Original Article

A three-phase trans-ethnic study reveals *B7-H3* expression is a significant and independent biomarker associated with colon cancer overall survival

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Background: There have been inconsistent results and conflicting conclusions among the existing prognostic studies of *B7-H3* expression in colon cancer patients. Therefore, the association between *B7-H3* expression and colon cancer survival has remained largely unclear.

Methods: We performed a three-phase and trans-ethnic prognostic study of *B7-H3* expression in colon cancer patients involving perhaps the largest population to date. In the discovery phase, we utilized a Cox proportional hazards model adjusted for covariates to test the association between *B7-H3* expression and colon cancer overall survival (OS) time in a European population from The Cancer Genome Atlas (TCGA) cohort (n=433). In the validation phase I, the association was replicated in a European population from Gene Expression Omnibus (GEO) cohort (n=811). In the validation phase II, we again confirmed the significant association in an Asian population from Fujian Medical University Union Hospital (UNION) cohort (n=179). Furthermore, a series of Kaplan-Meier analysis, bioinformatics analysis of tumor immune microenvironment (TIME), and immune checkpoint prognostic prediction analysis, as well as sensitivity analysis, were also conducted.

Results: Highly expressed *B7-H3* was a significant and robust biomarker to colon cancer survival, with a large hazard ratio (HR) [HR_{TCGA} =4.60, 95% confidence interval (CI): 2.15 to 9.83, $P=8.37\times10^{-05}$; HR_{GEO} =1.47, 95% CI: 1.12 to 1.94, P=0.0056; HR_{UNION} =1.63, 95% CI: 1.36 to 1.95, $P=7.91\times10^{-08}$]. We detected an involvement of B7-H3 in the tumor immune microenvironment (TIME). Meanwhile, *B7-H3* was significantly and weakly correlated with 6 out of 27 well-recognized immune checkpoint genes. Even after adjusting for effects of other immune checkpoint genes, *B7-H3* still exhibited a harmful effect on colon cancer survival using samples from TCGA and GEO cohorts (HR =1.47, 95% CI: 1.07 to 2.02, P=0.0184), indicating that it was an independent prognostic factor of colon cancer. We also proposed an immune checkpoint prognostic risk score which possessed the capability to identify colon cancers with high risk of mortality.

Conclusions: The expression of *B7-H3* is a significant, robust, and independent prognostic factor to colon cancer OS.

Keywords: *B7-H3*; gene expression; colon cancer; overall survival; prognostic factor

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Introduction

Colon cancer is a common malignant tumor of the digestive system, which is the third and second most commonly diagnosed cancer in men and women worldwide, respectively, with 1.15 million new cases and 576,858 deaths in 2020 (1). Even though the therapeutic effect of colon cancer has greatly improved with the development of medical technology, the prognosis of colon cancer is still poor (2). Immunotherapy, which boosts the immune system works to find and attack cancer cells, envisions a novel approach to cancer therapy (3). The immune system consists of a complex set of molecular interactions that are regulated by immune checkpoints and are named differentiated clusters based on their order of discovery (4,5). Immune checkpoint pathways are often activated to inhibit nascent anti-tumor immune responses to tumor cells, and immune checkpoint therapy enhances the immune activity against tumors by blocking or stimulating these pathways (6,7), including the most widely studied and well-recognized pathways of cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), programmed cell death receptor-1 (PD-1), and programmed cell death ligand 1 (PD-L1). Inhibitory immune checkpoints (e.g., PD-L1, PD-1, CTLA-4, and B7-H3) bind with their associated ligands to induce an inhibitory response and prevent the cascade of stimulatory immune checkpoint signals that activate T cell activity (8,9). Therefore, those immune checkpoints are consequentially associated with tumor prognosis and response to treatment.

Nevertheless, the association between *B7-H3* (B7 Homolog 3) and colon cancer survival still remains unclear. Also known as CD276, *B7-H3* is a costimulatory molecule belonging to the B7-CD28 family. It is a membrane protein encoded on chromosome 15 (10,11), and is widely expressed in heart, thymus, prostate, testis, uterus, placenta, spleen, liver, pancreas, small intestine, and colon in both normal and tumor tissues (12-14). An array of studies have indicated that highly expressed *B7-H3* promotes tumor progression and metastasis, as well as being associated with poor prognosis in various of cancers, including glioma (15), hepatocellular carcinoma (16), lung cancer (17), breast cancer (18), osteosarcoma (19), cutaneous melanoma (20), and pancreatic cancer (21). However, the results of existing prognostic studies of *B7-H3* expression and colon cancer (22-37) have been inconsistent and have presented conflicting conclusions; thus, we believe there is an urgent need to elaborate the effect of *B7-H3* on colon cancer survival. Besides, various technical bottlenecks of previous studies need to be addressed: (I) there have been very few European studies (22,23,29), and majority of these studies were conducted among Asian populations (24-28,30-37); (II) no study so far has focused on the non-linear effects of *B7-H3* on colon cancer survival, with previous studies simply having tested its linear effect; (III) there has been no independent validation of *B7-H3*, the association has consistently been evaluated in a single population; and (IV) there have been no trans-ethnic population studies of *B7-H3* thus far.

Hence, we performed a 3-phase designed and trans-ethnic study to test and confirm the prognostic effect of *B7-H3* expression on colon cancer survival using 3 independent cohorts comprising European and Asian populations, followed by a series of Kaplan-Meier (K-M) analysis, bioinformatics analysis of tumor immune microenvironment (TIME), and immune checkpoint prognostic prediction analysis, along with sensitivity analysis. We present the following article in accordance with the REMARK reporting checklist (available at https://dx.doi.org/10.21037/jgo-21-821) (38).

Methods

Study populations

We utilized European colon cancer patients from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) cohorts, as well as Asian colon cancer patients recruited from Fujian Medical University Union Hospital (UNION) to evaluate the association between gene expression level of *B7-H3* and overall survival (OS) of colon cancer (*Table 1*).

TCGA

Gene expression profiles (platform: Illumina HiSeq 2000 RNA Sequencing; San Diego, CA, USA) and clinical data of colon cancer patients were obtained from TCGA (https://portal.gdc.cancer.gov/) database in July 2021, including 471 tumor tissues and 41 adjacent-normal tissues. A total of 433 participants (95 deceased and 338 alive) with complete clinical and OS time were reserved for subsequent association analysis. The gene expression level was measured by fragments per kilobase of transcript per million fragments (FPKM) value and log2 transformed before analysis. Unqualified probes were excluded if they meet any of the quality control (QC) criteria: (I) high missing rates (>30%); or (II) coefficient of variance (CV)

Table 1 Demographic and clinical descriptions of colon cancer patients in TCGA, GEO, and UNION cohorts

Variable	TCGA (n=433)	GEO (n=811)	UNION (n=179)
Age (years), mean ± SD	66.33±12.83	66.28±13.31	55.87±15.22
Age group, n (%)			
<65 years	169 (39.03)	325 (40.07)	126 (70.39)
≥65 years	264 (60.97)	485 (59.80)	53 (29.61)
Unknown	18	1	0
Gender, n (%)			
Female	200 (46.19)	370 (45.62)	89 (49.72)
Male	233 (53.81)	441 (54.38)	90 (50.28)
Height (cm), mean ± SD	168.52±12.32	–	163.09±7.82
Weight (kg), mean ± SD	81.34±20.72	–	59.63±10.57
T stage, n (%)			
T0/Tis	1 (0.23)	4 (0.69)	0 (0.00)
T1	11 (2.54)	12 (2.07)	0 (0.00)
T2	75 (17.32)	48 (8.29)	0 (0.00)
T3	296 (68.36)	376 (64.94)	34 (18.99)
T4	50 (11.55)	119 (20.55)	145 (81.01)
Unknown	0	252	0
N stage, n (%)			
N0	254 (58.66)	311 (53.71)	32 (17.88)
N1	102 (23.56)	136 (23.49)	50 (27.93)
N2	77 (17.78)	100 (17.27)	97 (54.19)
N3	0 (0.00)	6 (1.04)	0 (0.00)
NX	0 (0.00)	6 (1.04)	0 (0.00)
Unknown	0	252	0
M stage, n (%)			
M0	320 (73.90)	496 (85.66)	0 (0.00)
M1	61 (14.09)	61 (10.54)	179 (100.00)
MX	45 (10.39)	2 (0.35)	0 (0.00)
Unknown	7	252	0
Clinical stage, n (%)			
I	73 (16.86)	65 (8.01)	0 (0.00)
II	165 (38.11)	341 (42.05)	0 (0.00)
III	123 (28.41)	285 (35.14)	0 (0.00)
IV	61 (14.09)	116 (14.30)	179 (100.00)
Unknown	11	4	0

