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ABOUT COVER

Editorial Board Member of *World Journal of Gastrointestinal Oncology*, Tomohide Hori, PhD, MD, FACS, is an Attending Surgeon at the Affiliated Hospital with Kyoto University (Kyoto, Japan). Dr. Hori obtained his medical doctor license from Tokyo Medical University (Japan) in 1997 and his Doctor of Philosophy degree from Mie University Graduate School of Medicine (Japan) in 2007. Dr. Hori is certified and qualified by many international and domestic societies, including the American College of Surgeons and Japan Society of Endoscopic Surgery. From 2009 to 2011, he worked at Mayo Clinic (Jacksonville, FL, United States). Dr. Hori's research career has spanned the fields of gastroenterology, hepatology, transplant immunology, and gastrointestinal, colorectal, hepatobiliary, pancreatic, transplant and pediatric surgeries, yielding more than 200 medical publications in international journals. (L-Editor: Filipodia)

AIMS AND SCOPE

The primary aim of *World Journal of Gastrointestinal Oncology* (*WJGO*, *World J Gastrointest Oncol*) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

   WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc.*

INDEXING/ABSTRACTING

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RESPONSIBLE EDITORS FOR THIS ISSUE

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Basic Study

Pathological significance of abnormal recepteur d'origine nantais and programmed death ligand 1 expression in colorectal cancer

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Abstract

BACKGROUND

Programmed death ligand 1 (PD-L1) immunotherapy remains poorly efficacious in colorectal cancer (CRC). The recepteur d'origine nantais (RON) receptor tyrosine kinase plays an important role in regulating tumor immunity.

AIM

To identify the patterns of RON and PD-L1 expression and explore their clinical significance in CRC.

Conflict-of-interest statement: The authors confirm that there are no known conflicts of interest associated with this publication. The manuscript has been read and approved by all authors and there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of authors.

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METHODS

Gene expression data from the Gene Expression Omnibus database (GEO; *n* = 290) and patients at the First Affiliated Hospital, Zhejiang University School of Medicine (FAHZUSM; *n* = 381) were analyzed to determine the prognostic value of RON and PD-L1 expression within the tumor microenvironment of CRC. HT29 cell line was treated with BMS-777607 to explore the relationship between RON activity and PD-L1 expression. Signaling pathways and protein expression perturbed by RON inhibition were evaluated by cellular immunofluorescence and Western blot.

RESULTS

In the GEO patient cohort, cut-off values for RON and PD-L1 expression were determined to be 7.70 and 4.3, respectively. Stratification of patients based on these cutoffs demonstrated that high expression of RON and PD-L1 was associated with a poor prognosis. In the FAHZUSM cohort, rates of high expression of RON in tumor cells, high PD-L1 expression in tumor cells and tumor infiltrating monocytes, and both high RON and high PD-L1 expression in the tumor microenvironment were 121 (32%), 43 (11%), 91 (24%), and 51 (13.4%), respectively. High expression of RON was significantly correlated with high expression of PD-L1 in the tumor cell compartment ($P < 0.001$). High expression of RON and that of PD-L1 were independent prognostic factors for poorer overall survival. Concurrent high expression of both RON and PD-L1 in the tumor microenvironment was significantly associated with a poor prognosis. *In vitro*, BMS-777607 inhibited the phosphorylation of RON, inhibited PD-L1 expression, and attenuated activation of the ERK1/2 and AKT signaling pathways in CRC cells.

CONCLUSION

RON, PD-L1, and their crosstalk are significant in predicting the prognostic value of CRC. Moreover, phosphorylation of RON upregulates PD-L1 expression, which provides a novel approach to immunotherapy in CRC.

Key Words: Colorectal cancer; Programmed death ligand 1; Prognosis; Recepteur d'origine nantais; Tumor infiltrating mononuclear cells; Tumor microenvironment

Core Tip: Recepteur d'origine nantais (RON) expression plays an important role in regulating tumor immunity, and the efficacy of programmed death ligand 1 (PD-L1) immunotherapy in colorectal cancer (CRC) is still not satisfactory. This inspired us to study the clinical significance and correlation of RON and PD-L1 in CRC. The major discoveries and findings in this study are: (1) The abnormal expression of RON and PD-L1 was not only correlated, but was also significant biomarkers for poor prognosis of CRC. This finding was identified in 389 primary CRC clinical samples *via* multiplex immunofluorescence staining and immunohistochemistry staining; and (2) In *in vitro* experiments, activation of RON phosphorylation up-regulated the expression of PD-L1 in CRC cells.

INTRODUCTION

Colorectal cancer (CRC) is the fourth most common malignancy worldwide in 2018[1].

Despite advances in CRC treatment methods, it still has a poor prognosis, and many patients will have local recurrence and/or distant metastasis within 5 years after treatment[2].

Immunotherapy has become a promising strategy for treating a wide variety of malignancies[3]. Therapeutic antibodies that block the activity of programmed death ligand 1 (PD-L1, also known as CD274) protein are effective against many cancer types[4]. PD-L1 is a transmembrane immune checkpoint protein that can be expressed on both immune cells and cancer cells, including CRC cells[5-7]. When expressed on immune cells, PD-L1 can be regulated by a variety of inflammatory mediators and cytokines. When expressed on the surface of tumor cells (TCs), PD-L1 enables TCs to evade the immune system[8,9]. Cancer cells can induce the expression of PD-L1 through activation of oncogenic pathways, such as the RAS-ERK or PI3K-AKT pathways, to facilitate immune escape[10-12].

However, the efficacy of PD-L1 immunosuppressive agents in CRC is poor[13,14]. The overall poor response of CRC to PD-L1 blockade may be due to the activity of multiple oncogenes[15,16]. For example, KRAS mutations can up-regulate the expression of PD-L1 in CRC cells, thereby reducing the efficacy of PD-L1 immunosuppressive agents[17]. RON (macrophage-stimulating 1 receptor, MST1R), a member of the MET proto-oncogene family and a receptor for MSP, is an effector of KRAS signaling, and exhibits "KRAS addiction", a phenomenon that plays an important role in the initiation and development of tumors[18-20]. RON is overexpressed pathologically in various types of cancer, including CRC, and many small-molecule inhibitors are being developed to target RON and its associated compounds[20-22]. BMS-777607 is a highly selective small molecule inhibitor of RON (IC50 1.8nM for RON)[23]. Activation of RON leads to RON phosphorylation, which then activates different signaling pathways in cancer cells, including the RAS-ERK and PI3K-AKT pathways[24-26]. Interactions between RON and other membrane receptors further promote pathway activation, enhancing the migration and invasion of CRC cells[20,22]. In addition, overexpression of RON can damage immune cells and inhibit anti-tumor immunity[27,28]. Thus, abnormal expression of RON may affect the efficacy of PD-L1 immunotherapy in CRC.

In this study, we investigated the clinical significance of expression patterns of RON and PD-L1 in primary CRC samples, and evaluated the potential for RON and PD-L1 as prognostic biomarkers in CRC. We treated CRC cells with BMS777607 *in vitro* to explore if RON inhibition affects the expression of PD-L1 and results in changes in related signaling pathways in CRC cells. Our data suggested that RON inhibition may be a novel approach to augment immunotherapy in CRC.

MATERIALS AND METHODS

Patient demographics

This study involved a GEO (Gene Expression Omnibus) cohort and a FAHZUSM (The First Affiliated Hospital, Zhejiang University School of Medicine) cohort. In the GEO cohort, the median age of all 287 CRC patients was 70 years (range: 24-97 years). There were 158 (55.1%) male patients and 129 (44.9%) female patients. The median survival time was 58 months, and 94 patients died during follow-up. In the FAHZUSM cohort, the median age of the CRC patients undergoing tumor resection was 61 years (range: 29-94 years). In this cohort, 215 (56.4%) patients were male and 166 (43.6%) were female. The median survival time was 100 months, and 91 patients died during follow-up. The primary site of a tumor, pathological grade, TNM stage, disease stage, histological type, and treatment status of CRC patients are shown in Table 1.

Cell lines and reagents

The CRC cell line HT29 was obtained from the American Type Cell Culture (ATCC, Manassas, VA, United States). Flow cytometric analysis revealed that RON and PD-L1 were strongly expressed in HT29 cells (Supplementary Figure 1). Cells were cultured in growth medium supplemented with 10% fetal bovine serum and incubated under standard cell culture conditions at 37 °C and 5% CO_2. Rabbit anti-RON antibody (5029) and mouse anti-RON monoclonal antibodies (Zt/f2 and Zt/g4) were used as previously described[28]. Macrophage-stimulating protein (MSP) is an activation factor for RON receptor tyrosine kinase phosphorylation and catalytic activity of a number of signal transduction proteins[29]. Human mature MSP was obtained from R&D Systems (Minneapolis, MN, United States). Antibodies against phospho-tyrosine (P-Tyr-100, Cat # 9411), AKT (Cat # 4685), phospho-AKT (Cat # 4060), extracellular signaling regulated kinase (ERK) 1/2 (Cat # 4695), and phospho-ERK1/2 (p44/42) (Cat # 4376)

Table 1 Comparison of RON and PD-L1 expression and clinicopathological characteristics of patients with colorectal cancer in the FAHZUSM and Gene Expression Omnibus cohorts, *n* (%)

