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

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Running Title: RON and PD-L1 expression in colorectal cancer

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Abstract:

Background: PD-L1 immunotherapy remains poorly efficacious in colorectal cancer. The RON receptor tyrosine kinase plays an important role in regulating tumor immunity. Here, we identify patterns of RON and PD-L1 expression and explore the clinical significance of these patterns in colorectal cancer.

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

Results: In the GEO cohort, cut-off values for RON and PD-L1 expression of 7.70 and 4.30, respectively, were determined. Stratification of patients based on these cutoffs demonstrated that high expression of RON and PD-L1 associated with poor prognosis. In the FAHZUSM cohort, rates of high expression of RON in tumor cells, high PD-L1 expression in tumor cells and in 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 and high expression of PD-L1 in the tumor cell compartment was significantly correlated (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 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 colorectal cancer cells.

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

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

BACKGROUND

In 2018, colorectal cancer (CRC) was ranked the fourth most common malignancy worldwide, accounting for 6.1% of the total number of cancers diagnosed, and responsible for 9.2% of total cancer deaths (1). The methods for treating CRC treatment are diverse, and include surgical resection, chemotherapy, and radiotherapy. Despite advances in these treatment methods, CRC often has a poor prognosis, and many patients will nevertheless have local recurrence and/or distant metastasis within five 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 and B7-H1) proteins are effective against many of cancer types (4). PD-L1 is a transmembrane immune checkpoint protein that can be expressed on both immune cells and on cancer cells, including CRC cells (5-7). When expressed in immune cells, PD-L1 can be regulated by a variety of inflammatory mediators and cytokines. When expressed on the surface of tumor cells, PD-L1 enables tumor cells 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 colorectal cancer 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 are most commonly seen in lung, pancreas, and colorectal adenocarcinoma, and KRAS mutations can up-regulate the expression of PD-L1 in colorectal cancer 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 over-expressed 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 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 Table 1. 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 μm 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, Carpentaria, 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
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 ≥ 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 μM and ≥10 μM, 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 μM BMS-777607 (72 h half-maximal inhibitory concentrations), and 2 nM MSP + 2 μM 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 μg 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 μM BMS-77677607, or 2 nM MSP + 2 μM 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 ± 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 Table 1.

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 $\chi^2$ 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
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.
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-regulate 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.
Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in the published paper. More details can be provided on request.

Abbreviations

TC = Tumor cells
TIMC = Tumor-infiltrating mononuclear cells
CRC = Colorectal cancer
RON = Recepteur d'origine nantais
Authors’ contributions

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

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 (χ² = 4.544, p = 0.033). b. The optimal cut-off value for PD-L1 was 4.30 (χ² = 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, ×100 and ×200). TC: tumor cells; TIMC: Tumor-infiltrating mononuclear cells.

Table 2

Bold entries indicate statistical significance (p < 0.05)