Table 1 (*continued*)

Table 1 (*continued*)

Variable	TCGA (n=433)	GEO (n=811)	UNION (n=179)
Adenocarcinoma, n (%)			
Yes	367 (84.76)	579 (100.00)	135 (75.42)
No	66 (15.24)	0 (0.00)	44 (24.58)
Unknown	0	232	0
Tumor location, n (%)			
Left	135 (31.18)	228 (39.38)	111 (62.01)
Right	204 (47.11)	351 (60.62)	68 (37.99)
Unknown	94	232	0
Complete tumor excision, n (%)			
Yes	53 (12.24)	–	57 (31.84)
No	13 (3.00)	–	122 (68.16)
Unknown	367	–	0
Carcinoma cell embolus, n (%)			
Negative	226 (52.19)	–	84 (46.93)
Positive	164 (37.88)	–	95 (53.07)
Unknown	43	811	0
Tumor size, n (%)			
<5 cm	–	–	66 (36.87)
≥5 cm	–	–	113 (63.13)
Unknown	433	811	0
Postoperative therapy, n (%)			
Yes	145 (33.48)	240 (41.45)	130 (72.63)
No	235 (54.27)	323 (55.79)	48 (26.82)
Unknown	53	248	1
CEA (median, Q_1–Q_3)	3.00 (1.80–7.52)	–	8.00 (2.70–33.4)
CEA group, n (%)			
Normal	178 (41.11)	–	73 (40.78)
Elevated	97 (22.40)	–	106 (59.22)
Unknown	158	811	0
CA199, n (%)			136.37±277.48
Normal	–	–	106 (59.22)
Elevated	–	–	73 (40.78)
Unknown	433	811	0

Table 1 (*continued*)

Table 1 (*continued*)

Variable	TCGA (n=433)	GEO (n=811)	UNION (n=179)
Median survival months			
Median (95% CI)	93 (66–NA)	135 (106–NA)	16 (11–20)
Deaths, n (%)	95 (21.94)	287 (35.39)	137 (76.54)

NA, not available; T, tumor; N, node; M, metastasis; CEA, carcinoembryonic antigen; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; UNION, Fujian Medical University Union Hospital.

<5% (Figure S1). The data used in this study comply with the requirements of TCGA official published data and are publicly available.

GEO

A total of 811 colon cancer patients with complete clinical and OS information, as well as gene expression data, were acquired from the GEO (https://www.ncbi.nlm.nih.gov/geo/) database, including GSE39582, GSE17536, and GSE17537. Gene expression data was profiled by GPL570 Affymetrix Human Genome U133 Plus 2.0 Array platform (Affymetrix, Santa Clara, CA, USA). The raw intensity values were background corrected, log2 transformed, and then quantile normalized. Next, the expression values were derived by robust multi-array average (RMA) method, an algorithm exclusively designed to create an expression matrix from Affymetrix platform. Redundant probes were collapsed, and then annotated to human gene symbols prior to analysis. We applied the same QC criteria for the GEO data.

UNION

Colon cancer patients in the UNION cohort were recruited from January 2010 to January 2018. Patients were admitted for surgical treatment of colorectal cancer. Routine preoperative examinations were performed to exclude patients with organic lesions of the heart, lung, liver, and others. Additionally, patients (I) who did not receive surgery but only a simple ileostomy; (II) who had intestinal obstruction, intestinal perforation, intestinal bleeding, or required emergency surgical resection; (III) who had simultaneous polygenic carcinoma of the colon, heterogeneous polygenic carcinoma of the colon, and familial adenomatous polyposis; (IV) who presented other malignant tumors or died due to postoperative complications, were further excluded. Finally, 179 patients were included in the study. The expression level of *B7-H3* in tumor cells was quantified by immunohistochemistry (IHC). Briefly, Slides (4-μm thick consecutive paraffin sections) from the blocks with the highest tumor content for each sample were used for IHC staining, immersed in xylene, rehydrated through graded concentrations of ethanol followed by phosphate-buffered saline (PBS) buffer, and deionized water for 5 min each. Slides were then heated to 100 ℃ for 20 min in a pH 9 Tris-based solution. All slides were incubated with the primary antibodies for 60 min at 37 ℃ for 1 h (dilutions: Human B7-H3 Antibody 1:300, R&D Systems, Minneapolis, MN, USA) and were then washed. A secondary antibody followed by incubation with rabbit anti-goat IgG and an avidin-biotin complex (Boster, Wuhan, China) was added for 30 min and the slides were again washed. The sections were processed with the universal SP Elivision-plus kit (Maixin Bio, Fuzhou, China). Finally, the sections were counterstained with hematoxylin (39). The percentages of *B7-H3* in 3 representative high-power fields of individual samples were analyzed for intensity of *B7-H3* membranous expression and were scored as 0 (<5% of *B7-H3*), 1 (5–25%), 2 (>25–50%), or 3 (>50%). Individual samples were evaluated by at least 2 pathologists in a blinded manner, and those samples with inconsistent scores were further discussed and decided. A score of 0 or 1 with *B7-H3* on IHC was regarded as low *B7-H3* expression group, and 2 or 3 staining as high *B7-H3* expression group. This study was approved by the institutional review board of Fujian Medical University Union Hospital (No. 2018YF024-01). All participants or their surrogates provided their written informed consent. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

Statistical analysis

Three-phase study of B7-H3 expression and colon cancer OS

The analysis workflow is presented in *Figure 1*. We adopted a 3-phase trans-ethnic study to evaluate the association

