The pathological significance of abnormal RON and PD-L1 expression in colorectal cancer

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Research

Keywords: Colorectal cancer, Tumor microenvironment, RON receptor tyrosine kinase, PD-L1 programmed cell death ligand-1, Tumor infiltrating mononuclear cells, Prognosis, The Gene Expression Omnibus

Posted Date: June 25th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-37737/v1>

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Submit to J Exp Clin Cancer Res

The pathological significance of abnormal RON and PD-L1 expression in colorectal cancer

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30 **Abstract:**

31 **Background:** PD-L1 immunotherapy remains poorly efficacious in colorectal cancer.

32 The RON receptor tyrosine kinase plays an important role in regulating tumor immunity.

33 Here, we identify patterns of RON and PD-L1 expression and explore the clinical
34 significance of these patterns in colorectal cancer.

35 **Methods:** Gene expression data were obtained from the Gene Expression Omnibus
36 database (GEO; n = 290) and patients at the First Affiliated Hospital, Zhejiang University
37 School of Medicine (FAHZUSM; n = 381) and were analyzed to determine the prognostic
38 value of RON and PD-L1 expression within the tumor cells and the tumor
39 microenvironment of colorectal cancer. Human colorectal cancer cell lines were treated
40 with BMS-777607 to explore the relationship between RON activity and PD-L1 protein
41 expression. Signaling pathways and protein expression perturbed by RON inhibition
42 were evaluated by cellular immunofluorescence and western blot.

43 **Results:** In the GEO cohort, cut-off values for RON and PD-L1 expression of 7.70 and
44 4.30, respectively, were determined. Stratification of patients based on these cutoffs
45 demonstrated that high expression of RON and PD-L1 associated with poor prognosis.
46 In the FAHZUSM cohort, rates of high expression of RON in tumor cells, high PD-L1
47 expression in tumor cells and in tumor infiltrating monocytes, and both high RON and
48 high PD-L1 expression in the tumor microenvironment were 121 (32%), 43 (11%), 91
49 (24%), and 51 (13.4%), respectively. High expression of RON and high expression of
50 PD-L1 in the tumor cell compartment was significantly correlated ($p < 0.001$). High
51 expression of RON and PD-L1 were independent prognostic factors for poorer overall

52 survival. Concurrent high expression of both RON and PD-L1 in the tumor
53 microenvironment was significantly associated with poor prognosis. *In vitro*,
54 BMS-777607 inhibited the phosphorylation of RON, inhibited PD-L1 expression, and
55 attenuated activation of the ERK1/2 and AKT signaling pathways in colorectal cancer
56 cells.

57 **Conclusions:** RON, PD-L1 and the crosstalk between these proteins plays an important
58 role in predicting the prognostic value of colorectal cancer. Moreover, phosphorylation of
59 RON upregulates the expression of PD-L1, which provides a novel approach to
60 immunotherapy in colorectal cancer.

61
62 **Keywords:** Colorectal cancer, Tumor microenvironment, RON receptor tyrosine kinase,
63 PD-L1 programmed cell death ligand-1, Tumor infiltrating mononuclear cells, Prognosis,
64 The Gene Expression Omnibus

66 BACKGROUND

67 In 2018, colorectal cancer (CRC) was ranked the fourth most common
68 malignancy worldwide, accounting for 6.1% of the total number of cancers diagnosed,
69 and responsible for 9.2% of total cancer deaths (1). The methods for treating CRC
70 treatment are diverse, and include surgical resection, chemotherapy, and radiotherapy.
71 Despite advances in these treatment methods, CRC often has a poor prognosis, and
72 many patients will nevertheless have local recurrence and/or distant metastasis within
73 five years after treatment (2).

74 Immunotherapy has become a promising strategy for treating a wide variety of

75 malignancies (3). Therapeutic antibodies that block the activity of programmed death
76 ligand 1 (PD-L1, also known as CD274 and B7-H1) proteins are effective against many
77 of cancer types (4). PD-L1 is a transmembrane immune checkpoint protein that can be
78 expressed on both immune cells and on cancer cells, including CRC cells (5-7). When
79 expressed in immune cells, PD-L1 can be regulated by a variety of inflammatory
80 mediators and cytokines. When expressed on the surface of tumor cells, PD-L1 enables
81 tumor cells to evade the immune system (8, 9). Cancer cells can induce the expression
82 of PD-L1 through activation of oncogenic pathways, such as the RAS-ERK or PI3K-AKT
83 pathways, to facilitate immune escape (10-12).