Characteristic	FAHZUSM cohort (n = 381)										GEO cohort (n = 287)						
	Cases	RON expression			PD-L1 expression						Cases No (%)	RON expression			PD-L1 expression		
		Low	High	[1]P value	Low	High (TIMC)	[2]P value	Low	High (TC)	[3]P value		Low	High	[1]P value	Low	High (TC)	[3]P value
No. of cases number	381	260	121		247	91		247	43		287	193	94		48	239	
Age (mean ± SD)	61.16 ± 12.419	60.40 ± 12.560	62.81 ± 11.997	0.859	60.94 ± 12.37	62.04 ± 13.080	0.771	60.94 ± 12.37	61.16 ± 12.419	0.938	68.44 ± 14.00	66.87 ± 13.18	71.65 ± 15.12	**0.006**	66.94 ± 14.33	68.74 ± 13.95	0.417
Sex																	
Man	215 (56.4)	157 (60.4)	58 (47.9)	**0.023**	143 (57.9)	54 (59.3)	0.811	143 (57.9)	18 (41.9)	0.051	158 (55.1)	111 (57.5)	47 (50)	0.230	30 (62.5)	128 (53.6)	0.256
Women	166 (43.6)	103 (39.6)	63 (52.1)		104 (42.1)	37 (40.7)		104 (42.1)	25 (58.1)		129 (44.9)	82 (42.5)	47 (50)		18 (37.5)	111 (46.4)	
Principal diagnosis																	
Rectum	229 (60.1)	158 (61.1)	71 (58.7)	0.698	153 (61.9)	47 (51.6)	0.088	153 (61.9)	29 (67.4)	0.491	165 (57.5)	111 (57.5)	54 (57.4)	0.920	34 (70.8)	131 (54.8)	**0.040**
Colon	152 (39.9)	102 (38.9)	50 (41.3)		94 (38.1)	44 (48.4)		94 (38.1)	14 (32.6)		122 (42.5)	82 (42.5)	40 (42.6)		14 (29.2)	108 (45.2)	
Pathological grade																	
Well/moderate	9 (2.4)	6 (2.3)	3 (2.5)	0.177	5 (2)	3 (3.3)	0.778	5 (2)	1 (2.3)	0.898	NA						
Poor	346 (90.8)	232 (89.2)	114 (94.2)		224 (90.7)	82 (90.1)		224 (90.7)	40 (93)								
Unknown	26 (6.8)	22 (8.5)	4 (3.3)		18 (7.1)	6 (6.3)		18 (7.3)	2 (4.7)								
T stage																	
Tis-T2	107 (28.1)	85 (32.7)	22 (18.2)	**0.001**	73 (29.6)	23 (25.3)	0.105	73 (29.6)	11 (25.6)	0.864	28 (9.7)	18 (9.3)	10 (10.6)	0.459	3 (6.3)	25 (10.5)	0.437
T3	177 (46.5)	121 (46.5)	56 (46.3)		118 (47.8) (46.8%)	37 (40.7)		118 (47.8)	22 (51.2)		208 (72.5)	144 (74.6)	64 (68.1)20		39 (81.3)	169 (70.7)	
T4	97 (25.5)	54 (20.8)	43 (35.5)		56 (22.7)	31 (34.1)		56 (22.7)	10 (23.3)		51 (17.8)	31 (16.1)	20 (21.3)		6 (12.5)	45 (18.8)	
N stage																	
N0	251 (65.9)	184 (70.8)	67 (55.4)	**0.003**	164 (66.4)	62 (68.1)	0.764	164 (66.4)	25 (58.1)	0.294	141 (49.1)	97 (50.3)	44 (46.8)	0.583	27 (56.3)	114 (44.7)	0.279
N (1-2)	91 (23.9)	76 (29.2)	54 (44.6)		83 (33.6)	29 (31.9)		83 (33.6)	12 (27.9)		146 (50.9)	96 (49.7)	50 (53.2)		21 (43.7)	125 (52.3)	
M stage																	
M0	368 (96.6)	252 (96.9)	116 (95.9) (96.3%)	0.822	241 (97.6)	85 (93.4)	0.133	241 (97.6)	42 (97.7)	1.000	268 (93.4)	183 (94.8)	85 (90.4)	0.160	47 (97.9)	221 (92.5)	0.216

M1	13 (3.4)	8 (3.1)	5 (4.1)		6 (2.4)	6 (6.6)		6 (2.4)	1 (2.3)		19 (6.6)	10 (5.2)	9 (9.6)		1 (2.1)	18 (7.5)	
Disease stage																	
0-II	246 (64.6)	180 (69.2)	66 (54.5)	**0.016**	162 (65.6)	59 (64.8)	0.897	162 (65.6)	25 (58.1)	0.346	139 (48.4)	95 (49.2)	44 (46.8)	0.701	27 (56.3)	112 (46.9)	0.235
III-IV	135 (35.4)	80 (30.8)	55 (45.5)		85 (34.4)	32 (35.2)		85 (34.4)	18 (41.9)		148 (51.6)	98 (50.8)	50 (53.2)		21 (43.8)	127 (53.1)	
Histological type																	
Adenocarcinoma	335 (87.9)	227 (87.3)	108 (89.3)	0.706	214 (86.6)	84 (92.3)	0.130	214 (86.6)	37 (86)	0.488	287 (100)	193 (100)	94 (100)		48 (100)	239 (100)	
Mucinous/SRCC	36 (9.4)	25 (9.6)	11 (9.1)		28 (11.3)	4 (4.4)		28 (11.3)	4 (9.3)		0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	
Other	10 (2.6)	8 (3.1)	2 (1.7)		5 (2.0)	3 (3.3)		5 (2.0)	2 (4.7)		0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	
Treatment																	
Yes	173 (45.4)	110 (42.3)	63 (52.1)	**0.043**	112 (45.3)	38 (41.8)	0.694	112 (45.3)	23 (32.6)	0.472	115 (40.1)	89 (46.1)	26 (27.7)	**0.003**	22 (45.8)	93 (38.9)	0.372
No	158 (41.5)	119 (45.8)	39 (32.2)		105 (42.5)	39 (42.9)		105 (42.5)	14 (53.5)		172 (59.9)	104 (53.9)	68 (72.3)		26 (54.2)	146 (61.1)	
Unknown	50 (13.1)	31 (11.9)	19 (15.7)		30 (12.1)	14 (15.4)		30 (12.1)	6 (14)		0 (0)	0 (0)					
PD-L1 (protein)																	
Low	247 (64.8)	177 (73.1)	70 (26.9)	0.976[4]							NA						
High (TIMCs)	91 (23.9)	65 (26.9)	26 (27.1)														
High (TCs)	43 (11.3)	18 (9.2)	25 (26.3)	**< 0.001**[5]													
PD-L1 (mRNA)																	
Low	NA										48 (16.7)	31 (16.1)	17 (18.1)	0.666			
High											239 (83.3)	162 (83.9)	77 (81.9)				

[1]Comparing RON high expression and RON low expression.

[2]Comparing PD-L1 low expression and PD-L1 expression in TIMCs.

[3]Comparing PD-L1 low expression and PD-L1 expression in TCs.

[4]PD-L1 low (both TCs and TIMCs) *vs* PD-L1 high (TIMCs).

[5]PD-L1 low (both TCs and TIMCs) *vs* PD-L1 high TCs. Bold entries indicate statistical significance (*P* < 0.05). GEO: The Gene Expression Omnibus; RON: Recepteur d'origine nantais; PD-L1: Programmed death ligand 1; TCs: Tumor cells; TIMCs: Tumor-infiltrating mononuclear cells; FAHZUSM: The First Affiliated Hospital, Zhejiang University School of Medicine.

were obtained from Cell Signaling Technology (Beverly, MA, United States). CK antibody was from ZSGB-BIO (Cat # ZM-0069, Beijing, China). The BMS-777607 RON inhibitor was from Medchem Express (Monmouth Junction, NJ, United States). BMS-777607 was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mmol/L and stored at -20 °C. The PD-L1 antibody (Cat # 13864) for immunohistochemistry and multiplex immunofluorescence was obtained from Cell

Signaling Technology (Beverly, MA, United States). The PD-L1 antibody (Cat # 205921) used for cell experiments was from Abcam (Burlingame, CA, United States). Opal tyramine signal amplification (TSA) staining kit was from PerkinElmer (Hopkinton, MA, United States).

The Gene Expression Omnibus database

Gene expression data for RON and PD-L1 and clinical data for CRC patients are available from the GEO database and accessed through the browser web site (https://geonames.org/)[30]. A total of 290 primary CRC tumors from patients with detailed RON and PD-L1 expression data were selected from the GEO database, based on the completeness of patient clinical data. Only patients with complete tumor characteristics, overall survival (OS), and RNA-seq information, and patients whose tumor samples were obtained prior to treatment were included. The clinicopathologic features of the patients included age, sex, tumor location, histological type, tumor-lymph node-metastasis (TNM) stage, treatment regimen, and OS.

Patients and tissue samples

The CRC specimens used for this study were obtained from 381 CRC patients who were pathologically diagnosed and treated between January 2006 and November 2009 at the FAHZUSM. Clinical data for patients included age, sex, tumor location, histological type, TNM stage, tumor differentiation, treatment pattern, and OS rate (Table 1). CRC tissues were fixed in 10% buffered formalin, embedded in paraffin, and were available for immunohistochemical (IHC) evaluation. Detailed demographic data and clinical features of the FAHZUSM cohort are provided in Table 1. This study was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine (reference number: 2017427-1).