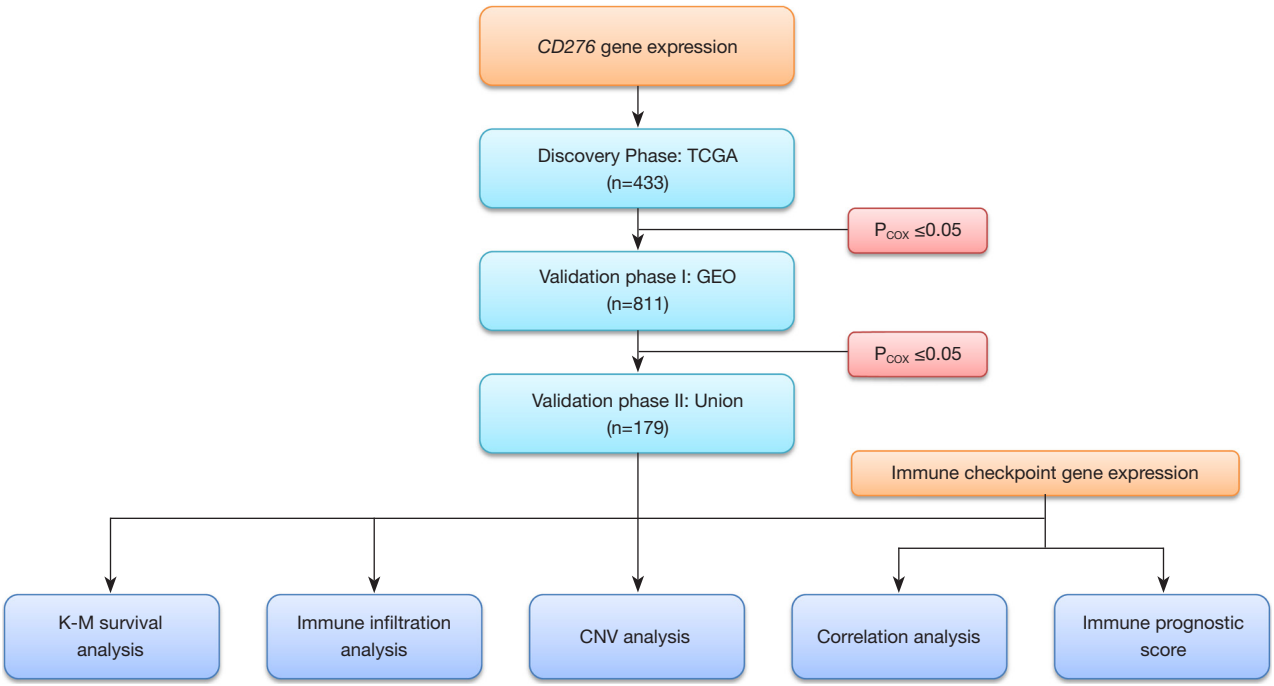

Figure 1 Flow chart of study design and statistical analysis.

between expression of *B7-H3* and colon cancer OS. In the discovery phase, we used a restricted cubic spline (RCS) regression model by setting the median expression of *B7-H3* as a reference point to investigate the potential non-linear effect of *B7-H3* on OS using a European population from the TCGA cohort. The RCS are a smoothly joined sum of polynomial functions that do not assume linearity of the relationship between variable and the response (40). This technique provides great flexibility for fitting data and modeling complex relationships between survival outcome and the variable of interest, while adjusting for other covariates. Furthermore, an RCS regression model can avoid arbitrary categorization of the continuous measures and permit identification of the risk function inflexion point. Then, the *B7-H3* expression was stratified by knot information into different groups (41). Finally, the association between *B7-H3* and survival was tested using a Cox proportional hazards model adjusted for covariates. These covariates were significant variables screened out by step forward regression model with P value of entry ≤0.05 and P value of remove >0.05, among a series of demographic and clinical variables. As a result, age, gender, and clinical stage were adjusted in the Cox proportional hazards model (Tables S1,S2). Proportional hazard assumption was confirmed based on Schoen's method (42). The association strength was measured by hazard ratio (HR) and 95% confidence interval (CI).

In the validation phase I, we used the RCS regression model again to evaluate the non-linear effect of *B7-H3* on colon cancer survival using a European population from the GEO cohort. The Cox proportional hazards model was utilized to test the association between *B7-H3* and survival, adjusted for the same covariates aforementioned in the discovery phase.

In the validation phase II, we directly tested the grade level of *B7-H3* using Cox proportional hazards regression model using an Asian population from the UNION cohort. Since all participants were in M1 stage, the model was adjusted for age, gender, tumor (T) stage, and node (N) stage. Finally, meta-analyses using both fixed-effects and random-effects models were applied in our study to combine results from 3 phases by R package *meta* (https://cran.r-project.org/web/packages/meta/index.html).

Supplementary analyses of B7-H3

Furthermore, we performed a series of supplementary analyses. First, K-M survival curves were drawn to represent the survival difference between patients with

different expression levels (43). The cutoff values of *B7-H3* expression were defined based on using R package *survMisc* (https://cran.r-project.org/web/packages/survMisc/index.html), which used log-rank test to find the optimal cut-off value, in TCGA and GEO cohorts, respectively. According to these cutoff values, these participants were divided into a high and low expression group, their survival differences were further compared using Cox proportional hazards regression model adjusted for covariates.

Second, bioinformatics analysis of *B7-H3* including immune infiltration analysis, somatic copy number alteration (SCNA) analysis, and correlation analysis were performed using TIMER2.0, which is a freely available web server to the research community (44). The TIMER2.0 resource uses RNA-seq expression profiles to detect and quantify the situation of immune cell infiltration in tumor tissues, so as to determine the relationship between tumor and immune cells in TCGA colon cancer database (45). (I) In immune infiltration analysis, the scatterplots were displayed, showing the association between *B7-H3* expression and immune infiltration level in colon cancer tumors using Spearman correlation analysis. (II) In SCNA analysis, SCNAs were categorized into different groups, including deep deletion (–2), arm-level deletion (–1), diploid/normal (0), arm-level gain (+1), and high amplification (+2). Box plots were presented to show the distributions of each immune subset (B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells) at each copy number status in colon cancer. The infiltration level for each category was compared with the normal using a 2-sided Wilcoxon rank-sum test. (III) In correlation analysis, we further evaluated the association between *B7-H3* and 27 well-recognized immune checkpoint genes (46).

Finally, we proposed an immune checkpoint prognostic score of colon cancer using overall participants from TCGA and GEO cohorts to distinguish patients at high risk of mortality. These well-recognized immune checkpoint genes associated with colon cancer survival were screened out by forward stepwise regression model adjusted for age, gender, clinical stage, study site and *B7-H3*, with P value of entry ≤0.05 and P value of remove >0.05. Patients were then categorized into high and low risk groups by median value, and K-M survival curves between 2 groups were tested. Sensitivity analyses were also performed stratified by age, gender, and clinical stage. The time-dependent receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) were used to measure the prediction ability (47). Statistical analyses were performed using R version 3.4.4 (The R Foundation of Statistical Computing, Vienna, Austria).

Results

After quality control, 3 datasets were composed of with 433 participants in TCGA cohort, 811 participants in the GEO cohort, and 179 participants in the UNION cohort for further association analysis. Demographic and clinical characteristics of these participants were described as mean ± SD for continuous variables and number (%) for categorical variables in *Table 1*.

Clinical experimental studies of B7-H3 and colon cancer

First, the expression level of B7-H3 in colon cancer and adjacent normal tissues were evaluated by differential analysis of gene expression data in TCGA and GEO cohort. Compared with the adjacent normal tissue, tumor tissue had high-expressed level of *B7-H3* ($P=1.18\times10^{-21}$). Besides, there was significant association between *B7-H3* expression and clinicopathological factors (age: P=0.029; clinical stage: P=0.002), which were shown in boxplot (Figure S2).

B7-H3 was significantly and robustly associated colon cancer OS

In the discovery phase, we observed the apparent non-linear association pattern between *B7-H3* and OS time of colon cancer patients from cubic spline regression model using European population in TCGA (*Figure 2*). This suggested that the inflection points were 3.7, 4.1, and 4.6. The covariates-adjusted Cox proportional hazards regression model indicated *B7-H3* was a significant risk factor of colon cancer survival (HR_{TCGA} =4.60, 95% CI: 2.15 to 9.83, $P=8.37\times10^{-05}$). In the validation phase I, there was an approximate linear association pattern between *B7-H3* and colon cancer survival derived from cubic spline regression model using European population in GEO. By Cox model, *B7-H3* was still significantly correlated with a worse prognosis (HR_{GEO} =1.47, 95% CI: 1.12 to 1.94, P=0.0056). In validation phase II, we again confirmed that *B7-H3* was a significant prognostic factor of colon cancer survival using an Asian population in UNION (HR_{UNION} =1.63, 95% CI: 1.36 to 1.95, $P=7.91\times10^{-08}$). Finally, meta-analysis of 3 phases ensured the significant association regardless of

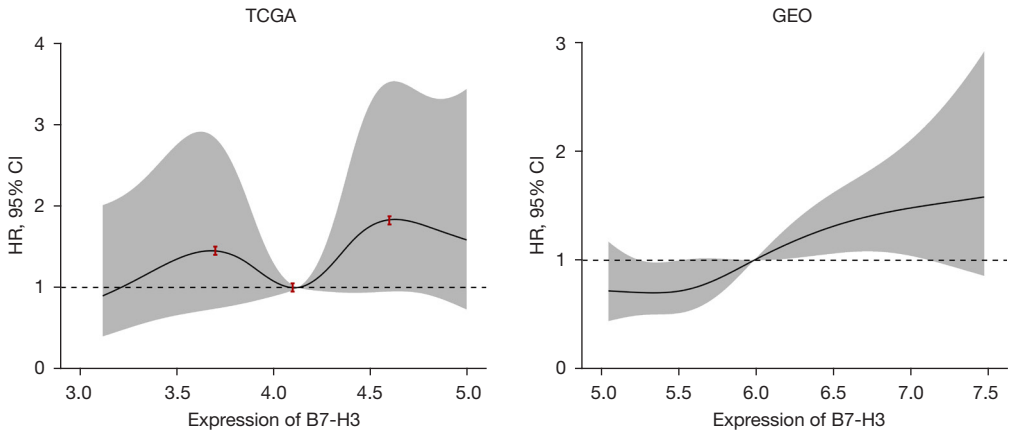

Figure 2 The apparent non-linear and approximate linear association pattern between *B7-H3* and OS time of colon cancer patients derived from cubic spline regression model. The inflection points of expression of *B7-H3* were 3.7, 4.1, and 4.6 in TCGA cohort. OS, overall survival; TCGA, The Cancer Genome Atlas.