84 However, the efficacy of PD-L1 immunosuppressive agents in colorectal cancer
85 is poor (13, 14). The overall poor response of CRC to PD-L1 blockade may be due to the
86 activity of multiple oncogenes (15, 16). For example, KRAS mutations are most
87 commonly seen in lung, pancreas, and colorectal adenocarcinoma, and KRAS mutations
88 can up-regulate the expression of PD-L1 in colorectal cancer cells, thereby reducing the
89 efficacy of PD-L1 immunosuppressive agents (17). RON (macrophage-stimulating 1
90 receptor, MST1R), a member of the MET proto-oncogene family and a receptor for MSP,
91 is an effector of KRAS signaling, and exhibits “KRAS addiction”, a phenomenon that
92 plays an important role in the initiation and development of tumors (18-20). RON is
93 over-expressed pathologically in various types of cancer, including CRC, and many
94 small-molecule inhibitors are being developed to target RON and its associated
95 compounds (20-22). BMS-777607 is a highly selective small molecule inhibitor of RON
96 (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 colorectal cancer cells (20, 22). In addition, over-expression 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 colorectal cancer.

In this study, we investigated the clinical significance of expression patterns in RON and PD-L1 in primary CRC samples, and we 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 effects changes in related signaling pathways in CRC cells. Our data suggest that RON inhibition may be a novel approach to augment immunotherapy in CRC.

MATERIALS AND METHODS

Cell lines and reagents

The HT29 and LoVo CRC cell lines were obtained from the American Type Cell Culture (ATCC, Manassas, VA, USA). Cell lines were cultured in growth medium supplemented with 10% FBS and incubated under standard cell culture conditions at 37°C and 5% CO₂. Rabbit anti-RON (5029) and mouse anti-RON mAb (Zt/f2 and Zt/g4) antibodies were used as previously described [28]. Human mature MSP was obtained from R&D Systems (Minneapolis, MN, USA). Phospho-tyrosine mouse monoclonal antibody (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) were obtained from Cell Signaling Technology (Beverly, MA, USA). CK antibody was from ZSGB-BIO (Cat # ZM-0069, Beijing, China). The BMS-777607 RON inhibitor was from Medchem Express (Monmouth Junction, NJ, USA). BMS-777607 was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mM and stored at -20°C. The PD-L1 antibody (Cat # 13864) for immunohistochemistry and multiplex immunofluorescence was obtained from Cell Signaling Technology (Beverly, MA, USA). The PD-L1 antibody (Cat # 205921) used for cell experiments was from Abcam (Burlingame, CA, USA). Opal® tyramine signal amplification (TSA) staining kit was from PerkinElmer (Hopkinton, MA, USA).

The Gene Expression Omnibus (GEO) 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/>)(29). 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 tumors characteristics, complete total survival (OS), complete 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, stage of lymph node metastasis (TNM), treatment regimen, and overall survival.

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 First Affiliated Hospital, Zhejiang University School of Medicine (FAHZUSM). Clinical data for patients included age, sex, tumor location, histological type, tumor lymph node metastasis (TNM) stage, tumor differentiation, treatment pattern, and overall survival 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 Table1. This study was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine (reference numbers: 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 mAb (1:150, 13864, CST) for PD-L1, 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, and a spectral library based on the single-stained slides was established. Paraffin-embedded sections were baked at 65°C for 2 h, dewaxed and rehydrated, and antigen-retrieval was performed with citric acid (pH = 6.0) in a microwave oven. After endogenous peroxidase was blocked, the tissues were incubated with the corresponding primary and secondary antibodies (PerkinElmer, USA) and opal diluent (PerkinElmer, USA). The primary antibodies/fluorescent dyes for PD-L1 /Opal 530, RON /Opal 690, and CK /Opal520 were applied to the CRC tissues in sequence. Finally, sections were stained with DAPI

and mounted with anti-fading mounting agent. PerkinElmer software version 2.1 was used to analyze multispectral images.