Multiplex immunofluorescence

Multiplex immunofluorescence staining of paraffin-embedded sections was performed using Zt/f2 (5 μg/mL) as the primary antibody for RON, rabbit anti-PD-L1 antibody (1:150, 13864, CST) for PD-L1, and mouse anti-CK mAb (1:200, ZM-0069, ZSGB-BIO) for CK, and visualized using the Opal tyramine signal amplification (TSA) staining kit. First, the concentration and sequence of the RON, PD-L1, and CK antibodies were optimized. The primary antibodies/fluorescent dyes for PD-L1/Opal 530, RON/Opal 690, and CK /Opal 520 were applied to the CRC tissues in sequence. Finally, sections were stained with DAPI and mounted with an anti-fading mounting agent. PerkinElmer software version 2.1 was used to analyze multispectral images.

Immunohistochemistry

IHC staining was performed according to standard protocols. Primary antibodies were incubated overnight using a dilution of 1:150 for PD-L1 (13684, CST, United States) and 1:800 for RON. Mouse anti-RON mAb (Zt/f2) was used as previously described[31]. The primary antibody was visualized with diaminobenzidine until a brown precipitate appeared at the antigenic site using EnVision + System-HRP (Dako, Carpentaria, CA, United States). Then, sections were counterstained with hematoxylin.

Evaluation of RON and PD-L1 expression in CRC samples

The IHC-stained sections were independently scored by two pathologists who were blinded to the clinicopathological data. RON expression was determined using a semi-quantitative system. According to the percentage of positive staining area in the whole cancer area, the scoring was as follows: 0 (< 5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (> 75%). The staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), or 3 (strong). The total score was obtained by adding the positive proportion score and the staining level score. Samples were categorized as follows: Samples with a final staining score ≤ 4 were considered to be low and those with a score of > 4 were considered to be high for RON expression.

PD-L1 expression was evaluated using an anti-PD-L1 rabbit antibody (13684, CST, United States). A cutoff of 5% for the total proportion of cells that were positive for PD-L1 was used, such that samples with < 5% PD-L1 positive cells were considered to be low and samples with ≥ 5% PD-L1 positive cells were considered to be high for PD-L1 expression. This standard has been established in various types of cancer[32-34].

Phosphorylation and Western blot analysis

The result of Cell Counting Kit 8 illustrated that the half-maximal inhibitory concentration (IC50) of BMS-777607 in HT29 cells at 72 h was 2 μmol/L (

Supplementary Figure 2). HT29 cells were treated with 2 nmol/L MSP, 2 μmol/L BMS-777607, or 2 nmol/L MSP + 2 μmol/L BMS-777607 for 24 h. The culture medium was removed and the cells were washed with ice-cold phosphate buffered saline (PBS). Cells were digested with trypsin, collected, and washed with PBS to remove residual trypsin. Cells were lysed in radioimmunoprecipitation (RIPA) buffer, and 25 μg of total protein per sample was separated by 8% SDS-PAGE. Western blot was performed using antibodies against RON, phospho-RON, PD-L1, ERK1/2, phospho-ERK1/2, AKT, and phospho-AKT; GAPDH protein was evaluated as an internal control to ensure equal sample loading. Immunoreactive protein bands were visually detected using enhanced chemiluminescence reagents.

Immunofluorescence analysis

HT29 cells were cultured overnight in a 6-well plate on a cover slide. After treatment with 2 nmol/L MSP, 2 μmol/L BMS-777607, or 2 nmol/L MSP + 2 μmol/L BMS-777607 for 24 h, cells were fixed with 4% paraformaldehyde at 37 °C for 20 min, then washed with PBS five times for 3 min. Cover slides were then blocked with 5% BSA in PBS at room temperature for 30 min. After that, the cells were incubated with primary antibody against PD-L1 (205921, Abcam, United States) and mouse anti-RON mAb (Zt/f2) overnight at 4 °C. Primary antibodies were then removed by washing with PBS five times for 3 min. The cells were then incubated with a fluorescently labeled secondary antibody in the dark at room temperature for 1 h. Excess secondary antibody was removed and the cells were counterstained with DAPI. Slides were mounted with an anti-quenching agent and sealed. The fluorescently labeled cells were observed using a LEICA DMi8 microscope (Leica). Representative images were selected and photographed.

Statistical analysis

Clinical quantitative data are expressed as the mean ± standard deviation (SD) and count data are expressed as constituent ratio (%) or ratio (%). Statistical evaluation was conducted with SPSS 25.0 (IBM Corp., Armonk, NY, United States) and GraphPad Prism v.8 (La Jolla, CA, United States). X-tile 3.6.1 software (Yale University, New Haven, CT, United States) was used to determine the optimal cut-off values for RON and PD-L1 expression in the GEO cohort[35]. χ^2 tests were used to analyze relationships between clinicopathological parameters and RON and PD-L1 expression. Differences between quantitative data with a normal distribution were compared using two independent samples *t*-tests between two groups and one-way analysis of variance among three groups and SNK-q test between groups. Kaplan-Meier method and log-rank test were used for OS analysis. Univariate and multivariate Cox regression analyses were used for identifying the risk factors for OS. A *P* value < 0.05 was considered statistically significant. All confidence intervals (CIs) are stated at the 95% confidence level.

RESULTS

Expression patterns of RON and PD-L1 in the GEO cohort

In the GEO cohort, X-tile was used to find the optimal cutoff values to divide the data of RON (the upper panel) and PD-L1 (the lower panel) mRNA expression into either low or high expression by selecting the highest χ^2 value and the smallest *P* value of a standard log-rank test. The cut-off values for RON and PD-L1 mRNA in TCs were determined as 7.70 and 4.30, respectively (Figure 1A and B), and the log-rank χ^2 values for RON and PD-L1 were 4.544 and 4.078, respectively. Patients in the GEO cohort were stratified according to RON and PD-L1 status: RON ≤ 7.70 and RON > 7.70, and PD-L1 ≤ 4.30 and PD-L1 > 4.30 (Figure 1A and B). We found that the high expression of RON and PD-L1 was associated with a poor prognosis.

Expression of RON and PD-L1 in CRC tumor tissue

In CRC tissue samples, multiple immunofluorescence staining was used to show the expression patterns of RON in TCs and PD-L1 in the tumor microenvironment. We found that both RON and PD-L1 exhibited membrane-enhanced expression in TCs (Figure 2A); elevated RON expression on the tumor cell membrane was accompanied by enhanced PD-L1 expression in tumor-infiltrating mononuclear cells (TIMCs) (Figure 2B). We also observed cases where PD-L1 expression was enhanced on the tumor cell membrane (Figure 2C) or in TIMCs (Figure 2D), but RON expression was
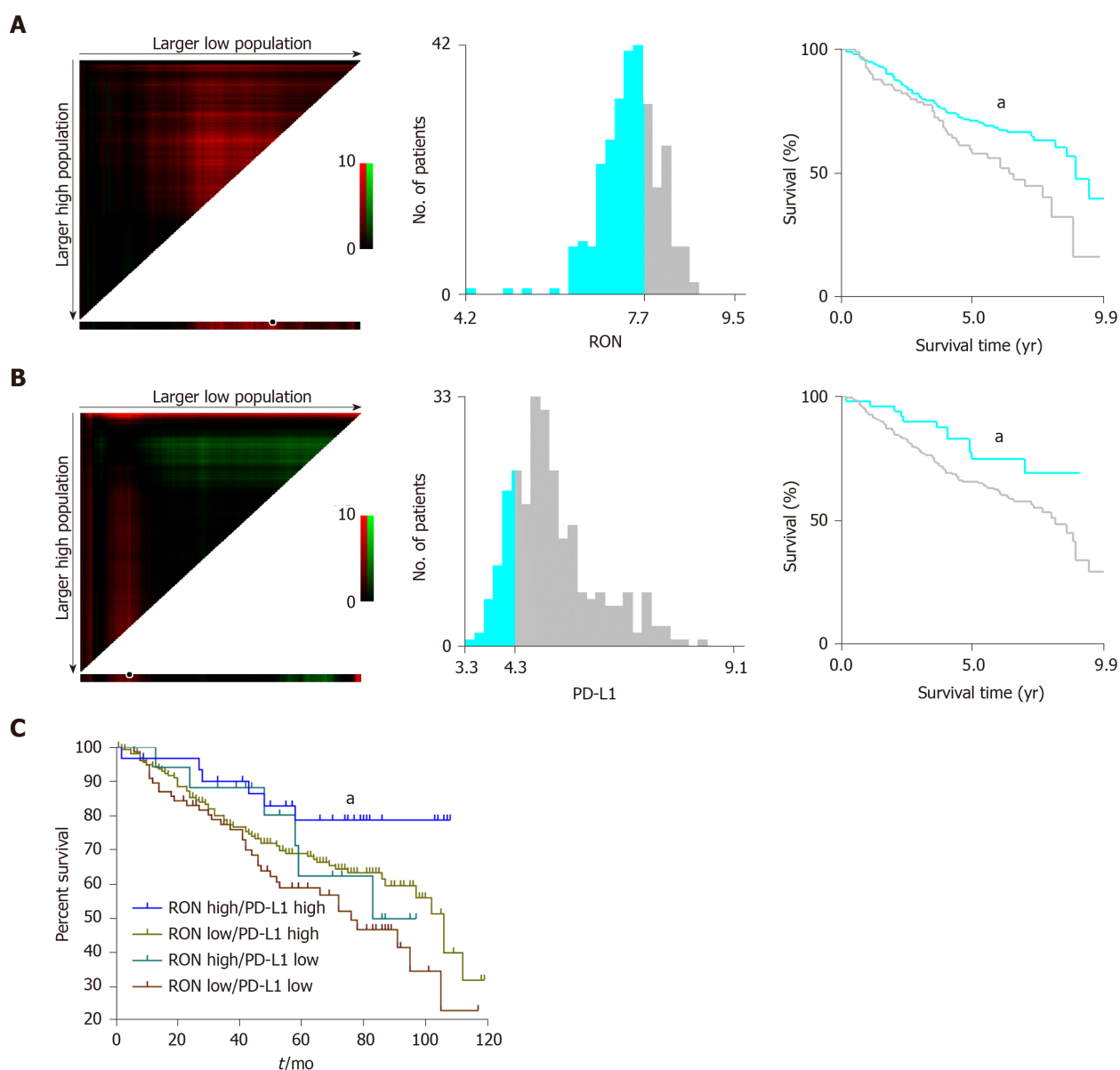
Figure 1 X-tile analysis to determine the optimal cut-off values for RON and PD-L1 expression and survival analysis in the GEO dataset.
The optimal cut-off values are highlighted by black circles (left panels) and shown in histograms for the entire cohort (middle panels). Kaplan-Meier survival plots are shown in right panels. A: The optimal cut-off value for RON was 7.50 (χ^2 = 4.544, P = 0.033); B: The optimal cut-off value for PD-L1 was 4.30 (χ^2 = 4.078, P = 0.043); C: Effect of different RON and PD-L1 expression states on patient survival. [a]P < 0.05.