Table 2 Association results of *B7-H3* expression and overall survival time of colon cancer patients derived from Cox proportional hazards model in TCGA, GEO, and UNION cohorts

Variable	Unadjusted model			Adjusted model		
	HR	95% CI	P value	HR	95% CI	P value
TCGA	3.240	1.514–6.933	0.0024	4.598	2.150–9.833	8.37E–05
GEO	1.332	1.016–1.746	0.0380	1.472	1.119–1.937	0.0056
UNION	1.553	1.317–1.831	1.7E–07	1.631	1.364–1.950	7.91E–08
Meta						
Fixed-effects model	1.529	1.331–1.756	1.91E–09	1.646	1.421–1.907	2.85E–11
Random-effects model	1.585	1.203–2.087	0.0011	1.846	1.281–2.661	0.0010
Test of heterogeneity			0.0919			0.0217

HR, hazard ratio; CI, confidence interval; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; UNION, Fujian Medical University Union Hospital.

a fixed-effects model (HR_{fixed} =1.65, 95% CI: 1.42 to 1.91, P=2.85×10^{-11}) or random-effects model (HR_{random} =1.85, 95% CI: 1.28 to 2.66, P=0.0010) (*Table 2*).

K-M survival analysis

The optimal cutoff value of *B7-H3* which corresponded to the highest log-rank test score was 4.317 and 5.864 in TCGA and GEO, respectively. Therefore, participants in TCGA and GEO were divided into high and low expression groups. In UNION, participants with the grade levels (0 and 1) of *B7-H3* expression were categorized into low expression group and the others were in high expression group. The survival curves were significantly separated between the 2 groups across 3 datasets (*Figure 3*), indicating that high expression of *B7-H3* had harm effect on colon cancer survival (HR_{TCGA} =1.63, 95% CI: 1.07 to 2.49, P=2.22×10^{-02}; HR_{GEO} =1.75, 95% CI: 1.26 to 2.42, P=7.62×10^{-04}; HR_{UNION} =2.36, 95% CI: 1.64 to 3.40, P=4.21×10^{-06}).

Bioinformatics analysis

Cancer cells within the TIME and neighboring tumor-associated noncancerous cells play an important role in tumor biology. The immune infiltration analysis indicated

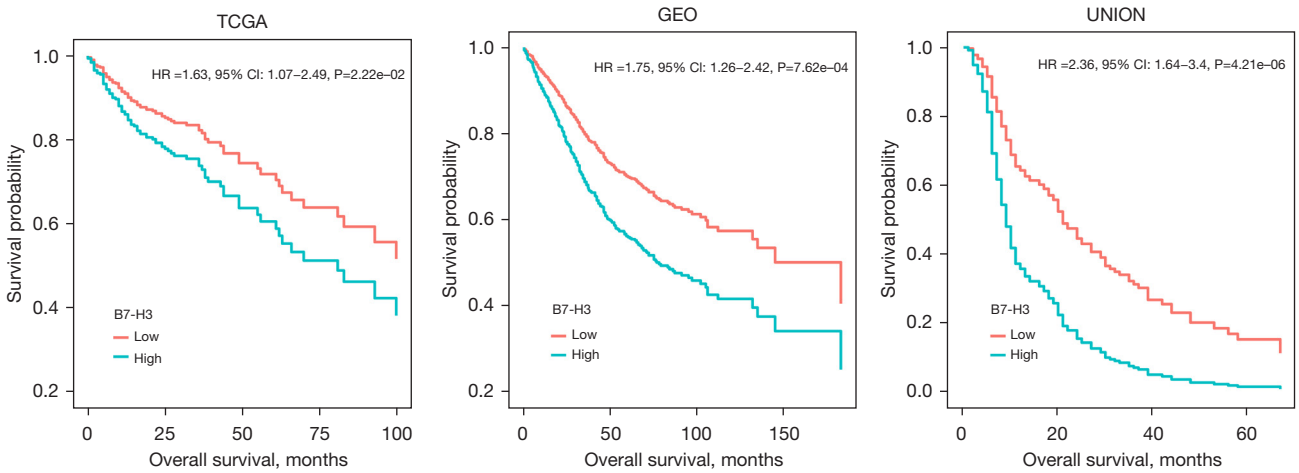

Figure 3 K-M survival curves of colon cancer patients with high and low gene expression level of *B7-H3* in TCGA, GEO, and UNION cohorts. K-M, Kaplan-Meier; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; UNION, Fujian Medical University Union Hospital.

that *B7-H3* expression had a significant negative correlation with immune infiltration level for tumor purity and B cells, but a significant positive correlation with that for $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells (*Figure 4*). Besides, the infiltration levels were significantly distributed among different groups of SCNAs of *B7-H3* (*Figure 5*), including B cell (P<0.05), $CD8^+$ T cell (P<0.001), neutrophil (P<0.01), and dendritic cell (P<0.001). In addition, *B7-H3* was significantly and weakly correlated with 6 out of 27 well-recognized immune checkpoint genes (Figure S3), including *PVRL2* (ρ=0.47, P=1.90×10^{-05}), *PVR* (ρ =0.43, P=8.31×10^{-5}), *IGSF11* (ρ=0.29, P=9.74×10^{-03}), *CEACAM1* (ρ=0.35, P=1.90×10^{-03}), *PTDSS1* (ρ=0.43, P=1.04×10^{-04}), and *HMGB1* (ρ=0.49, P=6.61×10^{-05}).

Immune checkpoint prognostic score of colon cancer

We performed stepwise regression model to screen other immune checkpoint genes using Cox model adjusted for covariates and *B7-H3* expression and identified 7 more genes associated with colon cancer survival (Table S3). In this multivariable Cox model, *B7-H3* was still significant (HR =1.47, 95% CI: 1.07 to 2.02, P=0.0184), indicating it was an independent risk factor of colon cancer prognosis. Then, we proposed an immune checkpoint prognostic score of colon cancer, which was a weighted linear combination of 8 significant genes. The weight of each gene was defined as the corresponding estimated log hazard ratio [ln(HR)] derived from the multivariable Cox model. The immune checkpoint prognostic score could distinguish patients at high risk of mortality in all subpopulations stratified by age (Figure S4), gender (Figure S5), and clinical stages (Figure S6). These 8 immune checkpoint genes slightly improved the prognostic prediction ability for 3-year survival (2.38%) and 5-year survival (1.63%), respectively. Hence, the prognostic model of colon cancer achieved an acceptable accuracy ($AUC_{3\text{-year}}$ =0.74 and $AUC_{3\text{-year}}$ =0.72) (Figure S7).