Table 3

a All variables are adjusted by the Cox proportional hazard model

b Bold 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
eexpression 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 μM BMS-777607, or 2 μM BMS-777607. a. Cellular immunofluorescence indicating the
eexpression of RON and PD-L1 after treatment of HT29 cells with 2 nM MSP, 2 nM MSP +
2 μM BMS-777607, or 2 μM 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. HT29 cells were treated with 2 nM
MSP, 2 nM MSP + 2 μM BMS-777607, or 2 μM 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 μM BMS-777607, or 2 μM
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 g Zt/g4 (yellow line) at room temperature for 60 minutes. Isotopes 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.
| Characteristics                  | Cases No (%) | RON expression | PD-L1 expression | Cases No (%) | RON expression | PD-L1 expression |
|----------------------------------|--------------|----------------|------------------|--------------|----------------|------------------|
| Cancer Number                    | 381 (100%)   | 360 (94.1%)    | 247 (64.4%)      | 287 (100%)   | 247 (85.8%)    | 143 (63.8%)      |
| Age (mean ± SD)                  | 61.9±12.4    | 60.9±12.3      | 60.5±11.9       | 61.6±12.4    | 60.8±12.3      | 60.6±11.9        |
| Sex                              | M            | 255 (66.8%)    | 244 (96.7%)      | 140 (61.5%)  | 255 (95.5%)    | 138 (98.0%)      |
|                                | F            | 126 (33.2%)    | 4 (3.9%)         | 107 (48.5%)  | 12 (4.5%)      | 6 (2.0%)         |
| Histological type                |              |                |                  |              |                |                  |
|                                 | Colon        | 215 (56.4%)    | 143 (66.4%)      | 104 (58.6%)  | 215 (95.2%)    | 138 (98.0%)      |
|                                | Rectum       | 176 (45.6%)    | 104 (58.6%)      | 71 (40.2%)   | 176 (94.5%)    | 147 (89.0%)      |
| Pathological stage               |              |                |                  |              |                |                  |
|                                 | N0           | 246 (64.6%)    | 162 (66.3%)      | 116 (63.4%)  | 246 (91.6%)    | 162 (90.6%)      |
|                                | N1 (2)       | 214 (55.8%)    | 144 (66.9%)      | 100 (57.4%)  | 214 (95.2%)    | 138 (98.0%)      |
|                                | N2           | 251 (65.9%)    | 164 (65.4%)      | 116 (65.6%)  | 251 (96.7%)    | 164 (96.7%)      |
|                                | N3           | 91 (23.9%)     | 62 (68.1%)       | 41 (45.1%)   | 91 (100%)      | 62 (100%)        |
|                                | Other        | 355 (93.6%)    | 241 (67.8%)      | 151 (42.8%)  | 355 (100%)     | 241 (100%)       |
| Disease stage                    |              |                |                  |              |                |                  |
|                                 | Stage I/II   | 381 (100%)     | 360 (93.8%)      | 247 (64.4%)  | 287 (100%)     | 247 (85.8%)      |
|                                | Stage III/IV | 355 (92.9%)    | 241 (67.8%)      | 151 (42.8%)  | 355 (100%)     | 241 (100%)       |
|                                | Unknown      | 35 (9.8%)      | 21 (63.6%)       | 9 (54.5%)    | 35 (100%)      | 21 (100%)        |
| PD-L1 status                     |              |                |                  |              |                |                  |
|                                 | Low          | 287 (75.6%)    | 177 (61.6%)      | 106 (43.4%)  | 287 (100%)     | 177 (100%)       |
|                                | High (TC)    | 65 (17.1%)     | 26 (26.0%)       | 19 (14.5%)   | 65 (100%)      | 26 (100%)        |
|                                | High (TIMC)  | 13 (3.4%)      | 6 (46.2%)        | 3 (23.1%)    | 13 (100%)      | 6 (100%)         |

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

FAHUSM cohort (N=381)

| Characteristic                  | Cases No (%) | RON expression | PD-L1 expression |
|----------------------------------|--------------|----------------|------------------|
| Cancer Number                    | 381 (100%)   | 360 (94.1%)    | 247 (64.4%)      |
| Age (mean ± SD)                  | 61.9±12.4    | 60.9±12.3      | 60.5±11.9       |
| Sex                              | M            | 255 (66.8%)    | 244 (96.7%)      |
|                                | F            | 126 (33.2%)    | 4 (3.9%)         |
| Histological type                |              |                |                  |
|                                 | Colon        | 215 (56.4%)    | 143 (66.4%)      |
|                                | Rectum       | 176 (45.6%)    | 104 (58.6%)      |
| Pathological stage               |              |                |                  |
|                                 | N0           | 246 (64.6%)    | 162 (66.3%)      |
|                                | N1 (2)       | 214 (55.8%)    | 144 (66.9%)      |
|                                | N2           | 251 (65.9%)    | 164 (65.4%)      |
|                                | N3           | 91 (23.9%)     | 62 (68.1%)       |
|                                | Other        | 355 (93.6%)    | 241 (67.8%)      |
| Disease stage                    |              |                |                  |
|                                 | Stage I/II   | 381 (100%)     | 360 (93.8%)      |
|                                | Stage III/IV | 355 (92.9%)    | 241 (67.8%)      |
|                                | Unknown      | 35 (9.8%)      | 21 (63.6%)       |
| PD-L1 status                     |              |                |                  |
|                                 | Low          | 287 (75.6%)    | 177 (61.6%)      |
|                                | High (TC)    | 65 (17.1%)     | 26 (26.0%)       |
|                                | High (TIMC)  | 13 (3.4%)      | 6 (46.2%)        |