low. Additionally, there were cases where RON expression was enhanced on the TC membrane, but PD-L1 was not expressed in the tumor microenvironment (Figure 2E). In addition, RON and PD-L1 were often accompanied by a cytoplasmic expression of cancer cells. In general, when RON and PD-L1 were enhanced in the tumor cell membrane and cytoplasm, PD-L1 was also expressed in TIMCs. The results of multiplex immunofluorescence are consistent with the evaluation of RON and PD-L1 by immunohistochemical staining (Figure 3A-C).

Association of RON and PD-L1 expression with clinicopathological features in the FAHZUSM cohort

In the FAHZUSM cohort, CRC patients were divided into two groups based on high or low expression of RON and PD-L1 detected by IHC staining. In 381 CRC patients, high and low RON expression in TCs was observed in 121 (31.8%) and 260 (68.2%), respectively. High and low expression of PD-L1 in TCs and TIMCs was noted in 43 (11.3%) and 91 (23.9%) patients, respectively, and PD-L1 was lowly expressed in TIMCs in 247 (64.8%) cases. None of the patients had high PD-L1 expression in both TCs and TIMCs. Among all samples expressing RON and PD-L1 in TCs, the proportion with both high RON and high PD-L1 expression was 8.6% (25/290), while
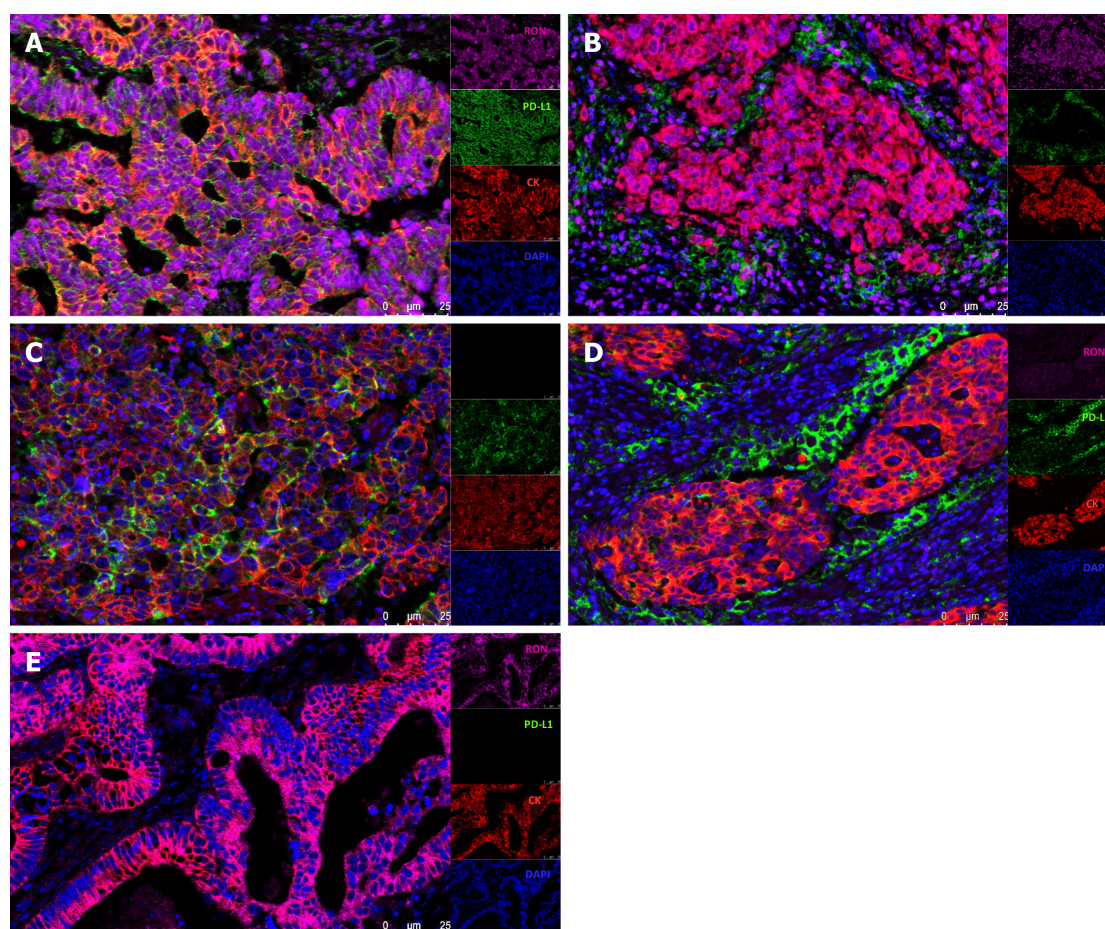
Figure 2 Four-color multiplex immunofluorescence staining of paraffin-embedded colorectal cancer tissues. A: High expression of RON and PD-L1 in tumor cells (TCs); B: High expression of RON in TCs and high expression of PD-L1 in tumor-infiltrating mononuclear cells (TIMCs); C: Lack of RON expression and high PD-L1 expression in TCs; D: Low expression of RON in TC and high PD-L1 expression in TIMCs; E: High RON expression in TCs and no PD-L1 expression in either TCs or TIMCs. Blue (DAPI) shows nuclei, green shows PD-L1, pink indicates RON, and red displays CK.

the proportion of samples expressing high PD-L1 in TIMCs and high RON in TCs was 7.7% (26/338). Chi-square tests were used to determine the correlation between the expression of RON and PD-L1 in tumor tissues. Compared to samples with low expression of RON, samples with high RON expression levels were more likely to have high levels of PD-L1 in TCs, demonstrating a significant correlation between high RON expression and high PD-L1 expression in TCs ($P < 0.001$, Table 1). The expression of PD-L1 in TIMCs was not significantly correlated with RON expression in TCs (Table 1).

We compared the clinical characteristics of patient groups categorized based on the expression of RON and PD-L1. High expression of RON was associated with gender, T stage, N stage, disease stage, and treatment. High RON and high PD-L1 expression in TCs was related to T stage (Table 2). High expression of RON and PD-L1 (TIMCs) was associated with T stage, principal diagnosis, histological type, and treatment ($P < 0.05$; Table 3).

Prognostic significance of RON and PD-L1 expression in CRC

In the GEO cohort, univariate Cox regression analysis showed that age, T stage, M stage, disease stage, RON, and PD-L1 (TCs) expression were significantly correlated with a poor prognosis in CRC patients ($P < 0.05$). Multivariate analysis after adjustment showed that only age, T stage, and M stage were independent prognostic factors for OS in CRC patients ($P < 0.05$); disease stage, RON expression, and PD-L1 expression were not significant factors in the multivariate analysis ($P > 0.05$; Table 4). Kaplan-Meier analysis showed that high expression of RON or PD-L1 (TCs), and both high RON and PD-L1 expression were all associated with a poor OS ($P < 0.05$; Figure 1A-C). High expression of RON was associated with patient age and treatment type, and high expression of PD-L1 (TCs) was associated with the primary site of the tumor ($P < 0.05$; Table 1).