Discussion

In recent years, previous studies of B7-H3 have mainly focused on the its expression and role in different types of cancers (48). Elevated expression of *B7H3* has been found in serum of patients with malignant tumors, which is related to clinical stage and progression of cancer (49,50), since *B7-H3* appears to be correlated with different proteins that contribute to tumor migration, invasion and angiogenesis (51,52). Besides, *B7-H3* blocking in combination with chemotherapy is a promising treatment option based on preclinical animal studies (53).

Currently, some previous studies have described a significant correlation between tumor expression of *B7-H3* and prognosis (14,54), whereas, majority of them reported that high tumor *B7-H3* expression was associated with more advanced disease (31), increased risk of recurrence (35) or

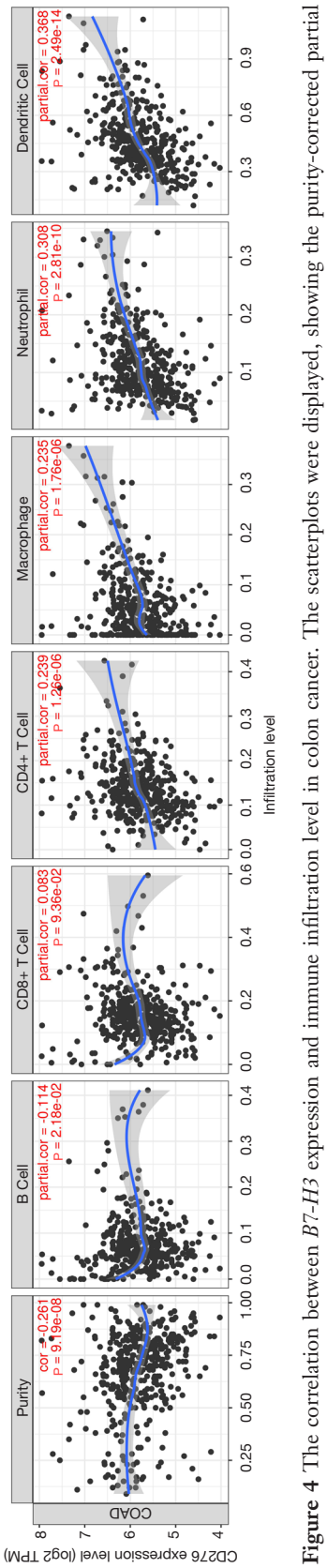

Figure 4 The correlation between *B7-H3* expression and immune infiltration level in colon cancer. The scatterplots were displayed, showing the purity-corrected partial Spearman's rho value and statistical significance.

shorter survival time (55), which was consistent with our results. Effective therapeutic strategies against colon cancer are scarce. Therefore, it is imperative to seek a promising therapeutic target for anti-tumor drugs and also important to develop a prognostic tool to identify patients at high risk of mortality who require more attention and aggressive treatment. By leveraging publicly available transcriptional data for European colon cancers in TCGA and GEO cohorts and our in-door data for Asian colon cancers in the UNION cohort, we performed a three-phase study, which perhaps was the first trans-ethnic prognostic study of *B7-H3* expression on this population level. We first tested the prognostic effect of *B7-H3* on survival in the European population, and then continuously validated its effect using another 2 independent European and Asian populations. Highly expressed *B7-H3* was a significant and independent harmful factor to colon cancer survival, even after adjusting for effects of other immune checkpoint genes, correlating with these genes, and joint involvement in TIME. We also proposed an immune checkpoint prognostic risk score which significantly stratified patients into high and low risk groups, regardless of their characteristics.

The mechanism of *B7-H3* expression affecting colon cancer survival still remains to be fully investigated. *B7-H3* expression is often induced on immune cells (56,57), which can mediate chemotherapeutic resistance and sensitivity to survival of patients (58,59). It was also shown that *B7-H3* promotes *VEGFA* expression and angiogenesis, which dependents on the NF-κB pathway in colorectal cancer (CRC), and CRC cell recruits regulatory T cells to promote chemoresistance via NF-κB signaling pathway (60). Besides, *B7-H3* is also regulated by Th1, IL-4, IFN-gamma and TNF-alpha, which is an important cancer-promoting inflammatory molecule and significantly increases the release of soluble B7-H3 in colon cancer cell lines (61,62). Meanwhile, the *B7-H3* pathway has a dual role in contributing to the regulation of innate immune responses. On the one hand, *B7-H3* co-stimulates innate immunity by augmenting proinflammatory cytokines release from lipopolysaccharide-stimulated monocytes or macrophages in both a Toll like receptor 4- and 2-dependent manner (49,50,63). On the other hand, *B7-H3* provides an additional mechanism allowing neuroblastoma cells, which protect them from natural killer cell-mediated lysis, to escape the control of immune response which may be associated with the expression of B7-H3 and multiple possibly related molecules, such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein

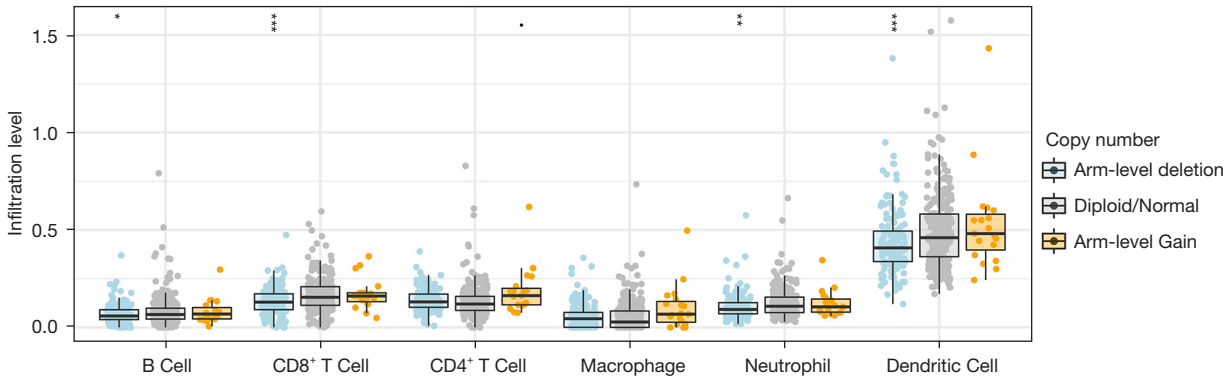

Figure 5 The comparison of tumor infiltration levels among tumors with different somatic copy number alterations of *B7-H3*. Copy number alternations were categorized into different groups, including deep deletion (–2), arm-level deletion (–1), diploid/normal (0), arm-level gain (+1), and high amplification (+2). Box plots are presented to show the distributions of each immune subset at each copy number status in COAD. The infiltration level for each category is compared with the normal using a two-sided Wilcoxon rank-sum test. •, P<0.1; *, P<0.05; **, P<0.01; ***, P<0.001. COAD, colon adenocarcinoma.

4 (CTAL-4) or the presence of different types of cells associated with immune responses (11,64). Nevertheless, the role of *B7-H3* in controlling the innate immunity is quite complex and requires more elucidation (65). Emerging evidence indicates that immune checkpoint blockade combined with radiotherapy acquires considerable success in multiple tumors (66,67). On account of its important role in immune evasion and cancer progression, *B7-H3* also possesses the capability to be a potential novel therapeutic target for colon cancer patients (68). Besides, *B7-H3* also plays a non-immunological role in promoting tumor invasion and progression (69,70). It was observed that the 3' UTR of B7-H3 transcript contains a target site for miR29 microRNA, and there is an inverse correlation between the expression of B7-H3 protein and miR29 levels, suggesting regulation of expression of this gene product by miR29. Experimental studies in mice showed that induction of colitis and water avoidance stress affect levels of Mir29a and Mir29b and intestinal permeability in wild-type mice (71). Despite inconsistent results and conflicting conclusions of existing prognostic studies of *B7-H3* (22,24-30,32-37), our study, with the largest sample size, confirmed that it was a significant and independent biomarker to colon cancer survival by several trans-ethnical validations.