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) staining was performed according to standard protocols. Briefly, paraffin-embedded samples were cut into 4 μ m sections, baked at 65°C for 2h, de-waxed in xylene, rehydrated by fractionated ethanol, and endogenous peroxidase blocked with 3% hydrogen peroxide. Sections for staining with RON antibodies underwent antigen retrieval with citrate buffer (pH 6.0) in a pressure boiling vessel at 97°C for 20 minutes. Sections for staining with PD-L1 antibody underwent antigen retrieval with EDTA buffer (pH = 8.0) in a pressure boiling vessel at 97°C for 30 minutes. Sections were blocked with 0.3% BSA solution for 30 minutes. Primary antibodies were incubated overnight using a dilution of 1:150 for PD-L1 (13684, CST, USA) and 1:800 for RON. Mouse anti-RON mAb (Zt/f2) antibody was used as previously described (30). The primary antibody was visualized with diaminobenzidine until a brown precipitate appeared at the antigenic site using EnVision + System-HRP (Dako, Carpinteria, CA, USA). Then, sections were counterstained with hematoxylin.

Evaluation of RON and PD-L1 expression in colorectal cancer 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 final staining score ≤ 4 were considered to be low and those with score of > 4 were considered to be high for RON expression.

PD-L1 expression was evaluated using an anti-PD-L1 rabbit monoclonal antibody clone (13684, CST, USA). A cutoff 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 $\geq 5\%$ PD-L1 positive cells were considered to be high. This standard has been established in various types of cancer (31-33).

Flow cytometry

CRC cell lines were lifted with trypsin and resuspended at 1×10^6 cells per tube. After conjugating anti-mouse immunoglobulin G (IgG) with Alexa Fluor 488, the Zt/g4 antibody was used to detect RON on the surface of CRC cells (BD, New York, USA). PD-L1 was detected using anti-PD-L1 mAb conjugated with Alexa Fluor 647 (205921, Abcam, USA). Normal rabbit or mouse IgG was used as an isotype control. All flow cytometry experiments were performed on a BD FACS Canto II instrument (BD, New York, USA). Flow cytometric analysis revealed that RON was strongly expressed in HT29 cells, but not in LoVo cells, while PD-L1 was strongly expressed in HT29 and LoVo cells (Figure S1).

Cell viability

Cell viability was determined using a Cell Counting Kit 8 (MedChem Express, Junction of Monmouth, USA), according to the manufacturer's instructions. HT29 and LoVo cells

were seeded at a concentration of 5000 cells per well in a 96-well plate in biological triplicate. At 24 h post-seeding, cells were treated with the BMS-777607 RON inhibitor at various concentrations for 72 hours. Absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA). The results of Cell Counting Kit 8 illustrated that the half-maximal inhibitory concentrations (IC₅₀) of BMS-777607 in HT29 and LoVo cells at 72 h were 2 μ M and >10 μ M, respectively. These data demonstrate that BMS-777607 significantly reduced CRC cell viability in a time-dependent and dose-dependent manner in HT29 cells, but not in LoVo cells (Figure S2).

Total protein and phosphorylated protein expression determined by western blot

HT29 cells were treated with 2 nM MSP, 2 μ M BMS-777607 (72 h half-maximal inhibitory concentrations), and 2 nM MSP + 2 μ M BMS-777607 (72 h half-maximal inhibitory concentrations) for 24 h. Culture medium was removed and the cells were washed with ice-cold phosphate buffered saline (PBS). Cells were lifted with trypsin, collected, and washed with PBS to remove residual trypsin. Cells were lysed in radioimmunoprecipitation (RIPA) buffer, and 25 μ g total protein per sample were separated by 8% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE). Western blotting 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 an enhanced chemiluminescence detection system HRP substrate reagent and the VersaDoc MP 5000 Imaging system (Bio- Rad).

