.231	

Table 2

The comparison of serum concentrations of B7-H1/IL-10 between CRC patients group and control group.

Group	B7-H1 ($\bar{x} \pm S$, ng/ml)	CI (95%)	P	IL-10 ($\bar{x} \pm S$, pg/ml)	CI (95%)	P	CEA ($\bar{x} \pm S$, ng/ml)	CI (95%)	P
Control	1.174 ± 0.328	0.215–2.368	.001	1.991 ± 0.694	0.447–2.214	.004	11.825 ± 10.019	0.809–2.651	.001
CRC Postoperative	4.015 ± 3.321*	0.145–9.316		4.316 ± 3.262*	0.863–8.567		68.879 ± 56.703*	35.124–106.118	
			.001			.003			.006
CRC Postoperative	1.925 ± 1.045†	0.136–2.376		2.076 ± 1.006†	0.704–3.011		15.253 ± 12.307†	2.674–20.358	

CI = confidence interval.

* $P < .01$ vs control group.

† $P < .01$ vs preoperative group.

tions. Peripheral blood samples were kept at room temperature for 22 hours, and serum was obtained by centrifugation at 3000 × g at 4°C for 15 minutes. The serum was removed immediately and frozen on dry ice and stored at −80°C until use. Samples were simultaneous determination under the same experimental conditions. The times period is 5 months to a year from the time of sample collection to the day of analysis.

2.2. B7-H1 and IL-10 serum expression measurement

B7-H1 and IL-10 serum expression were measured by enzyme-linked immunosorbent assay (ELISA) assay as described previously.[20,21] Detection ranges for B7-H1 and IL-10 were 0.156 to 10 ng/mL and 0.780 to 50 pg/mL, respectively (Rapidbio, Hayward, CA). Briefly, 100 μl of serum was placed into each well of an ELISA plate and incubated for 45 minutes at 37°C according to the protocol of assay kit. The plates were washed 4 times with buffer and incubated with 50 μL of detection antibody at 37°C for 1 hour. After 5 washes, the plates were incubated with 100 μL horseradish peroxidase solution (1:5000) for 30 minutes at 37°C, and then incubated with the enzyme substrate, p-nitrophenyl phosphate, for 30 minutes at room temperature. The absorbance was then read at 450 nm using an ELISA microplate reader (Multiskan MK3, Thermo Scientific, MA). A corresponding standard curve was generated using the provided standards and the quantities of B7-H1 and IL-10 in each serum sample were calculated.

2.3. Electrochemical luminescence detection of serum CEA

Expression levels of CEA in the serum samples were measured using a two-site microtiter plate-based immunoassay with electrochemical luminescence detection (Cobas601 Kit, Roche, Germany) according to the manufacturer's instructions. At least 3 independent experiments were conducted for each case. The accuracy of the results for the quality control samples and sensitivity met the experimental requirements.

2.4. Statistical analysis

Data are expressed as mean ± standard deviation. Tukey's post-hoc test and Student's *t* test were conducted to analyze comparisons among multiple groups and between 2 groups of variance, respectively. Variables following a nonnormal distribution were logarithmically transformed (natural logarithm) before use in parametric analyses. Pearson correlation, χ^2 test or Fisher exact test were used for comparison and estimation of correlations between B7-H1 or IL-10 serum expression and clinicopathological tumor parameters including infiltration, distant metastasis, lymph nodes metastasis or differentiation, etc. Bonferroni Data were analyzed using SPSS v.23.0 software. For all comparisons, all reported *P* values were the result of 2-sided tests and $P < .05$ was considered statistically significant. Graphs were plotted using GraphPad Prism v.6.0 software (GraphPad Inc., La Jolla, CA).

3. Results

3.1. Comparison of B7-H1 and IL-10 serum expression between CRC patients and controls

We analyzed B7-H1 and IL-10 protein levels in 89 CRC and 64 corresponding normal serum samples. The mean B7-H1 serum level in CRC patients (4.015 ± 3.321 ng/mL) was significantly higher than that of healthy controls (1.174 ± 0.328 ng/mL) ($P = .001$), Table 2, Fig. 1A and B. The mean IL-10 serum expression was also significantly higher in CRC samples compared with controls (4.316 ± 3.262 pg/mL vs 1.991 ± 0.694 pg/mL, $P = .004$). Summarized data for all markers are shown in Table 2.