Our study has several strengths. First, this was perhaps the largest prognostic study of *B7-H3* in colon cancer patients on a population level. A 3-phase design was applied to control false positives and ensure the robustness of our results. The significant association observed in the discovery phase was further replicated in the validation phases I and II. Therefore, the probability of reporting the false positive result was strictly controlled under 0.0125% (5%×5%×5%). Second, though population heterogeneity exists even among those of the same ethnicity, taking advantage of the RCS model, we successfully elucidated the nonlinear effect of *B7-H3* expression on colon cancer survival in the TCGA European population and its approximate linear effect in GEO European population. Third, by trans-ethnic validation using an Asian population, we confirmed the robust association between *B7-H3* expression and colon cancer survival again, meaning that this potential therapeutic target can be uniformly applied to all colon cancer patients worldwide. Fourth, by adjusting for other well-recognized immune checkpoint genes, *B7-H3* was still a significant biomarker to colon cancer survival, indicating it was an independent prognostic factor. Finally, we proposed an immune checkpoint prognostic risk score of colon cancer using 8 genes, which can identify patients at high risk of mortality regardless of age, gender, and clinical stages, possible providing an effective tool aiding in clinical interventions.

We also acknowledge some limitations. First, the censoring rate is high in the TCGA and GEO cohorts, which resulted in a low statistical power in time-to-event data analysis. Regardless, we still observed significant results, indicating the association between *B7-H3* expression and colon cancer survival was quite conservative. Second, several important clinical variables (e.g., status of complete tumor excision and tumor size) were not available in the TCGA and GEO cohorts, therefore a limited number of

covariates were adjusted in the Cox proportional hazards regression model. We envision a better model adjusted for more clinical variables to achieve more precise estimates of *B7-H3* effect and more accurate prognostic prediction ability in future studies. Finally, functional studies of *B7-H3* are warranted to confirm its biological effect.

Conclusions

In summary, in the largest prognostic study of *B7-H3* in colon cancer patients on population levels, we utilized a 3-phase design to confirm that *B7-H3* was a significant and independent biomarker of colon cancer prognosis. The proposed immune checkpoint prognostic risk score has the capability to identify colon cancers with high risk of mortality.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the institutional review board of Fujian Medical University Union Hospital (No. 2018YF024-01). All participants or their surrogates provided their written informed consent.

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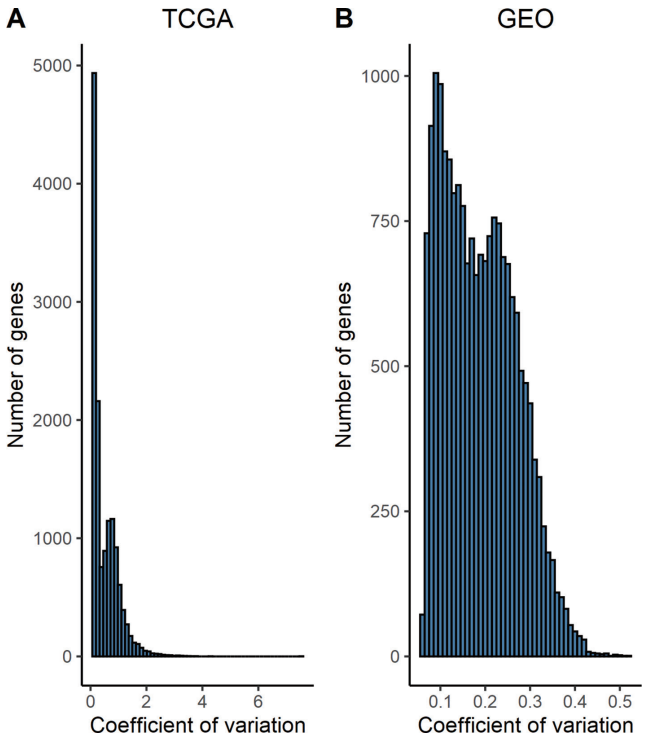

Figure S1 The distribution of coefficient of variation values of *B7-H3* expression in TCGA and GEO cohorts.

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Table S1 Association results of each variable derived from univariate Cox proportional hazards model in TCGA, GEO and UNION cohorts

Variable	TCGA			GEO			UNION		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age	1.021	1.003–1.039	0.0225	1.018	1.009–1.028	0.00018	1.006	0.994–1.018	0.3257
Age <65 years	1.462	0.944–2.263	0.0886	1.275	1.001–1.623	0.049	1.439	0.995–2.080	0.0529
Gender	1.124	0.749–1.687	0.5733	1.198	0.9479–1.515	0.130	1.072	0.764–1.505	0.0529
Height	1.001	0.977–1.026	0.9195	–	–	–	0.987	0.966–1.008	0.6864
Weight	0.986	0.971–1.001	0.0746	–	–	–	0.988	0.972–1.004	0.1376
T stage	2.588	1.732–3.867	3.44E–06	–	–	–	1.392	0.900–2.152	0.0125
N stage	2.023	1.592–2.571	8.014E–09	–	–	–	1.327	1.063–1.658	0.3257
M stage	4.753	3.005–7.519	2.70E–11	–	–	–	–	–	–
Clinical stage	2.201	1.728–2.804	1.67e–10	2.274	1.948–2.655	<2E–16	–	–	–
Adenocarcinoma	0.752	0.445–1.272	0.2879	–	–	–	0.927	0.633–1.358	0.6966
Tumor location	0.832	0.529–1.309	0.4263	–	–	–	1.078	0.759–1.532	0.6754
Complete tumor excision	1.229	0.339–4.462	0.7538	–	–	–	1.471	1.046–2.067	0.0265
Carcinoma cell embolus	2.133	1.387–3.281	5.62E–04	–	–	–	1.331	0.929–1.906	0.1189
Tumor size	–	–	–	–	–	–	1.471	1.046–2.067	0.0265
Postoperative therapy	1.16	0.722–1.863	0.5398	–	–	–	0.414	0.283–0.604	4.83E–06
CEA	3.146	1.770–5.591	9.34E–05	–	–	–	0.994	0.708–1.396	0.9744
CA199	–	–	–	–	–	–	1.071	0.761–1.505	0.6949

T, tumor; N, node; M, metastasis; CEA, carcinoembryonic antigen; CA199, carbohydrate antigen 199; HR, hazard ratio; CI, confidence interval; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; UNION, Fujian Medical University Union Hospital.

Table S2 Association results of covariates derived from multivariate Cox proportional hazards model in TCGA, GEO and UNION cohorts

Variable	TCGA			GEO			UNION		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age	1.033	1.014–1.052	6.38E–04	1.027	1.016–1.039	1.37E–06	1.004	0.992–1.016	0.4969
Gender	0.964	0.631–1.473	0.8663	1.378	1.080–1.757	0.0098	0.987	0.699–1.393	0.9412
T stage	–	–	–	–	–	–	1.214	0.772–1.907	0.4012
N stage	–	–	–	–	–	–	1.286	1.018–1.626	0.0351
Clinical stage	2.386	1.866–3.052	4.34E–12	2.455	2.068–2.916	<2E–16	–	–	–

Clinical stage which composed of T stage, N stage and M stage was included in the model in GEO and TCGA cohort, respectively. T stage and M stage were included in UNION cohort, since all subjects were in M1 stage. Significant variables were screened out by step forward regression model with P value of entry ≤0.05 and P value of remove >0.05 in TCGA, GEO and UNION, respectively. Covariates were defined as significant variables in any one of cohorts, along with two demographic variables (age and gender) common adjusted in COAD prognostic study. T, tumor; N, node; HR, hazard ratio; CI, confidence interval; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; UNION, Fujian Medical University Union Hospital.