Immunofluorescence Analysis

HT29 cells were cultured overnight in a 6-well plate on a cover slide. After treatment with 2 nM MSP, 2 μ M BMS-777607, or 2 nM MSP + 2 μ M BMS-777607 for 24 h, cells were fixed with 4% paraformaldehyde at 37°C for 20 minutes, then washed with PBS five times for three minutes. Cover slides were then blocked with 5% BSA in PBS at room temperature for 30 minutes. After that, the cells were incubated with primary antibody against PD-L1 (205921, abcam, USA) and mouse anti-RON (Zt/f2) overnight at 4°C. Primary antibodies were then removed by washing with PBS five times for three minutes. 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 were expressed as mean $\pm$ standard deviation (SD) and count data was expressed as constituent ratio (%) or ratio (%). Statistical evaluation was conducted with SPSS 25.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism v.8 (La Jolla, CA, USA). X-tile 3.6.1 software (34)(Yale University, New Haven, CT, USA) was used to determine the optimal cut-off values for RON and PD-L1 expression in the GEO cohort. Chi-square tests were used to analyze relationships between clinicopathological parameters and RON and PD-L1 expressions. Differences between quantitative data with normal distribution were compared using two independent samples T-tests in two groups, and one-way analysis of variance in three groups, and between groups with SNK.

Overall survival (OS) was evaluated using the Kaplan-Meier method, and survival differences between groups were assessed using the log-rank test. Univariate and multivariate Cox regression analysis was used to calculate the relationships between clinical statistics and the overall survival (OS) of patients. A p-value of $p < 0.05$ was considered to be statistically significant. All confidence intervals (CIs) are stated at the 95 % confidence level.

Results

Patient Demographics

In the GEO cohort, the median age of all 287 CRC patients was 70 years, ranging from 24 to 97 years. There were 158 male patients (55.1%) and 129 female patients (44.9%). The median survival time was 58 months, and 94 patients died during follow-up. In the FAHZUSM cohort, the median age of the 381 CRC patients undergoing tumor resection was 61 years and ranged from 29 to 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 tumor, pathological grade, TNM stage, disease stage, histological type, and treatment status of CRC patients are shown in Table1.

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

In the GEO cohort, the cut-off values for RON and PD-L1 (TC) mRNA in the tumor tissue were determined by the X-tile program to be 7.70 and 4.30, respectively (Figure 1a-b), and the χ^2 log-rank value for RON and PD-L1 were 4.544 and 4.078, respectively. Patients in the GEO cohort were divided into two groups according to RON and PD-L1

status: $RON \leq 7.70$ and $RON > 7.70$, and $PD-L1 \leq 4.30$ and $PD-L1 > 4.30$ (Figure 1a-b).

Expression of RON and PD-L1 in CRC tumor tissue

In colorectal cancer tissue samples, multiple immunofluorescence staining was used to evaluate expression patterns of RON in tumor cells (TC) and PD-L1 in the tumor microenvironment. We found that both RON and PD-L1 exhibited membrane-enhanced expression in tumor cells (Figure 2a); elevated RON expression on tumor cell membranes was accompanied by enhanced PD-L1 expression in tumor-infiltrating mononuclear cells (TIMC) (Figure 2b). We also observed cases where PD-L1 expression was enhanced in the tumor cell membranes (Figure 2c) or in the TIMC (Figure 2d), but RON expression was low. Additionally, there were cases where RON expression was enhanced in tumor cell membranes, but PD-L1 was not expressed in the tumor microenvironment (Figure 2e). In addition, RON and PD-L1 were often accompanied by 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 TIMC. The results of multiplex immunofluorescence are consistent with 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 based on IHC staining. In 381 CRC patients, high and low RON expression in tumor cells (TC) were 121 (31.8%) and 260 (68.2%), respectively. High and low expression of PD-L1 in TC and TIMC were 43 (11.3%) and 91 (23.9%),

respectively, and PD-L1 was low in TIMC in 247 (64.8%) cases. None of the patients had high PD-L1 expression in both TC and TIMC. Among all samples expressing RON and PD-L1 in TC, the proportion with both high RON and high PD-L1 expression was 8.6% (25/290), while the proportion of samples expressing high PD-L1 in TIMC and high RON in TC 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 TC, demonstrating a significant correlation between high RON expression and high PD-L1 expression TC ($p < 0.001$, Table 1). The expression of PD-L1 in TIMC was not significantly correlated with RON expression in TC (Table 1). We compared clinical characteristics of patient groups categorized based on 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 TC was related to T stage (Table 2A). High expression of RON and PD-L1 (TIMC) was associated with T stage, principal diagnosis, histological type, and treatment ($p < 0.05$; Table 2B).

Prognostic significance of RON and PD-L1 expression in CRC

In the GEO cohort, Univariate Cox regression modeling showed that age, T stage, M stage, disease stage, RON, and PD-L1 (TC) expression were significantly correlated with poor prognosis of 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 3). Kaplan-Meier

analysis showed that high expression of RON, PD-L1 (TC), and both high RON and PD-L1 were all associated with 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 (TC) was associated with the primary site of tumor ($P < 0.05$; Table 1).