3.2. Correlation between serum expressions of B7-H1, IL-10, and clinicopathological parameters of CRC

The correlations between serum B7-H1, IL-10, or CEA protein levels and clinicopathologic features are summarized in Table 1. There was no significant correlation between B7-H1, IL-10 expression and age or gender. High serum expression of B7-H1 ($P = .104$) and IL-10 ($P = .368$) were not correlated with tumor location or tumor mass. The data showed that the depth of tumor infiltration was not associated with B7-H1 ($P = .375$) or IL-10 ($P = .440$) levels. Further analysis also showed that B7-H1 ($P = .442$) and IL-10 ($P = .412$) levels were not associated with the degree of CRC tumor differentiation. However, B7-H1 and IL-10 levels were significantly associated with tumor metastasis (Table 1). B7-H1 expression was significantly higher in CRC patients with more lymph node metastases (≥4) (5.351 ± 1.024 ng/mL) compared with patients containing fewer lymph node metastases (3.367 ± 1.625 ng/mL) ($P = .0001$). This suggests that B7-H1 is related to tumor progression and metastasis in CRC. We also evaluated the association of serum B7-H1 and IL-10 levels with TNM staging in CRC patients. The mean B7-H4 serum level was significantly higher in patients with stage III/IV disease compared with early tumor stage I/II ($P = .0001$). Similarly, there was a strong relationship between serum IL-10 levels ($P = .069$) and CRC progression (Table 1). Serum CEA levels were significantly different between CRC patient and control groups ($P = .0001$, Table 2), but were not associated with the clinicopathological parameters.