Table 2 Comparison of RON (tumor cells) and PD-L1 (tumor cells) expression and clinicopathological characteristics of patients with colorectal cancer in the FAHZUSM cohort, *n* (%)

Characteristic	Cases	RON and PD-L1 (TCs) expression in the tumor microenvironment				
		RON low/PD-L1 low	RON high/PD-L1 low	RON low/PD-L1 high	RON high/PD-L1 high	*P* value
Number of cases	290	177	70	18	25	
Age (mean ± SD)	60.89 ± 12.215	60.37 ± 12.299	62.40 ± 12.518	58.39 ± 12.930	62.12 ± 10.179	0.495
Sex						
Man	161 (55.5)	108 (61)	35 (50.0)	9 (50.0)	9 (36.0)	0.061
Women	129 (44.5)	68 (39)	35 (50.0)	9 (50.0)	16 (64.0)	
Principal diagnosis				s		
Rectum	182 (62.8)	116 (65.5)	37 (52.9)	14 (77.8)	15 (60.0)	0.149
Colon	108 (37.2)	61 (34.5)	33 (47.1)	4 (22.2)	10 (40.0)	
Pathological grade						
Well/moderate	6 (2.1)	3 (1.7)	2 (2.9)	0 (0.0)	1 (4.0)	0.264
Poor	264 (9.1)	158 (89.3)	66 (94.3)	16 (88.9)	24 (96.0)	
Unknown	20 (6.9)	16 (9.0)	2 (2.9)	2 (11.1)	0 (0.0)	
T stage						
Tis-T2	84 (29)	64 (36.2)	9 (12.9)	5 (27.8)	6 (24.0)	**0.003**
T3	140 (48.3)	82 (46.3)	36 (51.4)	11 (61.1)	11 (44.0)	
T4	66 (22.8)	31 (17.5)	25 (35.7)	2 (11.1)	8 (32.0)	
N stage						
N0	238 (66.9)	125 (70.6)	39 (55.7)	12 (66.7)	13 (66.4)	0.072
N (1-2)	118 (33.1)	52 (29.4)	31 (44.3)	6 (33.3)	12 (33.6)	
M stage						
M0	283 (97.6)	173 (97.7)	68 (97.1)	18 (100)	24 (96.0)	0.735
M1	7 (2.4)	4 (2.3)	2 (2.9)	0 (0.0)	1 (4.0)	
Stage						
0-II	187 (64.5)	123 (69.5)	39 (55.7)	12 (66.7)	13 (52.0)	0.110
III-IV	103 (35.5)	54 (30.5)	31 (33.3)	6 (33.3)	12 (48.0)	
Histological type						
Adenocarcinoma	251 (86.6)	152 (85.9)	62 (88.6)	15 (83.3)	22 (88.0)	0.493
Mucinous/SRCC	32 (11)	21 (11.9)	7 (10.0)	1 (5.6)	3 (12.0)	
Other	7 (2.4)	4 (2.3)	1 (1.4)	2 (11.1)	0 (0.0)	
Treatment						
Yes	135 (46.6)	83 (46.9)	22 (31.4)	6 (33.3)	12 (48.0)	0.158
No	119 (41.0)	74 (41.8)	38 (54.3)	11 (61.1)	8 (32.0)	
Unknown	36 (12.4)	20 (11.3)	10 (14.3)	1 (5.6)	5 (20.0)	

Bold entries indicate statistical significance (*P* < 0.05). RON: Recepteur d'origine nantais; PD-L1: Programmed death ligand 1; TCs: Tumor cells; FAHZUSM: The First Affiliated Hospital, Zhejiang University School of Medicine.

In the FAHZUSM cohort, Kaplan-Meier analysis showed that high expression of RON in TCs, high PD-L1 expression in TCs, or high PD-L1 expression in TIMCs in CRC tissue samples was correlated with a lower OS ($P < 0.05$) (Figure 4A-C). Univariate Cox regression analysis showed that age, gender, TNM stage, disease stage, pathological grade, RON expression, and PD-L1 expression were associated with a poor OS in patients with CRC ($P < 0.05$). Multivariate analysis after adjustment showed that age, gender, T stage, M stage, pathological grade, RON expression, and PD-L1 expression remained independent prognostic factors for OS in CRC patients ($P < 0.05$), while N and disease stage were not significant ($P > 0.05$; Table 4). We further analyzed the relationship between OS and RON and PD-L1 expression in the tumor microenvironment. High expression of both RON and PD-L1 in TCs predicted a significantly worse OS, and high RON expression in TCs and high PD-L1 expression in TIMCs were associated with a significantly worse prognosis ($P < 0.001$; Figure 4D and E).

Effects of regulating RON phosphorylation on PD-L1 expression

In order to study the relationship between RON and PD-L1 in CRC cells, HT29 cells (following serum starvation) were treated with MSP, BMS-777607, or MSP + BMS-777607 for 24 h. The effects of treatment on RON and PD-L1 expression were analyzed by cellular immunofluorescence. Activating RON phosphorylation promoted PD-L1 expression, while inhibiting RON phosphorylation down-regulated PD-L1 expression (Figure 5A). This result was confirmed by analysis of protein expression by Western blot ($P < 0.05$; Figure 5B and C). In addition, we found that RON inhibition significantly reduced the phosphorylation of AKT and ERK1/2, which signal downstream of RON (Figure 6A-D).

DISCUSSION

The application of PD-L1 immunotherapy in cancer treatment is becoming more and more important, but its therapeutic effect has been limited in CRC. This may be due in part to the prevalence of the mismatch repair (MMR) proficient or microsatellite stabilization (MSS) subtypes of CRC[36]. In addition, c-MET plays an important role in regulating PD-L1 expression[37,38]. Therefore, RON may promote the expression of PD-L1 in tumor tissue to affect the efficacy of PD-L1 immunosuppressants.

RON and PD-L1 play important roles in cancer initiation and development and are important targets for tumor therapy. However, few studies have explored the relationship between RON and PD-L1 expression in CRC and the impact that RON and PD-L1 expression patterns may have on pathological characteristics and disease outcomes. It was our hypothesis that activation of the RON receptor tyrosine kinase may promote the expression of PD-L1 in CRC tissue and impair the efficacy of PD-L1 immunotherapy. The purpose of our study was to analyze the expression and clinical significance of RON and PD-L1 in the tumor microenvironment in CRC samples, and to determine whether RON can regulate the expression of PD-L1 in CRC. We discovered that high expression of both RON and PD-L1 in the tumor microenvironment was associated with the poorest OS rate compared to CRC tumors with other RON and PD-L1 expression states. RON and PD-L1 were independent prognostic factors for OS in CRC patients. In addition, we showed that RON phosphorylation led to increased expression of PD-L1 in CRC cells, and that inhibiting RON phosphorylation can reduce PD-L1 expression.

We used publicly available data from the GEO database to investigate the relationship between RON and PD-L1 expression and clinical outcomes in CRC. We used X-tile to determine the optimal cut-off values for RON and PD-L1 levels in the GEO cohort. Kaplan-Meier survival analysis revealed a positive correlation of high RON or PD-L1 expression with worse OS, and high expression of RON and PD-L1 together was associated with the worst OS for CRC patients. However, RON and PD-L1 levels in the GEO dataset were determined by RNA sequencing using RNA extracted from tumor tissue to quantify the expression level in TCs, and it is difficult to determine the interactions between RON and PD-L1 that may arise within the tumor microenvironment from the GEO dataset. Therefore, we used the FAHZUSM cohort, a larger patient cohort with pathological tumor samples, to further study the expression of RON and PD-L1 in CRC tissues and to determine the pathological significance of elevated RON and PD-L1 expression in the tumor microenvironment.

Dysregulation of RON and PD-L1 signal transduction is a key feature of many tumors. Our results and previous studies demonstrated that high expression of RON

Table 3 Comparison of RON (tumor cells) and PD-L1 (tumor-infiltrating mononuclear cells) expression and clinicopathological characteristics of patients with colorectal cancer in the FAHZUSM cohort, *n* (%)

Characteristic	Cases	RON and PD-L1 (TIMCs) expression in the tumor microenvironment				P value
		RON low/PD-L1 low	RON high/PD-L1 low	RON low/PD-L1 high	RON high/PD-L1 high	
Number of cases	338	177	70	65	26	
Age (mean ± SD)	61.24 ± 12.555	60.37 ± 12.299	62.40 ± 12.518	61.03 ± 13.286	64.58 ± 12.436	0.348
Sex						
Man	197 (58.3)	108 (61)	35 (50.0)	40 (61.5)	14 (52.8)	0.368
Women	141 (41.7)	68 (39)	35 (50.0)	25 (38.5)	12 (46.2)	
Principal diagnosis						
Rectum	200 (59.2)	116 (65.5)	37 (52.9)	28 (43.1)	19 (73.1)	**0.004**
Colon	138 (40.8)	61 (34.5)	33 (47.1)	37 (56.9)	7 (26.9)	
Pathological grade						
Well/moderate	8 (2.4)	3 (1.7)	2 (2.9)	3 (4.6)	0 (0)	0.492
Poor	306 (90.5)	158 (89.3)	66 (94.3)	58 (89.2)	24 (92.3)	
Unknown	24 (7.1)	16 (9.0)	2 (2.9)	4 (6.2)	2 (7.7)	
T stage						
Tis-T2	96 (28.4)	64 (36.2)	9 (12.9)	16 (2.6)	7 (26.9)	**0.002**
T3	155 (45.9)	82 (46.3)	36 (51.4)	28 (43.1)	9 (34.6)	
T4	87 (25.7)	31 (17.5)	25 (35.7)	21 (32.3)	10 (38.5)	
N stage						
N0	226 (66.9)	125 (70.6)	39 (55.7)	47 (72.3)	15 (57.7)	0.075
N (1-2)	112 (33.1)	52 (29.4)	31 (44.3)	18 (27.7)	11 (42.3)	
M stage						
M0	326 (96.4)	173 (97.7)	68 (97.1)	61 (93.8)	24 (92.3)	0.235
M1	12 (3.6)	4 (2.3)	2 (2.9)	4 (2.3)	2 (7.7)	
Stage						
0-II	221 (65.4)	123 (69.5)	39 (55.7)	45 (69.2)	14 (53.8)	0.104
III-IV	117 (34.6)	54 (30.5)	31 (33.3)	20 (30.8)	12 (46.2)	
Histological type						
Adenocarcinoma	287 (84.9)	152 (85.9)	62 (88.6)	60 (92.3)	13 (50)	**< 0.001**
Mucinous/SRCC	40 (11.8)	21 (11.9)	7 (10.0)	3 (4.6)	9 (34.6)	
Other	11 (3.3)	4 (2.3)	1 (1.4)	2 (3.1)	4 (15.4)	
Treatment						
Yes	298 (88.2)	83 (46.9)	22 (31.4)	30 (46.2)	24 (92.3)	**< 0.001**
No	32 (9.5)	74 (41.8)	38 (54.3)	25 (38.5)	1 (3.8)	
Unknown	8 (2.4)	20 (11.3)	10 (14.3)	10 (15.4)	1 (3.8)	

