Abstract. Tyrosine kinase receptor macrophage stimulating 1 receptor (MST1R, also known as RON) contributes to the transformation and malignant progression observed in epithelial cells. The purpose of the present study is to assess the value of RON as a potential target in bladder cancer (BC) therapeutics. The expression profile of RON in BC tissues and adjacent noncancerous tissues was detected via immunohistochemistry. The rate of positive RON expression differed significantly between bladder urothelial cancer tissues (54.7%) and paraneoplastic tissues (29.4%) (P<0.05). RON expression was positively associated with the number of tumors per patient, histological grading, pathological stage and distant metastasis (all P<0.05). Downregulation of RON expression using small interfering RNAs inhibited cell growth, cell migration and promoted cell apoptosis in the 5637 cell line. RON inhibition induced cell cycle arrest at the G1/S boundary following an increase of cyclin-dependent kinase inhibitor 1B and cyclin-dependent kinase inhibitor 1A, and a decrease of cyclin D1, cyclin D3 and cyclin-dependent kinase 4 expression. Furthermore, knockdown of RON significantly blocked signal transduction, including downstream protein kinase B and mitogen-activated protein kinase pathways. These results indicated that RON serves a notable role in BC and is a potential target of therapeutic intervention.

Introduction

Bladder cancer (BC) is one of the most common types of cancer globally, with high morbidity and mortality rates (1). The incidence of BC is increasing in much of the world (1); however, few novel approaches for the treatment of this disease have been developed in the past two decades (2). Only programmed cell death (PD) 1/PD-ligand 1 based immunotherapy has displayed promising results for the management of BC in recent years (3). Cisplatin-based combination chemotherapy remains the preferred first-line treatment for advanced or metastatic BC (4). Limited progress in improving outcomes has created a major incentive for the analysis of molecular alterations in BC, the identification of novel potential targets and to accelerate the identification of novel treatments for this disease (5).

Macrophage stimulating 1 receptor (MST1R, also known as RON) is a membrane tyrosine kinase receptor that has been considered to be a valuable target in cancer therapy (6). Elevated RON expression has been detected in a number of malignant tumor types, including those of the colon, breast and pancreas (7-9). RON overexpression has been reported to have prognostic value in predicting patient survival and clinical outcome in these types of cancer (7-9). The roles served by RON in BC have been studied extensively in vitro and in vivo, and results have demonstrated that RON and other tyrosine kinase receptors, including epidermal growth factor receptor and MET, exerted their effects collaboratively in BC carcinogenesis (10,11). Furthermore, macrophage-stimulating protein, which is the only known ligand of RON, was also detected in human urine samples (11). These results indicated that RON serves a key role in the development and progression of BC.

The results of the previous study indicated that the inhibition of RON by its specific monoclonal antibody (mAb) zt/g4 in human BC cell lines lead to reduced cell growth and motility, which provided evidence that RON is a potential target in BC (11). In the present study, the expression of RON tyrosine kinase receptor was further assessed via immunohistochemistry (IHC) in human BC tissues and adjacent noncancerous tissues. Furthermore, the effect of RON on BC cell proliferation, apoptosis and migration was also analyzed via the knockdown of RON expression with specific small interfering RNAs (siRNAs).
Materials and methods