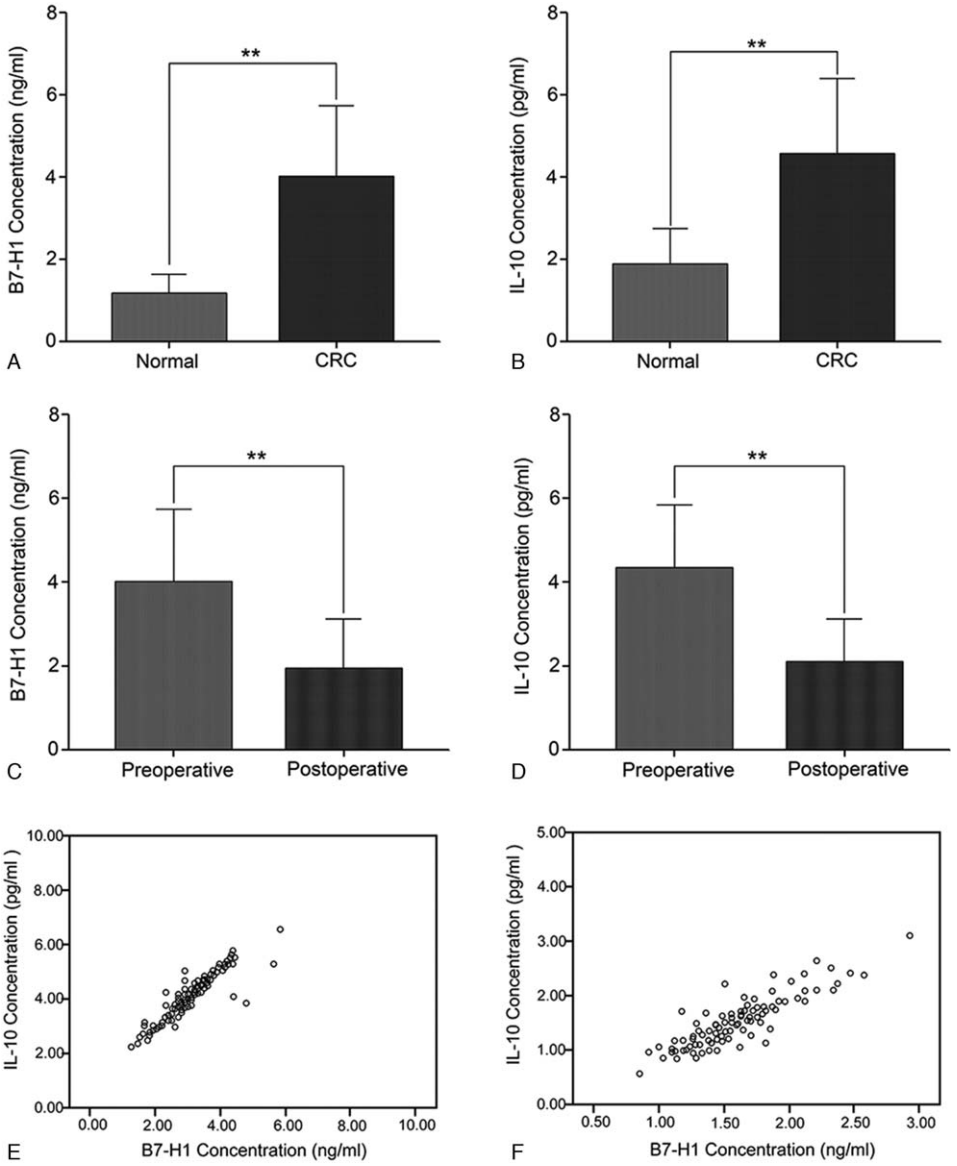

Figure 1. Comparison of B7-H1/IL-10 serum expression between preoperative and postoperative groups in CRC patients, and correlation analysis between B7-H1 and IL-10 serum expression. A&B: The comparison of serum concentrations of B7-H1/IL-10 between the CRC group and the control group. Both B7-H1 and IL-10 mean serum levels in CRC patients were significantly higher than that in healthy controls. C&D The comparison of serum concentrations of B7-H1/IL-10 between preoperative and postoperative groups in CRC patients. Serum expression of B7-H1 and IL-10 of the postoperative samples decreased significantly compared with preoperative levels. E&F: The correlation between preoperative serum B7-H1 and IL-10 levels was determined and the value of a Poisson's test indicated a positive correlation between them.

3.3. Assessment of preoperative and postoperative serum markers

All 89 CRC patients were evaluated preoperatively prior to radical surgical resection. Forty-three patients also underwent peripheral lymph node dissection. No patients experienced surgical or perioperative mortality, and there were no perioperative complications including hemorrhage or intestinal fistula. Serum expression of B7-H1 and IL-10, re-measured on the 7th postoperative day, had significantly decreased compared with preoperative levels ($P=.001$, $P=.003$ respectively), and were not different from levels in the healthy control group (Table 2, C and D).

3.4. Correlation analysis between serum B7-H1 and IL-10

We measured preoperative and postoperative serum levels of B7-H1 and IL-10 in CRC patients. A positive correlation ($r=0.976$, $P=.001$) was observed between preoperative serum B7-H1 and IL-10 levels as determined by a Poisson test. Postoperative serum IL-10 levels were also positively associated with that of B7-H1 ($r=0.83$, $P=.004$, Fig. E and F).

3.5. Diagnostic value by receiver operating characteristic curve analysis

We investigated the diagnostic potential of the markers in CRC by receiver operating characteristic (ROC) curve and area under

Table 3

The diagnosis value of serum B7-H1/IL-10/CEA in CRC patients.

Biomarkers	AUC(95%CI)	*P* value	Sensitivity (%)	Specificity (%)	Youden Index
B7-H1	70.63	.247	85.21	56.43	0.416
IL-10	57.06	.752	72.24	41.87	0.141
CEA	74.25	.457	57.09	81.65	0.387
B7-H1+IL-10	87.91	.022	90.63	75.18	0.658
B7-H1+CEA	75.84	.276	89.32	60.36	0.497
IL-10+CEA	73.72	.265	88.59	59.14	0.477

the curve (AUC) analyses. The cut-off values based on the ROC analysis were 3.46 ng/mL for B7-H4, 3.16 pg/mL for IL-10, and 3.4 ng/L for CEA. The ROC curve demonstrated optimal sensitivity (0.8521) of B7-H1 serum levels in distinguishing between CRC patients and healthy controls at a threshold of 3.46 ng/mL, with an AUC of 0.7063, while CEA levels showed the highest specificity (0.8165) (Table 3, Fig. 2). Combined ROC curve analysis using B7-H1 and IL-10 revealed an AUC of 0.8791 with a sensitivity of 90.63% and a specificity of 75.18% for discriminating CRC patients from healthy controls. Among these biomarkers, B7-H1 and IL-10 (Youden index value 0.658) represented the most accurate markers for diagnosing CRC (Table 3, Fig. 3). These results indicate that combined detection of B7-H1 and IL-10 provide a promising approach for diagnosing CRC (Fig. 2).