Bold entries indicate statistical significance (*P* < 0.05). RON: Recepteur d'origine nantais; PD-L1: Programmed death ligand 1; TCs: Tumor cells; TIMCs: Tumor-infiltrating mononuclear cells; FAHZUSM: The First Affiliated Hospital, Zhejiang University School of Medicine.

or PD-L1 in CRC tumor tissues correlated with the extent of primary tumor, lymph node metastasis, and distant metastasis[39,40]. Furthermore, high expression of RON or PD-L1 in the tumor microenvironment was associated with a poor survival in CRC patients[22,39-42].

We demonstrated that high expression of both RON and PD-L1 in TCs was associated with a poor OS in CRC patients. Interestingly, the expression levels of RON were positively correlated with those of PD-L1 in TCs, which has not been previously reported. This may be related to the fact that RON can regulate both the production of and response to IFN-γ[43]. In TCs, long-term continuous activation of IFN-γ can mediate adaptive resistance to PD-L1 tumor immunosuppression; high expression of RON may attenuate resistance, restore the efficacy of PD-L1 targeted therapy, and may even have a promoting effect on the expression of PD-L1 by inhibiting the production of IFN-γ[10,43-45].

MEK/ERK and PI3K/AKT signaling pathways play a central role towards understanding the mechanisms underlying tumorigenesis and tumor development, prevention, and treatment. For example, autophagy caused by the activation of PI3K/AKT pathway plays a significant role in tumors[46,47]. In our study, we demonstrated that RON phosphorylation increased the expression of PD-L1 in CRC cells, proving that RON activation promotes the expression of PD-L1, likely acting through the downstream AKT and ERK1/2 signaling pathways. Previous studies have shown that there is crosstalk between RON phosphorylation and other receptors, which can affect the surface expression of other receptors[22]. Phosphorylation of RON activates downstream oncogenic signaling pathways, such as the RAS-ERK and PI3K-AKT pathways, thereby promoting tumor initiation, growth, invasion, and metastasis[20]. Treatment with BRAF inhibitors or MEK inhibitors, or down-regulation of ERK1/2 can lead to down-regulation of PD-L1 expression in TCs[48]. Thus, RON phosphorylation may promote the expression of PD-L1 in TCs through the RAS-ERK signaling pathway. In addition, PTEN interacts with RTK-dependent signals at multiple levels[48], and has an antagonistic effect on PI3K, which plays an important role in mediating RTK-dependent cell signaling and tumor immune escape[10,48,49]. Therefore, RON phosphorylation may interact with PTEN to activate the PI3K-AKT signaling pathway, thereby promoting PD-L1 expression in TCs.

TIMCs are regarded as an indicator of host immune response to a tumor, and have long been considered an unfavorable prognostic marker in CRC[41,42]. Moreover, RON expression plays an important role in anti-tumor immune responses[27,50]. Previous studies also have shown that RON activation reduced the polarization of inflammatory M1 macrophages and induced the differentiation of immunosuppressive M2 macrophages. M2 macrophages promote PD-L1 expression through autocrine VEGF signaling[51]. In our study, we demonstrated that high expression of both RON and PD-L1 (TIMCs) was associated with a poor OS in patients with CRC. Thus, high expression of RON may impair the anti-tumor immune response by promoting the expression of PD-L1 in TIMCs, leading to tumor growth, invasion, and metastasis.

There were some limitations in this study. First, although we have analyzed a large patient cohort, this was a retrospective analysis and there is the potential for selection bias. Second, we did not evaluate which specific immune cell components in TIMCs expressed PD-L1. Third, although we have preliminarily studied that RON phosphorylation promotes the expression of PD-L1 in CRC cells, the mechanism by which RON promotes PD-L1 expression still needs to be further studied.

CONCLUSION

In summary, high expression of RON and/or PD-L1 in CRC samples is associated with a poor prognosis in CRC patients. The expression status of RON and PD-L1 may be helpful as biomarkers to evaluate the prognosis of patients with CRC. In addition, RON phosphorylation can promote the expression of PD-L1 in CRC cells, possibly through activation of the AKT and ERK1/2 signaling pathways, which provides a new idea for the immunotherapy of CRC.

Table 4 Univariate and multivariate Cox proportional hazard analyses of overall survival in patients with colorectal cancer in the FAHZUSM and Gene Expression Omnibus cohorts

Variable	FAHZUSM cohort				GEO cohort			
	Univariate analysis, HR (95%CI)	*P* value	Multivariate analysis, HR (95%CI)	*P* value	Univariate analysis, HR (95%CI)	*P* value	Multivariate analysis, HR (95%CI)	*P* value
Age (mean ± SD)	1.029 (1.011-1.048)	**0.002**	1.031 (1.011-1.050)	**0.002**	1.025 (1.009-1.041)	**0.002**	1.028 (1.012-1.045)	**0.001**
Gender								
Male	1.000	**0.011**	1.000	**0.008**	1.000	0.293	1.000	0.076
Female	0.562 (0.362-0.874)		0.527 (0.328-0.847)		0.814 (0.555-1.194)		0.698 (0.469-1.039)	
Principal diagnosis								
Rectum	1.000	0.298	1.000	0.318	1.000	0.688	1.000	0.544
Colon	0.796 (0.518-1.224)		0.790 (0.497-1.255)		1.081 (0.738-1.585)		0.882 (0.589-1.322)	
Histological type								
Adenocarcinoma	1.000	0.408	1.000	0.387	NA			
Mucinous/SRCC	2.464 (0.343-17.710)		2.720 (0.263-28.122)					
Unknown	3.355 (0.433-25.986)		1.232 (0.129-11.795)					
T stage								
Tis-T2	1.000	**< 0.001**	1.000	**0.003**	1.000	**0.001**	1.000	**0.007**
T3	0.211 (0.108-0.413)		0.298 (0.141-0.632)		0.333 (0.145-0.766)		0.381 (0.159-0.914)	
T4	0.503 (0.325-0.781)		0.596 (0.361-0.897)		0.455 (0.292-0.709)		0.476 (0.295-0.769)	
N stage								
N0	1.000	**< 0.001**	1.000	0.111	1.000	0.062	1.000	0.141
N (1-2)	3.156 (2.083-4.783)		5.376 (0.678-42.660)		1.435 (0.982-2.097)		0.306 (0.063-1.479)	
M stage								
M0	1.000	**< 0.001**	1.000	0.039	1.000	**< 0.001**	1.000	**0.008**
M1	4.338 (2.094-8.985)		2.445 (1.048-5.701)		3.546 (2.013-6.247)		2.481 (1.269-4.852)	
Stage								
0-II	1.000	**< 0.001**	1.000	0.406	1.000	**0.029**	1.000	0.099
III-IV	3.102 (2.044-4.708)		0.409 (0.050-3.366)		1.530 (1.045-2.242)		3.924 (0.774-19.888)	
Pathological grade								
Well/moderate	1.000	**< 0.001**	1.000	**0.003**	NA			
Poor	7.689 (2.434-24.290)		4.044 (1.103-14.830)					
Unknown	1.266 (0.513-3.126)		0.375 (0.104-1.357)					
RON (protein)								
Low	1.000	**0.001**	1.000	**0.012**	1.000	**0.035**	1.000	0.466
High	2.060 (1.364-3.112)		1.788 (1.138-2.808)		1.515 (1.030-2.228)		1.170 (0.767-1.785)	
RON (mRNA)								
Low	NA				1.000	**0.035**	1.000	0.466
High					1.515 (1.030-2.228)		1.170 (0.767-1.785)	
PD-L1 (protein)								
Low	1.000	**< 0.001**	1.000	**0.001**	NA			

High (TIMCs)	0.544 (0.293-1.011)	0.676 (0.350-1.304)					
High (TCs)	1.325 (0.669-2.511)	1.654 (0.819-3.338)					
PD-L1 (mRNA)							
Low	NA			1.000	**0.047**	1.000	0.083
High				1.835 (1.007-3.345)		1.719 (0.931-3.173)	