Main reagents. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Dojindo laboratories, Inc., Kumamoto, Japan). Mouse mAb zuf/2 specific to RON immunoglobulin, plexins and transcriptional factor domain and rabbit antibody R5029 (specific to the RON C-terminal peptide) were provided by Professor Yao (Laboratory of Cancer Biology and Therapeutics, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China). The primary antibodies rabbit anti-β-actin (cat. no. 4970; dilution, 1:1,000), anti-p38 (cat. no. 8690; dilution, 1:1,000), anti-phospho-p38 (cat. no. 4511; dilution, 1:1,000), anti-extra-cellular signal-regulated kinase 1/2 (ERK1/2) (cat. no. 9102s; dilution, 1:1,000), anti-phospho-ERK1/2 (cat. no. 4370s; dilution, 1:1,000), anti-protein kinase B (Akt) (cat. no. 4685s; dilution, 1:1,000), anti-phospho-Akt (cat. no. 4060s; dilution, 1:1,000), anti-cyclin D1 (cat. no. 2978; dilution, 1:1,000), anti-cyclin D3 (cat. no. 2936; dilution, 1:2,000), anti-cyclin-dependent kinase 4 (CDK4; cat. no. 12790; dilution, 1:1,000), anti-cyclin-dependent kinase inhibitor 1A (p21) (cat. no. 2947; dilution, 1:1,000), and rabbit antibody R5029 (specific to the RON C-terminal immunoglobulin, plexins and transcriptional factor domain and rabbit antibody R5029 (specific to the RON C-terminal peptide) were provided by Professor Yao (Laboratory of Cancer Biology and Therapeutics, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China). The primary antibodies rabbit anti-β-actin (cat. no. 4970; dilution, 1:1,000), anti-phospho-p38 (cat. no. 4511; dilution, 1:1,000), anti-extra-cellular signal-regulated kinase 1/2 (ERK1/2) (cat. no. 9102s; dilution, 1:1,000), anti-phospho-ERK1/2 (cat. no. 4370s; dilution, 1:1,000), anti-protein kinase B (Akt) (cat. no. 4685s; dilution, 1:1,000), anti-phospho-Akt (cat. no. 4060s; dilution, 1:1,000), anti-cyclin D1 (cat. no. 2978; dilution, 1:1,000), anti-cyclin D3 (cat. no. 2936; dilution, 1:2,000), anti-cyclin-dependent kinase 4 (CDK4; cat. no. 12790; dilution, 1:1,000), anti-cyclin-dependent kinase inhibitor 1A (p21) (cat. no. 2947; dilution, 1:1,000) and anti-cyclin-dependent kinase inhibitor 1B (p27) (cat. no. 3686; dilution, 1:1,000) were all purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Fetal bovine serum (FBS), RPMI-1640, L-glutamine and penicillin were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Tissue collection and ethics statement. Specimens from patients with BC (n=106) were obtained during surgical tumor resection in the Ningbo First Hospital (Ningbo, China) and with pathological identification in the Ningbo Diagnostic Pathology Center (Ningbo, China) between March 2011 and July 2014. Of the total patients, there were 87 males and 19 females, aged between 35 and 84 years old (mean age, 68 years old). The available adjacent non-cancerous samples were also obtained 1.5 cm away from the cancer tissues and confirmed by pathologists. None of the patients had undergone chemotherapy or radiotherapy prior to surgery. The present study was reviewed and approved by the Ethical Committee of Ningbo First Hospital (Ningbo, China) and all patients provided written informed consent prior to sample collection.

IHC and scoring. The tissue samples were fixed with 4% paraformaldehyde for 24 h at room temperature, embedded in paraffin and 4-μm thick sections were prepared. Following deparaffinization with xylene and ethanol (100% ethanol for 5 min, 95% ethanol for 3 min, 85% ethanol for 3 min and 70% ethanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 3 min), the tissue sections were incubated for 30 min at room temperature in 0.3% H2O2 to block endogenous peroxidase activities. For antigen retrieval, samples were incubated with 0.1 M citrate buffer (pH 6.0) (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) in boiling water for 10 min. Following rinsing in PBS, non-specific antigens were blocked by 3% normal bovine serum (Beijing Solarbio Science & Technology Co., Ltd.) for 15 min at room temperature, the slides were incubated with the anti-RON antibody zuf/2 used at a 1:200 dilution at 4°C overnight and subsequently incubated with anti-mouse IgG (H+L) antibody (cat. no. ab6789, dilution, 1:500; Abcam, Cambridge, MA, USA) for 1 h at room temperature. Detection was performed using an EnVision system according to the manufacturers protocol (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and visualized with diaminobenzidine as substrate.

Six views were observed per slide and 100 cells were examined per view at x400 magnification under a light microscope (Nikon Corporation, Tokyo, Japan). The stain of RON was scored depending on the staining proportion (0, 0-4%; 1, 5-24%; 2, 25-49%; 3, 50-74%; and 4, 75-100%) and staining intensity (the intensity of RON membrane staining: No staining, 0; weak staining, 1; moderate staining, 2; and strong staining, 3). Finally, a cumulative evaluation of scores based on the addition of the two results ranging from 0-7 was conducted. The total expression of RON was considered as overexpression with a score ≥4 or normal expression with a score <4.

Cell line and cell culture. The human 5637 BC cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO2 in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin.