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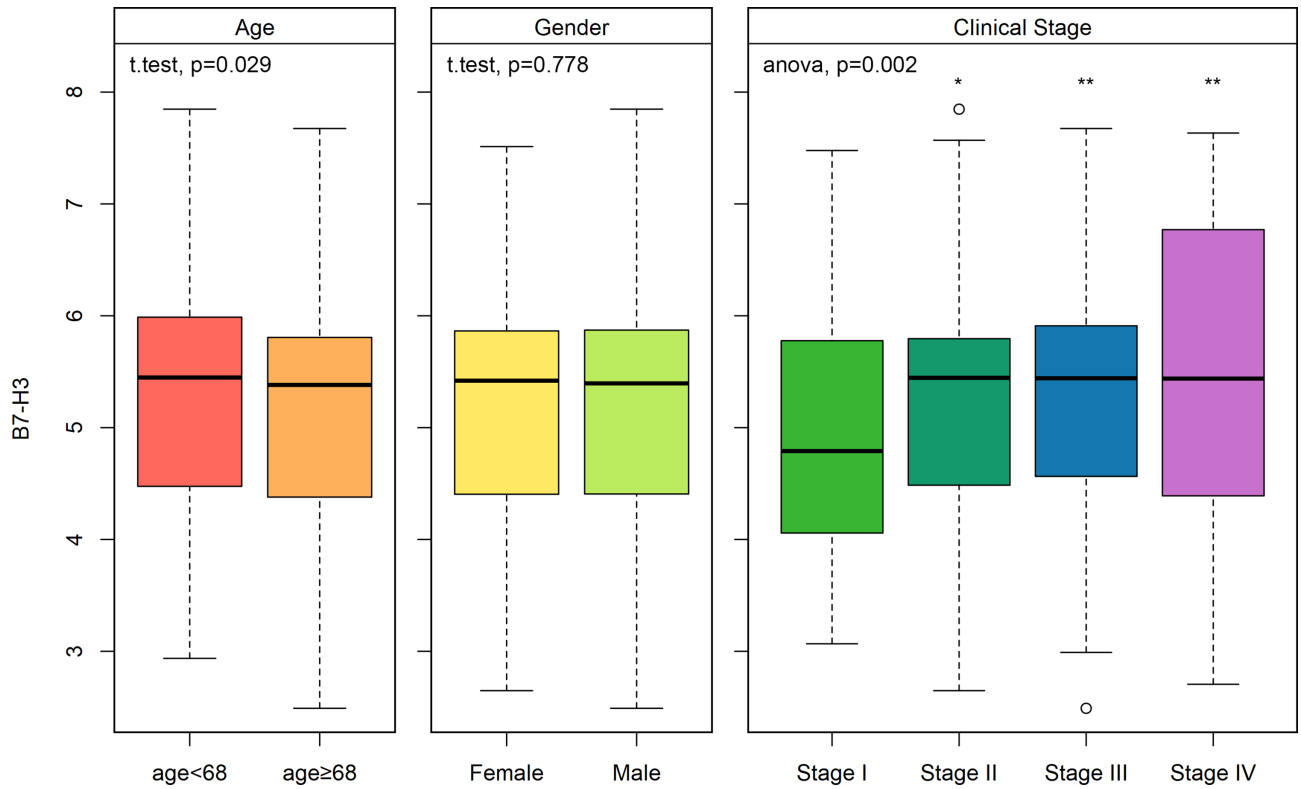

Figure S2 Boxplot of B7-H3 versus clinicopathological factors using all subjects from TCGA and GEO cohorts. The clinical stage for each category was compared with the stage I using a two-sided t test. *, P<0.05; **, P<0.01.

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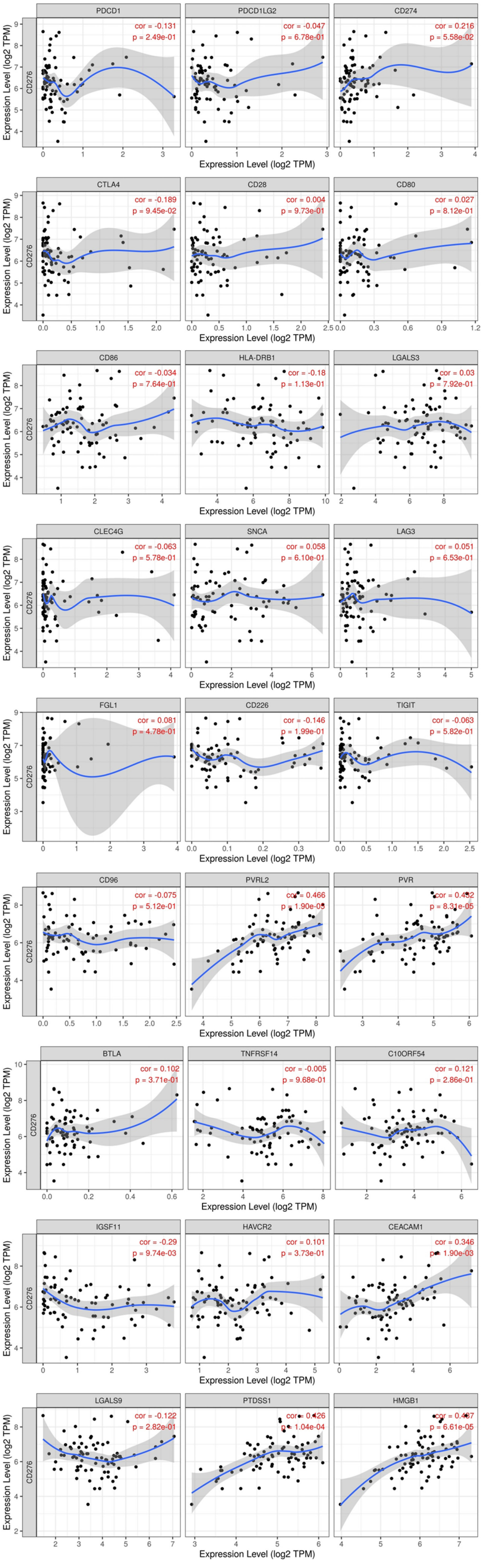

Figure S3 The correlation of between *B7-H3* expression and immune checkpoint genes in colon cancer patients.

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Table S3 The multivariate Cox proportional hazards model using demographic, clinical characteristics and immune checkpoint genes

Variable	TCGA and GEO		
	HR	95% CI	P
Age	1.028	1.018–1.038	3.03E−08
Gender	1.302	1.031–1.642	0.0264
Clinical stage	2.187	1.880–2.543	3.05E−24
Study site	0.874	0.685–1.115	0.2779
B7-H3	1.469	1.067–2.022	0.0184
CTLA4	0.416	0.255–0.681	4.79E−04
LAG3	1.464	1.128–1.900	0.0042
CD80	1.762	1.080–2.875	0.0233
LGALS9	0.725	0.611–0.861	2.53E−04
HMGB1	0.795	0.64–0.988	0.0383
HLA-DOA	1.512	1.110–2.061	0.0088
HLA-DPB1	0.768	0.612–0.964	0.0226

All immune checkpoint genes were screened out by back forward stepwise regression model adjusted for age, gender, clinical stage, study site and B7-H3, with P value of entry ≤0.05 and P value of remove >0.05. HR, hazard ratio; CI, confidence interval; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; UNION, Fujian Medical University Union Hospital.