All variables are adjusted by the Cox proportional hazard model. Bold entries indicate statistical significance (*P* < 0.05). GEO: The Gene Expression Omnibus; RON: Recepteur d'origine nantais; PD-L1: Programmed death ligand 1; TCs: Tumor cells; TIMCs: Tumor-infiltrating mononuclear cells; FAHZUSM: The First Affiliated Hospital, Zhejiang University School of Medicine.
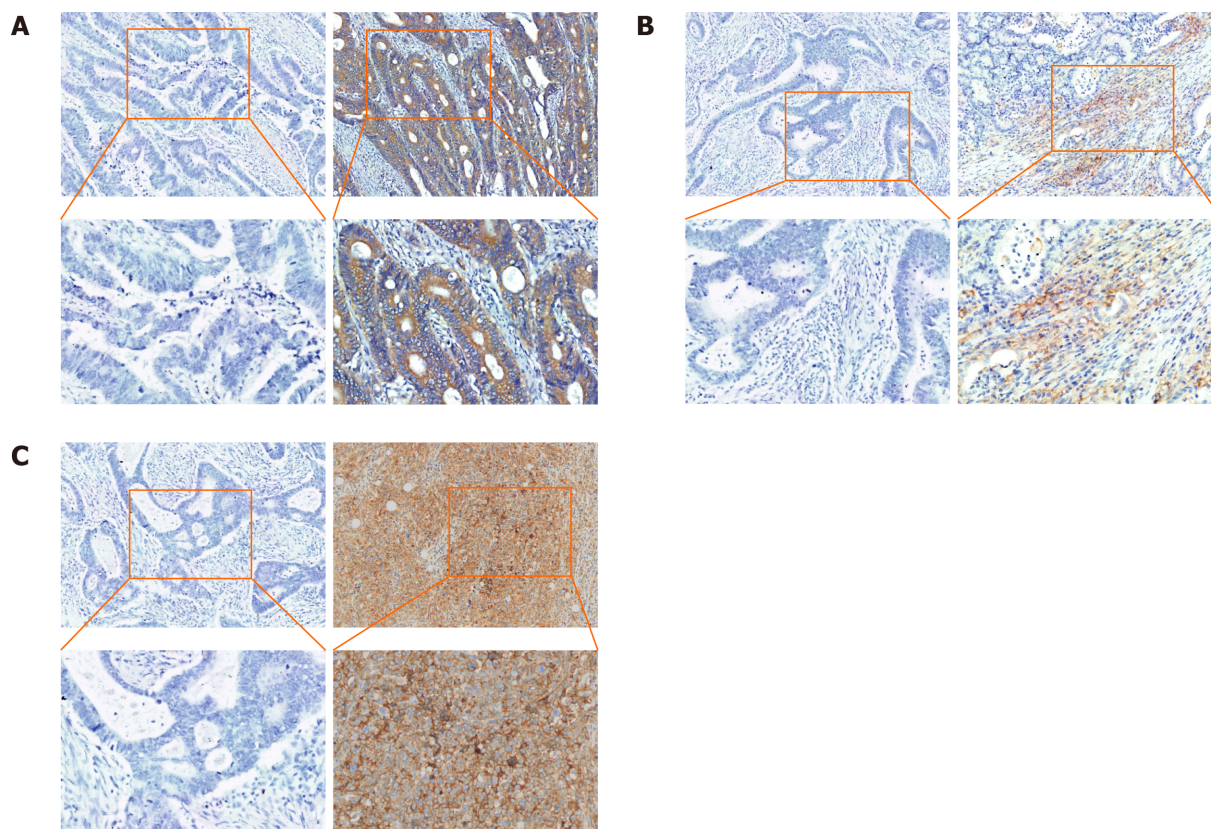