In the FAHZUSM cohort, Kaplan-Meier analysis showed that high expression of RON in TC, high PD-L1 in TC, or high PD-L1 in TIMC in colorectal cancer tissue samples was correlated with lower overall survival (OS) ($p < 0.05$) (Figure 4a-c). Univariate Cox regression modeling showed that age, gender, TNM stage, disease stage, pathological grading, RON, and PD-L1 expression were associated with poor OS in patients with CRC ($p < 0.05$). Multivariate analysis after adjustment showed that age, gender, T stage, M stage, pathological grading, RON, 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 3). 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 TC predicted a significantly worse overall survival, and high RON in TC and high PD-L1 in TIMC was associated with significantly worse prognosis ($p < 0.001$; Figure 4d-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 can down-regulated PD-L1 expression (Figure 5a). This result was confirmed by analysis of protein expression by western blot ($p < 0.05$; Figure 5b-c). In addition, we found that RON inhibition significantly reduces 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 colorectal cancer (CRC). This may be due in part to the prevalence of the mismatch repair (MMR) proficient or microsatellite stabilization (MSS) subtypes of CRC (35). However, even CRC tumors with microsatellite instability (MSI) can selectively up-regulate the expression of multiple immune checkpoints in the tumor microenvironment, such as PD-L1, to counterbalance the anti-tumor responses of active cytotoxic T lymphocytes/Th1 cell immune microenvironment, thereby reducing the efficacy of PD-L1 immunotherapy (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 colorectal cancer 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 overall survival rate compared to CRC tumors with other RON and PD-L1 expression states. We report that RON and PD-L1 are independent prognostic factors for OS in CRC patients. In addition, we show that RON phosphorylation leads 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 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 and high 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 of TC, 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 complete with pathological tumor samples, to further study the expression of RON and PD-L1 in colorectal cancer tissues and to determine the pathological significance of

elevated RON and PD-L1 expression in TC and in the cells of the tumor microenvironment.

Dysregulation of RON and PD-L1 signal transduction is a key feature of many tumors. Our results and previous studies demonstrate that high expression of RON or PD-L1 in CRC tumor tissues correlates 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 poor survival in CRC patients (22, 39-42).

Colorectal cancer is a heterogeneous disease caused by the interaction of many different oncogenes(4). And the immune system is also regulated by these oncogenes, which promotes tumor immune escape and reduces the effectiveness of immunosuppressive agents (43).

We demonstrate that high expression of both RON and PD-L1 in TC is associated with poor overall survival in CRC patients. Interestingly, the expression levels of RON are positively correlated with PD-L1 expression in TC, which has not been previously reported. This may be related to the fact that RON can regulate both the production and response to IFN- γ (44). In tumor cells, 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, 44-46).

In our study, we demonstrate that RON phosphorylation increases 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, MEK inhibitors, or down-regulation of ERK1/2 can lead to down-regulation of PD-L1 expression in tumor cells (47). Thus, RON phosphorylation may promote the expression of PD-L1 in tumor cells through the RAS-ERK signaling pathway. In addition, PTEN interacts with RTK-dependent signals at multiple levels (47), and has an antagonistic effect on PI3K, which plays an important role in mediating RTK-dependent cell signaling and tumor immune escape (10, 47, 48). Therefore, RON phosphorylation may interact with PTEN to activate the PI3K-AKT signaling pathway, thereby promoting PD-L1 expression in tumor cells.

Tumor-infiltrating mononuclear cells (TIMC) are regarded as an indicator of host immune response to a tumor, and have long been considered as an unfavorable prognostic marker in CRC (41, 42). Moreover, RON expression plays an important role in anti-tumor immune responses (27, 28). 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 (49, 50). In our study, we

demonstrate that high expression of both RON and PD-L1 (TIMC) is associated with poor overall survival in patients with CRC. Thus, high expression of RON may impair the anti-tumor immune response by promoting the expression of PD-L1 in TIMC, leading to tumor growth, invasion, and metastasis.