GEO cohort (N=287)

| Characteristic                  | Cases No (%) | RON expression | PD-L1 expression |
|----------------------------------|--------------|----------------|------------------|
| Cancer Number                    | 287 (100%)   | 287 (100%)     | 287 (100%)       |
| Age (mean ± SD)                  | 60.8±12.4    | 60.8±12.3      | 60.8±12.3        |
| Sex                              | M            | 167 (58.1%)    | 167 (58.1%)      |
|                                | F            | 114 (39.1%)    | 114 (39.1%)      |
| Histological type                |              |                |                  |
|                                 | Colon        | 132 (45.6%)    | 132 (45.6%)      |
|                                | Rectum       | 156 (54.4%)    | 156 (54.4%)      |
| Pathological stage               |              |                |                  |
|                                 | N0           | 194 (67.7%)    | 194 (67.7%)      |
|                                | N1 (2)       | 110 (38.4%)    | 110 (38.4%)      |
|                                | N2           | 26 (9.1%)      | 26 (9.1%)        |
|                                | Other        | 10 (3.3%)      | 10 (3.3%)        |
| Disease stage                    |              |                |                  |
|                                 | Stage I/II   | 287 (100%)     | 287 (100%)       |
|                                | Stage III/IV | 287 (100%)     | 287 (100%)       |
|                                | Unknown      | 35 (12.1%)     | 35 (12.1%)       |
| PD-L1 status                     |              |                |                  |
|                                 | Low          | NA             | NA               |
|                                | High (TC)    | 65 (17.1%)     | 26 (26.0%)       |

Note: 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 FAH7USIM cohort

| Characteristics | Cases (No.% | RON low/PD-L1 low | RON high/PD-L1 low | RON high/PD-L1 high | PD-L1 low/PD-L1 low | PD-L1 high/PD-L1 high | P value |
|-----------------|-------------|-------------------|-------------------|-------------------|-------------------|-------------------|---------|
| Cases Number    | 250         | 198 (86.8%)       | 42 (16.8%)        | 10 (4%)           | 135 (54.0%)      | 113 (45.0%)      | 0.034   |
| Age (mean ± SE) | 60 ±13.3 (2) | 60 ±13.3 (2)      | 62 ±13.3 (2)      | 59 ±13.3 (2)      | 60 ±13.3 (2)      | 60 ±13.3 (2)      | 0.008   |
| Sex             | 191 (89.0%) | 59 (29.0%)        | 29 (69.2%)        | 9 (20.0%)         | 141 (70.4%)       | 9 (20.0%)         | 0.001   |
| Primary tumor   | 128 (51.2%) | 66 (26.4%)        | 36 (87.0%)        | 9 (20.0%)         | 92 (44.8%)        | 9 (20.0%)         | 0.004   |
| Stage           | 152 (60.8%) | 71 (28.3%)        | 57 (68.1%)        | 14 (17.8%)        | 134 (65.5%)       | 19 (23.9%)        | 0.148   |
| Pathological grade | 103 (41.2%) | 45 (18.0%)        | 37 (35.8%)        | 6 (5.8%)          | 88 (43.3%)        | 11 (10.6%)        | 0.250   |
| Performance     | 289 (83.1%) | 134 (54.0%)       | 154 (77.2%)       | 24 (27.8%)        | 238 (90.3%)       | 20 (8.2%)         | 0.003   |
| **T Stage**     |              |                   |                   |                   |                   |                   |         |
| T1              | 0.00         |                   |                   |                   |                   |                   |         |
| T2              | 62 (25.0%)   | 32 (51.6%)        | 20 (62.5%)        | 6 (19.4%)         | 36 (55.1%)        | 16 (24.6%)        | 0.493   |
| T3              | 138 (55.2%)  | 66 (47.8%)        | 48 (62.5%)        | 8 (10.2%)         | 92 (66.7%)        | 20 (14.5%)        | 0.115   |
| **N Stage**     |              |                   |                   |                   |                   |                   |         |
| N0              | 230 (88.0%)  | 112 (49.1%)       | 56 (72.5%)        | 8 (10.2%)         | 170 (73.7%)       | 21 (15.4%)        | 0.673   |
| N1              | 32 (12.0%)   | 11 (34.4%)        | 10 (31.3%)        | 3 (9.4%)          | 22 (68.8%)        | 5 (15.6%)         | 0.058   |
| **Histological Type** |        |                   |                   |                   |                   |                   |         |
| Adenocarcinoma  | 287 (82.0%)  | 152 (85.9%)       | 71 (69.6%)        | 10 (9.9%)         | 202 (70.1%)       | 25 (8.7%)         | 0.439   |
| Mucinous/SRCC   | 64 (18.0%)   | 15 (23.4%)        | 20 (62.5%)        | 4 (6.3%)          | 49 (76.6%)        | 7 (10.9%)         | 0.111   |
| **Treatment**   |              |                   |                   |                   |                   |                   |         |
| Yes             | 239 (96.0%)  | 129 (53.6%)       | 70 (77.8%)        | 10 (4.2%)         | 207 (83.3%)       | 21 (8.5%)         | 0.014   |
| No              | 14 (6.0%)    | 10 (71.4%)        | 3 (21.4%)         | 3 (21.4%)         | 10 (71.4%)        | 3 (21.4%)         | 0.014   |