4. Discussion

Human anti-tumor immune mechanisms are complex, involving a variety of immune components, including humoral and cellular immunity.[22,23] Tumor and stromal cells at the tumor site exploit multiple immunoregulatory pathways including the production of immunosuppressive cytokines (e.g., IL-10) and enzymes, down-regulation of tumor cell surface MHC, or favoring conversion of immune effector cells into an immunosuppressive cell population.[24]

It is widely accepted that B7-H1 is involved in the down-regulation of cell-mediated immune responses in the priming state by inducing inhibitory cytokines such as IL-10. In addition, the engagement of fully activated T cells by B7-H1 may lead to increased programmed cell death.[25] The B7-H1/programmed cell death 1 (PD-1) pathway represents a class of immunomodulatory targets. Current data indicate that this pathway works selectively at the tumor site with minimal expression in other organs.[26] This pathway is accepted as one of the major mechanisms of tumor immune escape by stimulating inhibitory signals in T cells. Moreover, B7-H1 acts not only as a ligand for PD-1, but can also serve as a receptor, transmitting reverse signals that protect cancers cells from apoptosis mediated by the FAS/FASL pathway or anticancer agents.[27]

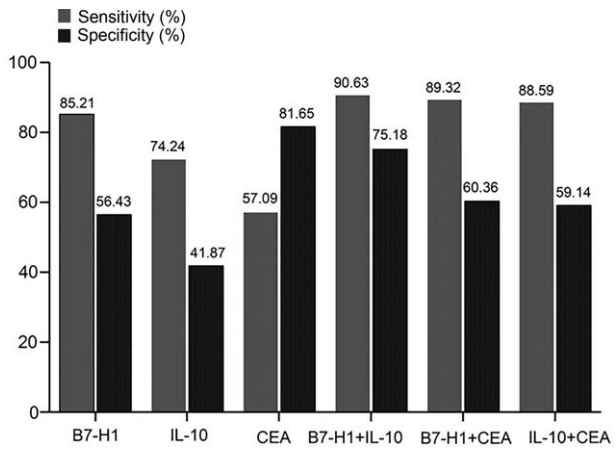

Figure 2. Diagnostic value of serum B7-H1/IL-10/CEA in CRC patients. Combined analysis using B7-H1 and IL-10 revealed a sensitivity of 90.63% and a specificity of 75.18% for discriminating CRC patients from healthy controls.

Theoretically, blockade of the B7-H1 pathway could be used in conjunction with all active and passive immunotherapy aiming to stimulate the activation of T cells. The importance of this pathway has been confirmed in the clinic with an unprecedented tumor response ratio observed when this pathway is blocked.[28–30] Predictably, tumor-targeted therapy via specific blockage of the inhibitory PD-1/PD-L1 pathway is expected. In fact, early PD-1/PD-L1 pathway researchers won the Nobel Prize in 2018. Although therapies targeting these mechanisms have demonstrated promising results in some clinical trials, there are still no effective immunotherapies for CRC.[31] Since many important questions remain to be clarified with respect to immunization therapy, one area of focus should be on the development of diagnostic and prognostic markers. Although serum B7-H1 levels have been considered to have diagnostic value in several human cancers,[32] studies demonstrating a role in early diagnosis or prognosis in CRC is currently lacking.

In this study, we demonstrate that changes in serum IL-10 levels are associated with response to B7-H1 therapy in patients with CRC. We further analyzed the correlations between serum B7-H1 or IL-10 and clinicopathologic features of CRC and showed that overexpression of both are closely associated with tumor metastasis and TNM staging, but not with gender, age or tumor infiltration. Moreover, we found that B7-H1 and IL-10 levels decreased significantly during the first postoperative week. We also determined if the combined detection of B7-H1 with IL-10 or CEA could increase the specificity and sensitivity of a CRC diagnosis. B7-H1 alone exhibited high sensitivity and low specificity, while CEA and IL-10 exhibited the opposite. We performed ROC analysis using the two markers combined, and showed that the combination provided relatively high sensitivity and specificity for discriminating CRC patients from healthy controls. No reliable independent biomarker has yet been established for CRC screening. These results suggest that measurement of serum B7-H1 or IL-10 expression following surgery may provide a tool for assessing the success and curative effect of surgery in CRC patients.

In conclusion, our pilot study contributes to our further understanding of the clinical significance of combined serum