There were some limitations in our 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 the TIMC expressed PD-L1. Further studies are needed to determine the characteristics of immune cells expressing PD-L1 to study the relationship between RON expression and which specific immune cells express PD-L1. Third, although we have preliminarily studied that RON phosphorylation promotes the expression of PD-L1 in colorectal cancer cells, possibly due to activation of the ERK1/2 and AKT signaling pathways, the mechanism by which RON promotes PD-L1 expression still needs to be further studied.

Conclusion

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

450

451 **Declarations**

452

453 **Acknowledgements**

454 We thank Elixigen Corporation for reading our manuscript and providing native English
455 professional support.

456

457 **Funding**

458 The work was supported by National Natural Science Foundation of China grant
459 #81872883 (to HPY) and Zhejiang Major Medical Health & Sciences Technology
460 Foundation Projects #WKJ-ZJ-13 (to HPY), and Zhejiang Provincial Natural Science
461 Foundation of China grant #LY18H160014 (to XMX). The funders had no role in study
462 design, data collection and analysis, decision to publish, or preparation of the
463 manuscript.

464

465 **Availability of data and materials**

466 All data generated or analyzed during this study are included in the published paper.

467 More details can be provided on request.

468

469 **Abbreviations**

470 TC = Tumor cells

471 TIMC = Tumor-infiltrating mononuclear cells

472 CRC = Colorectal cancer

473 RON = Recepteur d'origine nantais

474 PD-L1 = Programmed death ligand 1
475 GEO = The Gene Expression Omnibus
476 FAHZUSM = The First Affiliated Hospital, Zhejiang University School of Medicine
477 PI-3K = Phosphatidylinositol 3-kinases
478 ERK = extracellular signal-regulated kinase
479 DAPI = 4',6-diamidino-2-phenylindole
480 IC50 = half maximal inhibitory concentration
481 FBS = Fetal bovine serum Fetal bovine serum
482 IC50 = Half-maximal inhibitory concentrations
483 CI = Confidence interval
484 IHC = Immunohistochemistry
485 mAb = monoclonal antibody
486 MSP = macrophage-stimulating protein
487 OS = Overall survival
488

489 **Authors' contributions**

490 Experimental design and supervision were carried out by HPY, XMX and MHW. Sample
491 collection, resources, immunohistochemistry, and multiplex immunofluorescence
492 analysis were conducted by HPY, YZL, BH, YQ, TMT, ZGW, SHY and XMX. YZL, DRS
493 and DTH performed cellular experiments *in vitro* and data analysis. The manuscript was
494 drafted by HPY, MHW and YZL. All authors read and approved the final manuscript.

495

496 **Ethics approval and consent to participate**

The Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine, approved the present study (Reference Number: 2017427-1).

Consent for publication

Not applicable.

Competing interests

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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Figure legends

Table 1

Bold entries indicate statistical significance ($p < 0.05$)

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 ($\chi^2 = 4.544$, $p = 0.033$). b. The optimal cut-off value for PD-L1 was 4.30 ($\chi^2 = 4.078$, $p = 0.043$). c. Evaluation of different RON and PD-L1 expression states on patient survival.

Figure 2

4-color multiplex immunofluorescence staining of paraffin-embedded colorectal cancer

tissue. a. High expression of RON and PD-L1 in TC. b. High expression of RON in TC and high expression of PD-L1 in TIMC. c. Lack of RON expression and high PD-L1 expression in TC. d. Low expression of RON in TC and high PD-L1 expression in TIMC. f. High RON expression in TC and no PD-L1 expressed in either TC or TIMC. Blue was used to visualize nuclei (DAPI), green shows PD-L1, pink indicates RON, and red displays CK. TC: tumor cells; TIMC: Tumor-infiltrating mononuclear cells.

Figure 3

Immunohistochemical expression of RON and PD-L1 in colorectal cancer tissue. a. High (right) and negative (left) expression of RON in TC. b. High (right) and negative (left) expression of PD-L1 in TIMC. c. High and negative expression of PD-L1 in TC (original magnification, $\times 100$ and $\times 200$). TC: tumor cells; TIMC: Tumor-infiltrating mononuclear cells.

Table 2

Bold entries indicate statistical significance ($p < 0.05$)

Table 3

^aAll variables are adjusted by the Cox proportional hazard model

^bBold entries indicate statistical significance ($p < 0.05$)

Figure 4

Kaplan-Meier survival analysis of overall survival of patients with colorectal cancer stratified by RON and/or PD-L1 expression. a. OS according to RON expression in TC; b.