### Table 2B
Comparison of RON (TC) and PD-L1 (TC) expression and clinicopathological characteristics of patients with colorectal cancer in the FAH7USIM cohort

| Characteristics | Cases (No.% | RON low/PD-L1 low | RON high/PD-L1 low | RON high/PD-L1 high | PD-L1 low/PD-L1 low | PD-L1 high/PD-L1 high | P value |
|-----------------|-------------|-------------------|-------------------|-------------------|-------------------|-------------------|---------|
| Cases Number    | 329         | 236 (71.4%)       | 42 (13.3%)        | 51 (15.5%)        | 256 (77.8%)       | 53 (16.2%)        | 0.036   |
| Age (mean ± SE) | 60 ±13.3 (2) | 60 ±13.3 (2)      | 60 ±13.3 (2)      | 60 ±13.3 (2)      | 60 ±13.3 (2)      | 60 ±13.3 (2)      | 0.036   |
| Sex             | 257 (78.6%) | 183 (71.2%)       | 32 (12.9%)        | 42 (16.5%)        | 230 (86.5%)       | 37 (13.5%)        | 0.004   |
| Primary tumor   | 206 (62.8%) | 143 (56.0%)       | 36 (17.5%)        | 25 (12.4%)        | 181 (73.7%)       | 25 (12.4%)        | 0.004   |
| Stage           | 171 (52.1%) | 120 (70.3%)       | 21 (23.0%)        | 10 (11.1%)        | 151 (88.5%)       | 16 (9.2%)         | 0.001   |
| Pathological grade | 152 (46.0%) | 107 (67.6%)       | 37 (24.2%)        | 8 (5.3%)          | 133 (87.2%)       | 19 (12.8%)        | 0.001   |
| Performance     | 326 (82.1%) | 216 (66.0%)       | 60 (18.5%)        | 50 (15.5%)        | 276 (84.8%)       | 40 (12.8%)        | 0.001   |
| **T Stage**     |              |                   |                   |                   |                   |                   |         |
| T1              | 24 (24.0%)  | 16 (66.7%)        | 4 (16.7%)         | 4 (16.7%)         | 19 (79.2%)        | 5 (20.8%)         | 0.004   |
| T2              | 83 (25.3%)  | 53 (63.7%)        | 17 (20.5%)        | 13 (15.7%)        | 50 (59.5%)        | 32 (40.5%)        | 0.002   |
| T3              | 113 (34.2%) | 72 (63.6%)        | 27 (23.8%)        | 20 (17.6%)        | 87 (76.4%)        | 26 (23.6%)        | 0.001   |
| **N Stage**     |              |                   |                   |                   |                   |                   |         |
| N0              | 220 (67.0%) | 152 (69.1%)       | 57 (65.9%)        | 10 (9.1%)         | 202 (91.9%)       | 25 (10.1%)        | 0.014   |
| N1              | 109 (33.0%) | 64 (58.7%)        | 34 (30.3%)        | 20 (18.3%)        | 89 (81.3%)        | 24 (21.3%)        | 0.014   |
| **Histological Type** |        |                   |                   |                   |                   |                   |         |
| Adenocarcinoma  | 287 (82.0%) | 152 (53.1%)       | 71 (69.6%)        | 10 (9.9%)         | 202 (70.1%)       | 25 (8.7%)         | 0.439   |
| Mucinous/SRCC   | 64 (18.0%)  | 15 (23.4%)        | 20 (62.5%)        | 4 (6.3%)          | 49 (76.6%)        | 7 (10.9%)         | 0.111   |
| **Treatment**   |              |                   |                   |                   |                   |                   |         |
| Yes             | 239 (96.0%) | 129 (53.6%)       | 70 (77.8%)        | 10 (4.2%)         | 207 (83.3%)       | 21 (8.5%)         | 0.014   |
| No              | 14 (6.0%)   | 10 (71.4%)        | 3 (21.4%)         | 3 (21.4%)         | 10 (71.4%)        | 3 (21.4%)         | 0.014   |