Western blot analysis. Western blot analysis was performed as previously described (11). Cellular proteins (100 μg/sample) were separated by 8% or 12% SDS-PAGE under reduced conditions. Individual proteins were detected using the aforementioned primary antibodies followed by horseradish peroxidase-coupled secondary antibodies (cat. no. ab205718, dilution, 1:5,000; Abcam,Cambridge,MA,USA). Densitometry was analyzed using Quantity One software (version 4.4.02; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Transfection of BC cells. Gene silencing was performed using scrambled siRNA (cat. no. sc-37007, nominated as si-Scr) and humanRON sequence-specific duplex siRNA (cat. no. sc-36434, nominated as si-RON), which were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The 5637 cells were seeded into a plate when they were 50-60% confluent at the time of transfection. The 5637 cells were transfected with 75 pmol si-RON and si-Scr using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. At 48 h after transfection, the expression of RON was analyzed via western blot analysis as previously stated.

Cell viability assay. Sensitivity of the cells to RON knockdown by si-RON was analyzed using the CCK-8. Briefly, a total of 1x10^4/well cells were seeded in 96-well plates in 100 μl culture medium (RPMI-1640 with 10% FBS) for 24 h at 37°C and then transfected with si-RON and si-Scr as previously described. Following transfection, cells were cultured for 0, 24, 48, 72 h individually. The cells were then treated with 10 ul/well of CCK-8 solution and incubated for another 2 h at 37°C. The absorbance values of each well were measured using a Multiskan Go plate reader (Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm. The experiment was performed in triplicate.
Flow cytometric assays for cell cycle distribution and apoptosis. Transfected cells (2x10^5) were harvested and fixed with 75% ethanol overnight at -20°C. The fixed cells were treated with 1 mg/ml RNase A (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in darkness at 37°C for 30 min and with 50 µg/ml propidium iodide (PI; Sigma-Aldrich; Merck KGaA) for 30 min at 37°C. The cells were analyzed using a flow cytometer (FACScan; BD Biosciences, Franklin Lakes, NJ, USA) to investigate cell cycle distribution. Quadrant analysis was performed using the CellQuest Pro software (version 5.1; BD Biosciences, Franklin Lakes, NJ, USA).

The cell apoptosis assay was performed using an Annexin-V/Fluorescein Isothiocyanate Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Transfected cells were collected and washed twice with cold PBS prior to staining with Annexin V and PI solution for 15 min in darkness at room temperature. The ratios of apoptotic cells were determined using a flow cytometer (FACScan; BD Biosciences).

Wound healing assay. Cells from the si-RON and the si-Scr groups were incubated in RPMI-1640 with 10% FBS in 6-well plates. Following transfection with si-RON or si-Scr, a small wound area was produced in the 90% confluent monolayer using a 200-µl pipette tip in a lengthwise stripe. Following incubation at 37°C for 24 h, the area covered by migrated cells was examined under a light microscope (Nikon Corporation) and images were captured at x100 magnification. Experiments were repeated in triplicate.

Statistical analysis. Statistical analyses were performed using the SPSS software package (version 20; IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation. Statistical analysis was conducted using the Student’s t-test for paired samples and the χ²-test was used for univariate analysis of RON proteins. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of RON protein in BC and paraneoplastic tissues. The presence of RON protein was observed in the plasma membrane of BC cells, whereas the paraneoplastic tissues exhibited negative or low staining (Fig. 1). The association between total RON expression and clinical parameters was analyzed. As summarized in Table 1, the positive expression rates of the RON was 54.7% (58/106) in BC cases and 17.6% (6/34) in paraneoplastic tissues. RON overexpression was positively associated with the number of tumors the patient had, histological grading, pathological stage and distant metastasis. No statistical differences were observed between RON overexpression and characteristics of age and sex.

Knockdown of RON inhibits cell proliferation in the 5637 cells. It was previously determined that the level of RON expression in 5637 cells was high (11), therefore 5637 cells were selected for future studies. To confirm the knockdown efficiency of si-RON, si-RON was transiently transfected into the 5637 cells. As depicted in Fig. 2A and B, at 48 h following transfection, the protein expression level of RON was significantly downregulated by transfection with si-RON, compared with si-Scr (P<0.01). A CCK-8 assay was performed to analyze the proliferation rate of the 5637 cells following silencing of RON. The results of the CCK-8 assay demonstrated that si-RON notably inhibited cell proliferation, compared with cells treated with si-Scr (Fig. 2C, P<0.05).

Downregulation of RON expression inhibits 5637 cell migration. To assess whether the downregulation of RON affected the cellular migration, a wound-healing assay was conducted on the 5637 cells in the si-RON and si-Scr groups. As depicted in Fig. 3, the number of migrated cells in the si-RON group (47.37±7.02%) was significantly lower than that in the si-Scr group (65.00±5.57%) at 24 h (P<0.05) once the wound was produced on the cell monolayer, which indicated that the downregulation of RON expression could significantly decrease the migration of 5637 cells.