OS according to PD-L1 expression in TC; c. OS according to PD-L1 expression in TIMC;
d. OS according to RON and PD-L1 expression in TC; f. OS according to RON
expression in TC and PD-L1 expression in TIMC. TC: tumor cells; TIMC:
Tumor-infiltrating mononuclear cells.

Figure 5

Expression of RON and PD-L1 in HT29 cells after treatment with 2 nM MSP, 2 nM MSP +
2 μ M BMS-777607, or 2 μ M BMS-777607. a. Cellular immunofluorescence indicating the
expression of RON and PD-L1 after treatment of HT29 cells with 2 nM MSP, 2 nM MSP +
2 μ M BMS-777607, or 2 μ M 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 $\times 400$ (all photomicrographs). b. HT29 cells were treated with 2 nM
MSP, 2 nM MSP + 2 μ M BMS-777607, or 2 μ M BMS-777607 for 24 h, and the
expression of RON and PD-L1 was detected by western blot and quantified according to
the immunoblots. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6

Expression of RON, PD-L1, and activation of signaling pathways in HT29 cells. a. HT29
cells were treated with 2 nM MSP, 2 nM MSP + 2 μ M BMS-777607, or 2 μ M
BMS-777607 for 1 hour. 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 were detected by
western blot and quantified. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure S1

Flow cytometric determination of RON and PD-L1 expression in CRC cells. CRC cells (1×10^6 cells/mL) resuspended in 1 mL PBS were incubated with 5.0 μ g Zt/g4 (yellow line) at room temperature for 60 minutes. Isotypes without antibodies matching mouse IgG (blue line) and blank (red line) were used as controls. CRC cells (1×10^6 cells/mL) resuspended in 1 mL PBS were incubated with rabbit anti-human PD-L1 (green line) for 30 minutes. Rabbit IgG without antibodies (blue line) and blank (red line) were used as controls.

Figure S2

Effects of BMS-777607 on the viability of colorectal cancer cells. LoVo and HT29 cells (in triplicates of 1×10^4 cells per well in 96-well plates) were treated with BMS-777607 at different concentrations for 24 h, 48 h and 72 h. The viability of colorectal cancer cells treated with BMS-777607 was measured by CCK-8 assay. The data shown are from one of three representative experiments, all with similar results.

Table 1
Comparison of RON and PD-L1 expression and clinicopathological characteristics of patients with colorectal cancer in FAHZUSM and GEO cohort

FAHZUSM cohort (N=381)											GEO cohort (N=287)						
Characteristics	Cases No	RON expression			PD-L1 expression						Cases No	RON expression			PD-L1 expression		
	(%)	Low	High	P Value	Low	High (TIMC)	P Value	Low	High (TC)	P Value	(%)	Low	High	P Value	Low	High (TC)	P Value
Cases Number	381	260	121		247	91		247	43		287	193	94		48	239	
Aqe (mean ± SD)	61.16±12.419	60.40±12.56	62.81±11.99	0.859	60.94±12.37	62.04±13.080	0.771	60.94±12.37	61.16±12.41	0.938	68.44±14.00	66.87±13.18	71.65±15.1	0.006	66.94±14.3	68.74±13.9	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	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	
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	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	
Pathological																	
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%)	37 (40.7%)		118 (47.8%)	22 (51.2%)		208 (72.5%)	144 (74.6%)	64 (68.1%)		39 (81.3%)	169	
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	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	
M stage																	
M0	368 (96.6%)	252 (96.9%)	116 (95.9%)	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	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																	
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	0.235
Stage (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	
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	
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 ^a							NA						
High (TIMC)	91 (23.9%)	65 (26.9%)	26 (27.1%)														
High (TC)	43 (11.3%)	18 (9.2%)	25 (26.3%)	<0.001^b													
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%)				

a. PD-L1 low (both TC and TIMC) vs PD-L1 high (TIMC). b. PD-L1 low (both TC and TIMC) vs PD-L1 high (TC)

Table 2A
Comparison of RON (TC) and PD-L1 (TC) expression and clinicopathological characteristics of patients with colorectal cancer in the FAHZUSM cohort