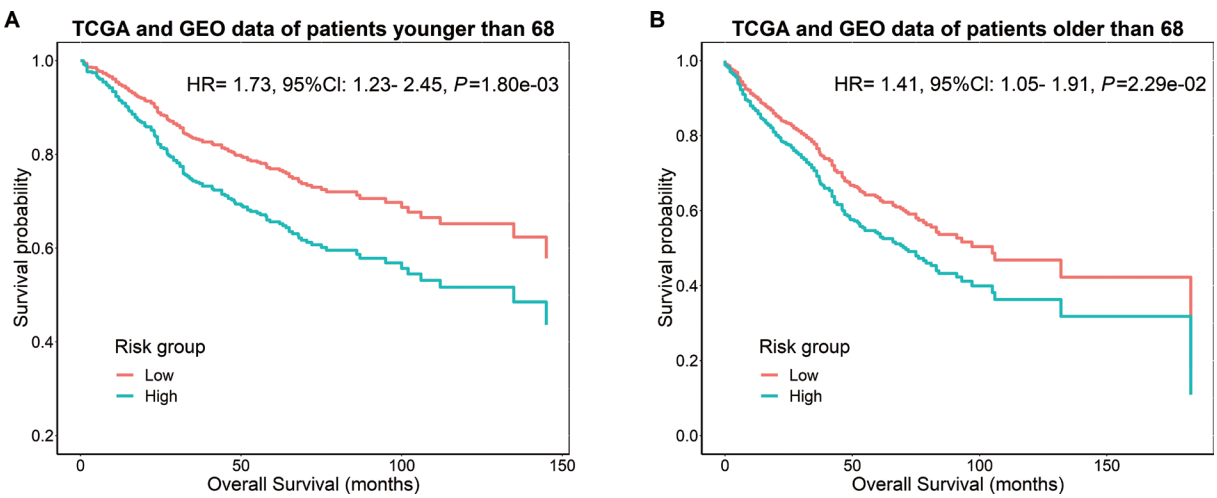

Figure S4 Kaplan-Meier survival curves for patients with high and low level of immune checkpoint prognostic risk score stratified by age using all subjects from TCGA and GEO cohorts.

https://dx.doi.org/10.21037/jgo-21-821

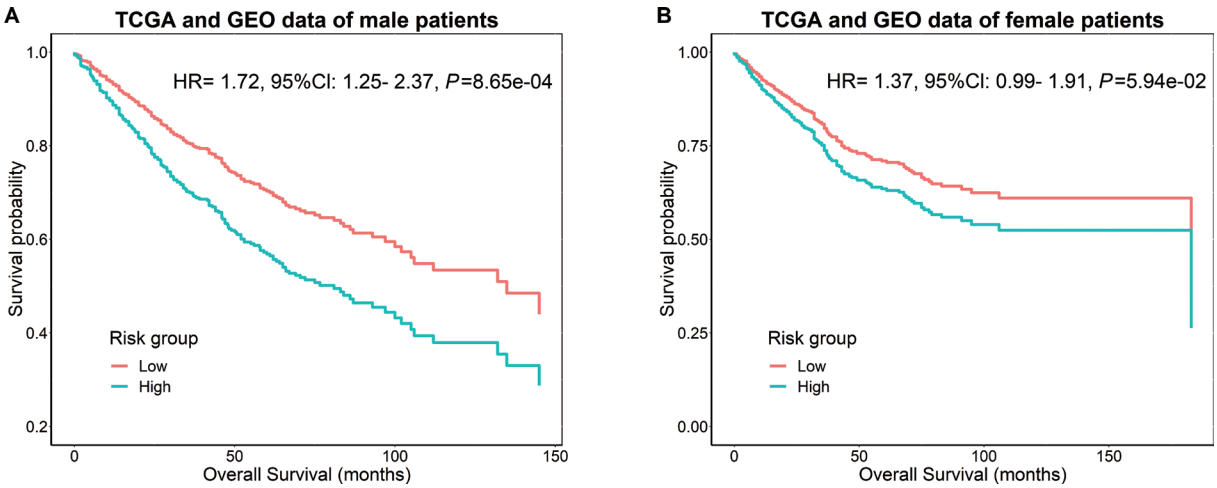

Figure S5 Kaplan-Meier survival curves for patients with high and low level of immune checkpoint prognostic risk score stratified by gender using all subjects from TCGA and GEO cohorts.

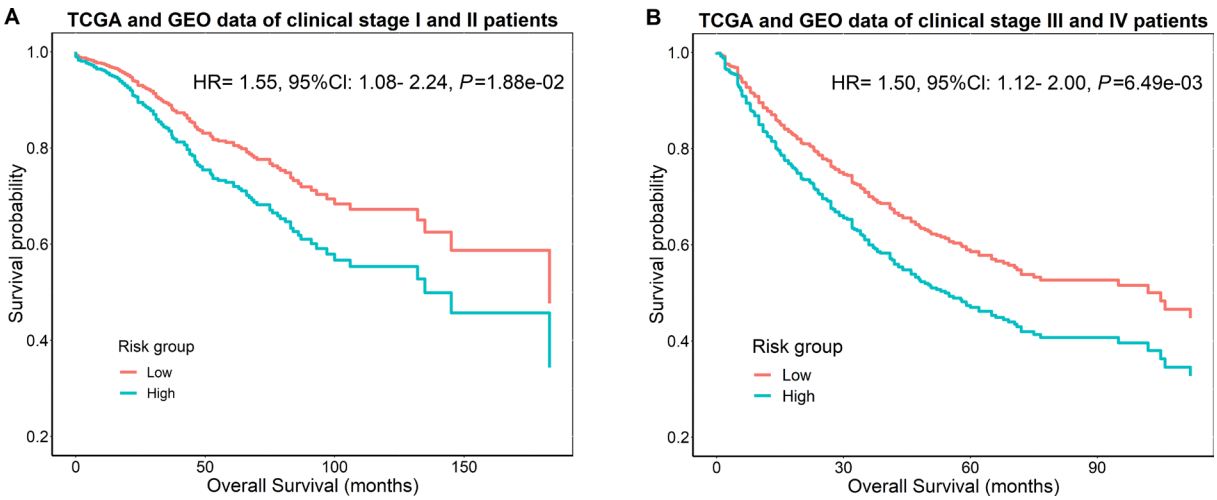

Figure S6 Kaplan-Meier survival curves for patients with high and low level of immune checkpoint prognostic risk score stratified by clinical stage using all subjects from TCGA and GEO cohorts.

https://dx.doi.org/10.21037/jgo-21-821

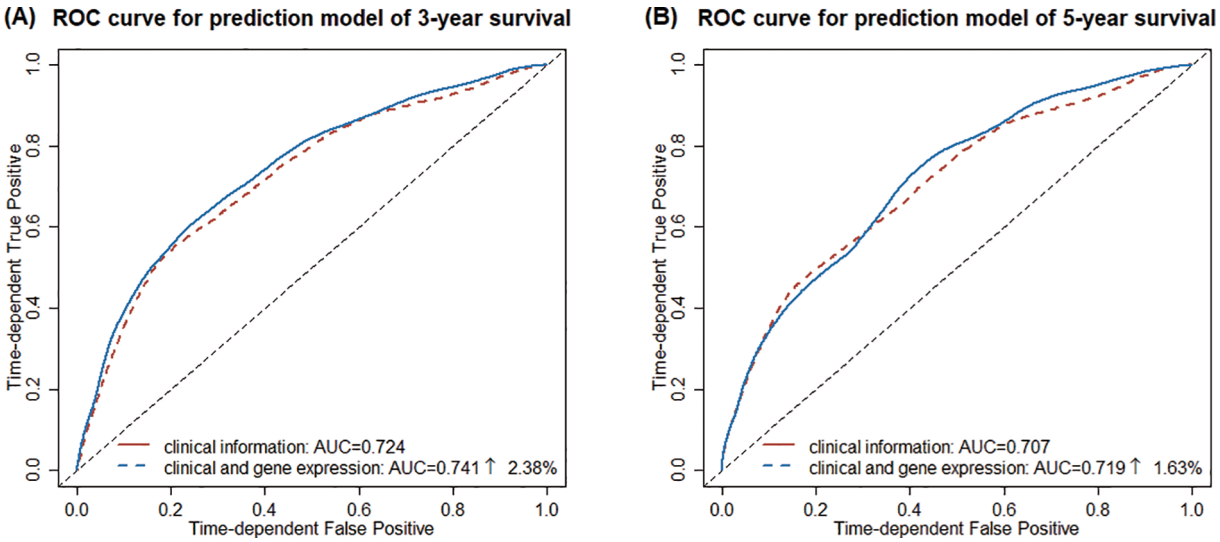

Figure S7 The time-dependent ROC of prognostic prediction model of 3- and 5-year overall survival, respectively.

https://dx.doi.org/10.21037/jgo-21-821