Knockdown of RON promotes apoptosis in the 5637 cells. Flow cytometry was performed to investigate whether the knockdown of RON increased the proportion of apoptotic 5637 cells. Flow cytometric analysis indicated that the proportion of early and late apoptotic cells were significantly increased in cells in the si-RON group, compared with those in si-Scr group (Fig. 4A and B; P<0.01).

Knockdown of RON induces cell cycle arrest at the G1/S phase in the 5637 cells. Owing to the growth inhibitory response of si-RON treatment in 5637 cells, the effect of this treatment on the cell cycle distribution was studied. The 5637 cells were incubated with si-RON or si-Scr for 48 h, following which cell cycle analysis was performed. The proportions of si-RON group cells were significantly reduced in S phase and increased in G1 phase, compared with those of si-Scr cells (Fig. 5A and B; P<0.05). Furthermore, the effects of RON on CDKs and CDK inhibitors (CDKIs) were evaluated, which are involved in regulation of cell cycle arrest in BC cells (11). The 5637 cells treated with si-RON exhibited an increased expression of p27 and p21, whereas the expression of cyclin D1, cyclin D3 and CDK4 were decreased compared with cells transfected with si-Scr (Fig. 5C).

Knockdown of RON decreases the phosphorylation of Akt and mitogen-activated protein kinases (MAPKs) in 5637 cells. To investigate the potential signaling pathways involved...
in RON-mediated cellular migration, apoptosis and cell cycle arrest, the phosphoinositide 3-kinase (PI3K)-Akt and MAPK pathways were selected for further study as the two pathways are responsible for cell proliferation, migration, apoptosis and cell cycle arrest (12). As depicted in Fig. 6, the phosphorylation levels of Akt, ERK1/2 and p38 in the si-RON group of 5637 cells were lower than those in the si-Scr group at 48 h. The levels of total Akt, ERK1/2 and p38 appeared unchanged.

Discussion

The aberrant expression of RON has been verified to serve a notable role in the development and progression of colon, breast, bladder and pancreatic cancer, and other types of epithelial cancer (7-14). Overexpression of RON is associated with poor pathological characteristics in these cancer types (15). In the present study, 106 BC clinical specimens were examined for RON expression. The majority of patient specimens (54.7%) were determined to be positive for RON expression, and the level of RON expression in the cancer tissues was significantly higher than that in the adjacent tissues (Fig. 1). Subsequently, analysis of the association between the expression of RON and clinicopathological features revealed that the positive expression rate of RON protein was closely associated with the number of tumors, distant metastasis, histological grading and pathological stage (Table I). Cheng et al (9) previously reported that RON was overexpressed in only 32.8% of analyzed BC samples. The disparity in results between the study by Cheng et al (9) and the present study could be attributed to the difference in antibodies used to detect RON expression, and different criteria used to interpret the results. In the present study, the level of RON expression was evaluated in a standardized and semi-quantitative manner using the mAb zt/f2, which is frequently regarded as an effective tool in immunochemistry for its high reactivity, specificity and minimal background in human tissue sections (15).

Overexpression of RON is associated with oncogenic properties, including the promotion of cellular proliferation, migration, invasion and survival in several human cancer cell lines (16-19). To verify the potential role of RON in BC cells, its expression level in BC cell lines was analyzed and the 5637 cells were selected for further study as they have a relatively high RON expression (11). Subsequently, si-RON was used to knockdown RON expression in the 5637 cells (Fig. 2A). The results indicated that knockdown of RON could notably inhibit cellular proliferation (Fig. 2B) and decrease migration (Fig. 3).