Characteristics	Cases No (%)	RON and PD-L1 (TC) 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	
Cases Number	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 grading						
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						
Stage (0-II)	187 (64.5%)	123 (69.5%)	39 (55.7%)	12 (66.7%)	13 (52.0%)	0.110
Stage (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%)	

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Table 2B
Comparison of RON (TC) and PD-L1 (TIMC) expression and clinicopathological characteristic of patients with colorectal cancer in the FAHZUSM cohort

Characteristics	Cases No (%)	RON and PD-L1 (TIMC) 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	
Cases Number	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 grading						
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						
Stage (0-II)	221 (65.4%)	123 (69.5%)	39 (55.7%)	45 (69.2%)	14 (53.8%)	0.104
Stage (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%)	

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Table 3
Univariate and multivariate Cox proportional hazard analysis of OS in patients with CRC in FAHZUSM and GEO cohorts

Variables ^a	FAHZUSM cohort				GEO cohort			
	Univariate analysis HR (95 % CI)	<i>p</i> ^b	Multivariate analysis HR (95 % CI)	<i>p</i> ^b	Univariate analysis HR (95 % CI)	<i>p</i> ^b	Multivariate analysis HR (95 % CI)	<i>p</i> ^b
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								
Stage (0-II)	1.000	<0.001	1.000	0.406	1.000	0.029	1.000	0.099
Stage 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 grading								
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								
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)	
PD-L1 (protein)								
Low	1.000	<0.001	1.000	0.001	NA			
High (TIMC)	0.544 (0.293-1.011)		0.676 (0.350-1.304)					
High (TC)	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)

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. S1

Fig. S2

Figures

Figure 1

Expression of RON, PD-L1, and activation of signaling pathways in HT29 cells. a. HT29 cells were treated with 2 nM MSP, 2 nM MSP + 2 μ M BMS-777607, or 2 μ M BMS-777607 for 1 hour. 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 were detected by western blot and quantified. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

Expression of RON and PD-L1 in HT29 cells after treatment with 2 nM MSP, 2 nM MSP + 2 μ M BMS-777607, or 2 μ M BMS-777607. a. Cellular immunofluorescence indicating the expression of RON and PD-L1 after treatment of HT29 cells with 2 nM MSP, 2 nM MSP + 2 μ M BMS-777607, or 2 μ M 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 $\times 400$ (all photomicrographs). b. HT29 cells were treated with 2 nM MSP, 2 nM MSP + 2 μ M BMS-777607, or 2 μ M BMS-777607 for 24 h, and the expression of RON and PD-L1 was detected by western blot and quantified according to the immunoblots. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3

Kaplan-Meier survival analysis of overall survival of patients with colorectal cancer stratified by ROR and/or PD-L1 expression. a. OS according to ROR expression in TC; b. OS according to PD-L1 expression in TC; c. OS according to PD-L1 expression in TIMC; d. OS according to ROR and PD-L1 expression in TC; e. OS according to ROR expression in TC and PD-L1 expression in TIMC. TC: tumor cells; TIMC: Tumor-infiltrating mononuclear cells.

Figure 4

Immunohistochemical expression of RON and PD-L1 in colorectal cancer tissue. a. High (right) and negative (left) expression of RON in TC. b. High (right) and negative (left) expression of PD-L1 in TIMC. c. High and negative expression of PD-L1 in TC (original magnification, $\times 100$ and $\times 200$). TC: tumor cells; TIMC: Tumor-infiltrating mononuclear cells.

Figure 5

4-color multiplex immunofluorescence staining of paraffin-embedded colorectal cancer tissue. a. High expression of RON and PD-L1 in TC. 681 b. High expression of RON in TC and high expression of PD-L1 in TIMC. c. Lack of RON expression and high PD-L1 expression in TC. d. Low expression of RON in TC and high PD-L1 expression in TIMC. f. High RON expression in TC and no PD-L1 expressed in either TC or TIMC. Blue was used to visualize nuclei (DAPI), green shows PD-L1, pink indicates RON, and red displays CK. TC: tumor cells; TIMC: Tumor-infiltrating mononuclear cells.

Figure 6

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 ($\chi^2 = 4.544$, $p = 0.033$). b. The optimal cut-off value for PD-L1 was 4.30 ($\chi^2 = 4.078$, $p = 0.043$). c. Evaluation of different RON and PD-L1 expression states on patient survival.