Figure 3 Immunohistochemical expression of RON and PD-L1 in colorectal cancer tissues. A: High (right) and negative (left) expression of RON in tumor cells (TCs); B: High (right) and negative (left) expression of PD-L1 in tumor-infiltrating mononuclear cells; C: High and negative expression of PD-L1 in TCs (original magnification, × 100 and × 200).
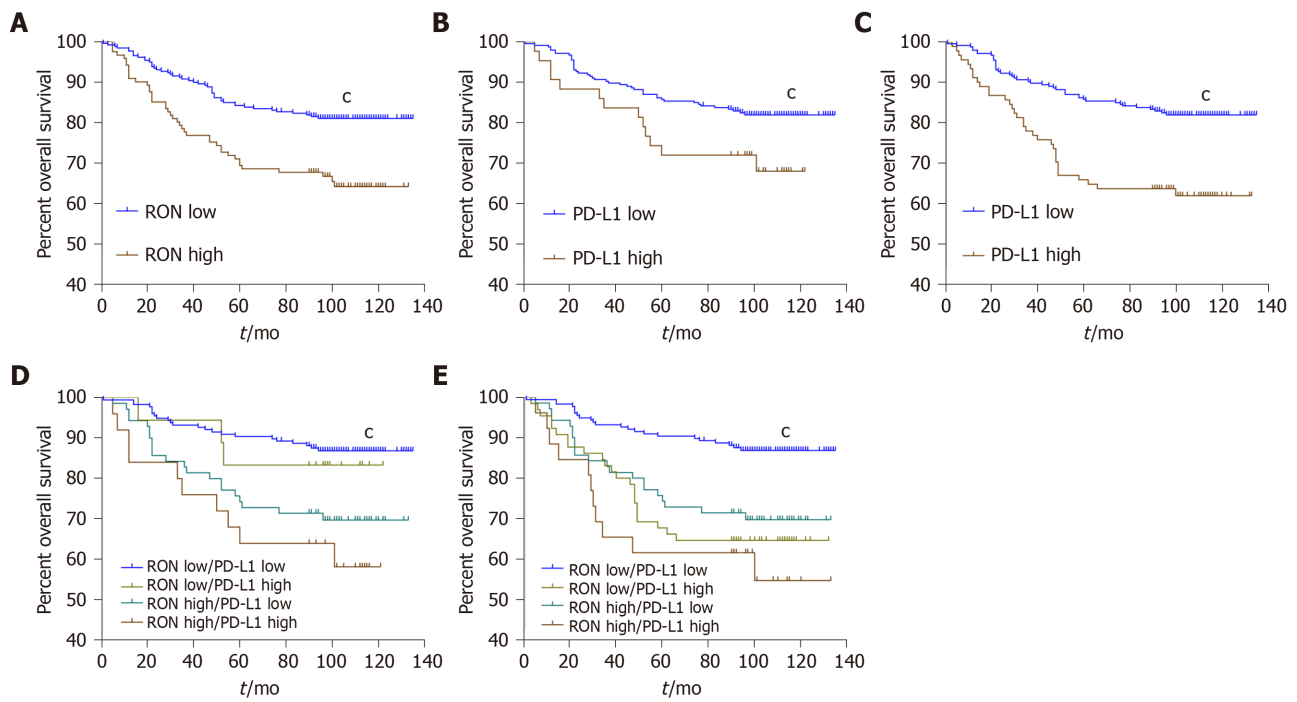
Figure 4 Kaplan-Meier analysis of overall survival of patients with colorectal cancer stratified by RON and/or PD-L1 expression. A: Overall survival (OS) according to RON expression in tumor cells (TCs); B: OS according to PD-L1 expression in TCs; C: OS according to PD-L1 expression in tumor-infiltrating mononuclear cells (TIMCs); D: OS according to RON and PD-L1 expression in TCs; E: OS according to RON expression in TCs and PD-L1 expression in TIMCs. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.
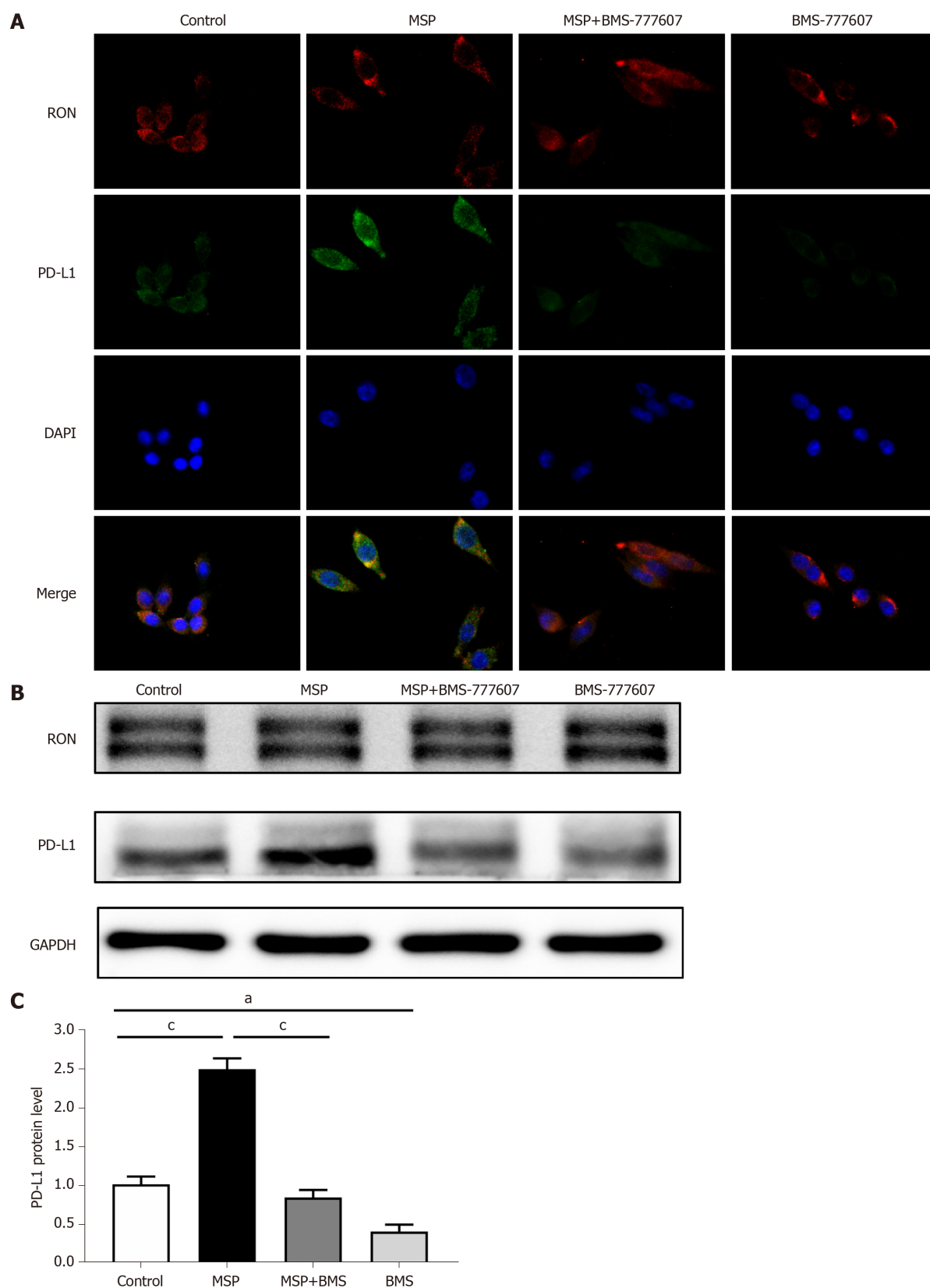
Figure 5 Expression of RON and PD-L1 in HT29 cells after treatment with 2 nmol/L MSP, 2 nmol/L MSP + 2 μmol/L BMS-777607, or 2 μmol/L BMS-777607. A: Cellular immunofluorescence indicating the expression of RON and PD-L1 after treatment of HT29 cells with 2 nmol/L MSP, 2 nmol/L MSP + 2 μmol/L BMS-777607, or 2 μmol/L BMS-777607 for 24 h, respectively. DAPI indicates nuclei (blue color), FITC indicates PD-L1 (green color), and PE indicates RON (red color). Original magnification × 400 (all photomicrographs). B-C: HT29 cells were treated with 2 nmol/L MSP, 2 nmol/L MSP + 2 μmol/L BMS-777607, or 2 μmol/L BMS-777607 for 24 h, and the expression of RON and PD-L1 was detected by Western blot and quantified according to the immunoblots. [a]$P <$ 0.05, [b]$P < 0.01$, [c]$P < 0.001$.
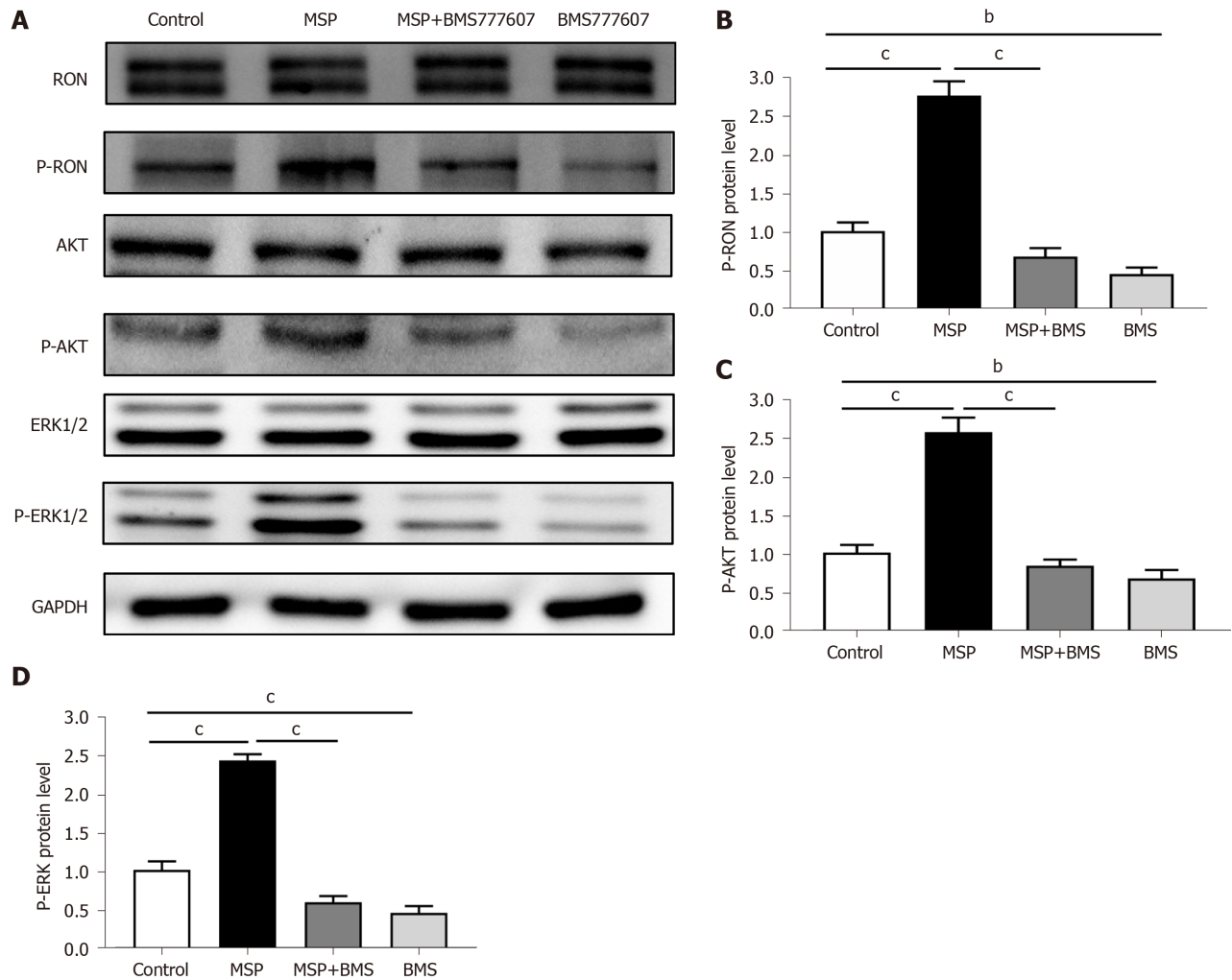
Figure 6 Expression of RON and PD-L1, and activation of signaling pathways in HT29 cells. A: HT29 cells were treated with 2 nmol/L MSP, 2 nmol/L MSP + 2 μmol/L BMS-777607, or 2 μmol/L BMS-777607 for 1 h. The proteins analyzed include RON, phosphorylated RON, AKT, ERK1/2, phosphorylated AKT, and phosphorylated-ERK1/2. GAPDH was used as a loading control; B-D: The expression of p-RON, p-AKT, and p-ERK1/2 was detected by Western blot and quantified. [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

ARTICLE HIGHLIGHTS

Research background

Programmed death ligand 1 (PD-L1) protein expression on immune cells enables tumor cells to evade the immune system in a wide variety of malignancies. However, the efficacy of PD-L1 immunosuppressive agents in colorectal cancer (CRC) is poor. The recepteur d'origine nantais (RON) receptor tyrosine kinase plays an important role in regulating tumor immunity.

Research motivation

The poor anti-tumor effect of PD-1 inhibitors in CRC promoted the research on the mechanism of PD-1 expression, so as to improve the therapeutic effect of PD-1 inhibitors on CRC.

Research objectives

The present study aimed to identify patterns of RON and PD-L1 expression and explore the clinical significance of these patterns in CRC.

Research methods

The gene expression data from the Gene Expression Omnibus database (GEO; $n = 290$) and patients at the First Affiliated Hospital, Zhejiang University School of Medicine (FAHZUSM; $n = 381$) were analyzed to determine the prognostic value of RON and PD-L1 expression in the tumor microenvironment of CRC. HT29 cells were treated

with BMS-777607 to explore the relationship between RON activity and PD-L1 expression. Signaling pathways and protein expression perturbed by RON inhibition were evaluated by cellular immunofluorescence and Western blot.

Research results

In the GEO patient cohort, the cut-off values for RON and PD-L1 expression were determined to be 7.70 and 4.30, respectively. Stratification of patients based on these cutoffs demonstrated that high expression of RON and PD-L1 was associated with a poor prognosis. In the FAHZUSM cohort, rates of high expression of RON in tumor cells, high PD-L1 expression in tumor cells and tumor infiltrating monocytes, and both high RON and high PD-L1 expression in the tumor microenvironment were 121 (32%), 43 (11%), 91 (24%), and 51 (13.4%), respectively. High expression of RON was significantly correlated with high expression of PD-L1 in the tumor cell compartment ($P < 0.001$). High expression of RON and PD-L1 were independent prognostic factors for poorer overall survival. Concurrent high expression of both RON and PD-L1 in the tumor microenvironment was significantly associated with a poor prognosis. *In vitro*, BMS-777607 inhibited the phosphorylation of RON and PD-L1 expression, and attenuated the activation of the ERK1/2 and AKT signaling pathways in CRC cells.

Research conclusions

RON, PD-L1, and their crosstalk are significant in predicting the prognostic value of CRC. Moreover, phosphorylation of RON upregulates PD-L1 expression, which provides a novel approach to immunotherapy in CRC.

Research perspectives

The study of RON and PD-L1 expression will help improve the efficacy of PD-L1 immunosuppressive agents for CRC.

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