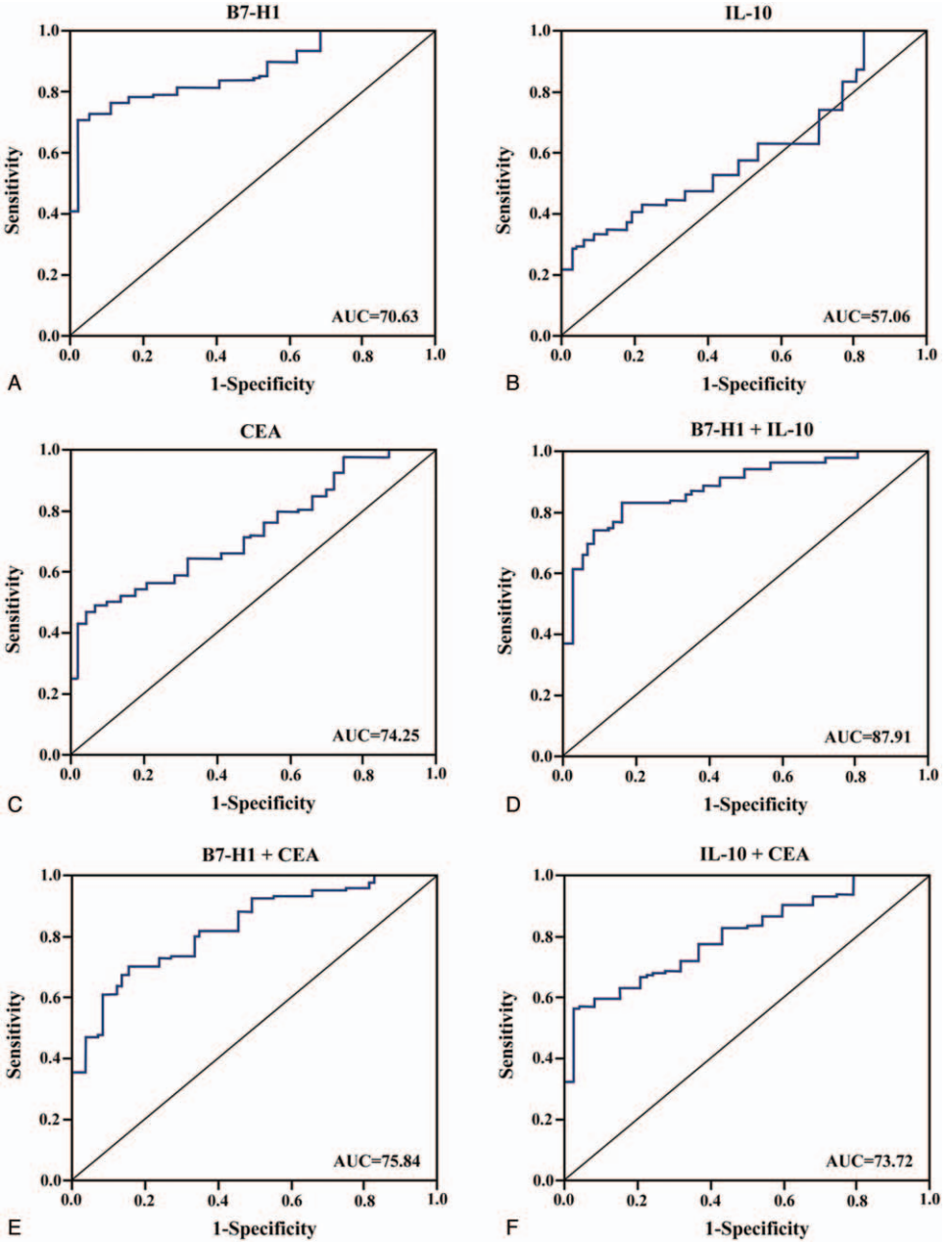

Figure 3. ROC Curve of B7-H1/IL-10/CEA in CRC Patients. Receiver operating characteristic (ROC) curve analysis using 4 biomarkers to differentiate patients. Diagnostic accuracy of the biomarkers was determined by obtaining the largest possible area under the curve (AUC) in ROC analysis. A, B7-H1; B, IL-10; C, CEA; D, B7-H1+IL-10; E, B7-H1+CEA; F, IL-10+CEA.

B7-H1 and IL-10 levels in CRC patients. Elevated serum expression of B7-H1 and IL-10 may play a critical role in the development and progression of CRC. Detection of B7-H1 may thus serve as a diagnostic marker or predict outcome in patients with CRC. A limitation to the present study is the relatively small numbers of patients enrolled. Therefore, we propose that our findings should be validated in a larger cohort of patients. Furthermore, larger scale studies should include patients representing other diseases, such as chronic enteritis, to differentiate with CRC. This is needed to confirm the enhanced diagnostic and prognostic values obtained from the combined detection of serum B7-H1 and IL-10 in CRC patients.

5. Conclusions

Serum expression of B7-H1 and IL-10 was positively correlated and may play a critical role in the development of CRC. Assessment of serum B7-H1 or IL-10 expression may provide an additional tool for assessing the success and curative effect of surgery, and also provide an effective monitoring method for tumor metastasis.

Author contributions

Conceptualization: Peng Wang, Xiaodong Gai
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Writing – original draft: Peng Wang
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