Note: L1 (TC) expression and clinicopathological characteristics of patients with colorectal cancer.
Table 3
Univariate and multivariate Cox proportional hazard analysis of OS in patients with CRC in FAHZUSM and GEO cohorts

| Variable                  | FAHZUSM cohort               | GEO cohort               |
|---------------------------|------------------------------|--------------------------|
|                           | Univariate analysis          | Multivariate analysis    | Univariate analysis          | Multivariate analysis    |
|                           | HR (95% CI)                  | P                         | HR (95% CI)                  | P                         |
|                           | Univariate analysis          | Multivariate analysis    | Univariate analysis          | Multivariate analysis    |
|                           | HR (95% CI)                  | P                         | HR (95% CI)                  | P                         |
| Age (Mean ± SD)           | 1.029 (1.011-1.048)          | 0.002                     | 1.031 (1.011-1.050)          | 0.002                     |
| Gender                    | Male                         | 1.000                     | 0.891                       | 1.000                     | 0.808                     |
|                           | Female                       | 0.562 (0.382-0.874)       | 0.527                       | 0.326-0.847               | 0.614 (0.525-1.194)       | 0.054 (0.449-1.039)       |
| Principal Diagnosis       | Rectum                       | 1.000                     | 0.208                       | 1.000                     | 0.318                     | 1.000                     |
|                           | Colon                        | 0.796 (0.518-1.224)       | 0.290 (0.467-1.235)         | 1.081 (0.738-1.508)       | 0.882 (0.549-1.322)       |
| Histological type         | Adenocarcinoma               | 1.000                     | 0.408                       | 1.000                     | 0.267                     | NA                       |
|                           | Mucinous/SRCC                | 2.494 (0.343-17.710)      | 2.720 (0.263-28.122)        |
|                           | Unknown                      | 3.323 (0.433-25.988)      | 1.232 (0.129-11.756)        |
| T stage                   | T1-T2                        | 1.000                     | <0.001                      | 1.000                     | 0.003                     | <0.001                   | 1.000                     | 0.007                     |
|                           | T3                           | 0.211 (0.108-0.413)       | 0.288 (0.141-0.632)         | 0.333 (0.145-0.766)       | 0.381 (0.193-0.751)       |
|                           | T4                           | 0.553 (0.325-0.781)       | 0.586 (0.361-0.907)         | 0.450 (0.292-0.709)       | 0.476 (0.298-0.769)       |
| N stage                   | N0                           | 1.000                     | <0.001                      | 1.000                     | 0.003                     | <0.001                   | 1.000                     | 0.006                     |
|                           | Stage III-IV                 | 3.156 (2.083-4.783)       | 5.376 (0.678-42.607)        | 1.435 (0.382-2.097)       | 0.396 (0.083-1.479)       |
| M stage                   | M0                           | 1.000                     | <0.001                      | 1.000                     | 0.039                     | <0.001                   | 1.000                     | 0.008                     |
|                           | Stage (0-1)                  | 4.338 (2.094-8.685)       | 2.445 (1.048-5.701)         | 3.546 (0.213-6.427)       | 2.460 (1.280-4.835)       |
|                           | Stage III-IV                 | 3.102 (2.044-4.708)       | 0.459 (0.050-3.393)         | 1.520 (1.045-2.242)       | 3.924 (0.774-19.888)      |
| Pathological grading      | Well/moderate                | 1.000                     | 0.001 (0.050-3.386)         | 1.520 (1.045-2.242)       | 3.924 (0.774-19.888)      |
|                           | Poor                         | 7.989 (2.434-24.295)      | 4.046 (1.023-15.320)        |
|                           | Unknown                      | 1.286 (0.313-5.526)       | 0.375 (0.104-1.287)         |
| RON                       | Low                          | 1.000                     | 0.001                       | 1.000                     | 0.005                     | 1.000                     | 0.007                     |
|                           | High                         | 2.060 (1.364-3.112)       | 1.788 (1.136-2.808)         | 1.515 (1.030-2.238)       | 1.170 (0.776-1.795)       |
| PD-L1 (protein)           | Low                          | 1.000                     | <0.001                      | 1.000                     | <0.001                    | 1.000                     | <0.001                    |
|                           | High                         | 0.546 (0.293-1.011)       | 0.674 (0.320-1.504)         |
|                           | High (TIMC)                  | 1.325 (0.669-2.611)       | 1.654 (0.819-3.334)         |
|                           | Low (mRNA)                   | NA                        | 1.000                      | 0.047                     | 1.000                     | 0.083                     |
|                           | High                         | 1.823 (1.607-3.348)       | 1.719 (0.931-3.173)         |
Fig. 1
Fig. 6
Fig. S1

Fig. S2