### Table I. Association between the RON expression and the clinical pathological features in bladder cancer.

<table>
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<tr>
<th>Variables</th>
<th>Patients, n</th>
<th>Low</th>
<th>High</th>
<th>(\chi^2)-value</th>
<th>P-value</th>
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<td></td>
<td></td>
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<td>58</td>
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</tr>
<tr>
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<td>14</td>
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<tr>
<td>&lt;70</td>
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<td>(\geq70)</td>
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<td>25</td>
<td>30</td>
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<td>Number of tumor nodules</td>
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<tr>
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<tr>
<td>Multiple</td>
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<td>11</td>
<td>32</td>
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<td>Distant metastasis</td>
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<td>M1</td>
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RON, macrophage stimulating 1 receptor; T, tumor; M, metastasis.
Figure 2. Silencing RON impaired BC cell proliferation. Silencing RON impaired BC cell proliferation. Western blot analysis results indicated RON specific siRNA significantly downregulated the protein expression level. (A) The effect of RON siRNA on RON protein expression level using western blotting and (B) quantification of RON protein expression level in 5637 cells. (C) CCK-8 assay was performed following RON siRNA treatment. A reduction in absorbance was observed upon treatment with si-RON, when compared with si-Scr. Values are presented as the mean ± standard deviation from three independent experiments. *P<0.05 and **P<0.01, compared with si-Scr group. RON, macrophage stimulating 1 receptor; si-Scr, scramble small interfering RNA; si-RON, human RON sequence-specific duplex siRNA; OD, optical density.

Figure 3. Downregulation of RON expression inhibited the migration of the 5637 cells. (A) A wound-healing assay was performed on si-RON- and si-Scr-treated 5637 cells. (B) The proportion of migrated cells in the si-RON group (47.33±7.02%) was significantly less than those of si-Scr group (65.00±5.57%) at 24 h (*P<0.05) following the wound being produced on the monolayer cells. The percentage of the wounded area covered by the migrated cells was measured. Data depicted here are from one of three experiments with similar results. RON, macrophage stimulating 1 receptor; si-Scr, scramble small interfering RNA; si-RON, human RON sequence-specific duplex siRNA.
Figure 4. Silencing RON induced apoptosis in 5637 cells. Flow cytometry was used to detect the proportion of cells in early and late apoptosis following transfection with si-RON, and compared with si-Scr. (A) Cells were transfected with si-RON or si-Scr for 48 h, collected, stained with Annexin V and PI and then analyzed by flow cytometry. (B) Quantitative results were obtained using Annexin V/PI staining. Data are derived from three independent experiments and are expressed as the mean ± standard deviation. **P<0.01, compared with si-Scr group. FITC, fluorescein isothiocyanate; RON, macrophage stimulating 1 receptor; si-Scr, scramble small interfering RNA; si-RON, human RON-targeting small interfering RNA; PI, propidium iodide.

Figure 5. Silencing RON induced cell cycle arrest in 5637 cells. Silencing RON induced cell cycle arrest in the 5637 cells. (A) Representative results of flow cytometric analysis. (B) Quantitative analysis of cell cycle distribution of the 5637 cells treated with si-RON or si-Scr. (C) Representative western blot analysis demonstrated changes in the expression of cyclins, cyclin-dependent kinases and cyclin-dependent protein kinase inhibitors following si-RON or si-Scr treatment in the 5637 cells. *P<0.05, compared with si-Scr group. Values are presented as the mean ± standard deviation from three independent experiments. RON, macrophage stimulating 1 receptor; si-Scr, scramble small interfering RNA; si-RON, human RON sequence-specific duplex siRNA; CDK4, cyclin dependent kinase 4.
The potential functions of RON in the regulation of cell apoptosis and cell cycle progression have been investigated in several cancer cell lines (20-22). However, there is limited information regarding the influence of RON on apoptosis and cell cycle progression in human BC. In the present study, RON knockdown promoted apoptosis and induced G₁/S arrest in the 5637 cells (Figs. 4 and 5). CDKs and cyclins serve a crucial role in the regulation of cell cycle progression (23,24). The G₁ cyclin-CDK complex cyclin D-CDK4/6 induces a transition from G₁ to S phase (25). CDKIs have been regarded as putative tumor suppressors (26). p21 and p27 are CDKIs that bind to cyclin-CDK complexes to inactivate them, inhibiting cell cycle progression (26). The results indicated that RON knockdown promoted G₁/S arrest via the downregulation of cyclin D1, cyclin D3 and CDK4, and the upregulation of p21 and p27, indicating that RON serves a vital role in regulating cell cycle-associated proteins in BC (Fig. 5C).

RON signaling is conventionally transmitted by the RAS-MAPK cascade and the PI3K-Akt pathway (12). Activation of the PI3K-Akt pathway is involved in RON-mediated cell apoptosis, proliferation, migration and cell matrix invasion (12,27,28). Furthermore, RON-mediated MAPK signaling cascade directs various cellular programs, including cell growth, migration, survival and differentiation (12,19,28,29). To observe whether si-RON inhibited downstream signal transduction, si-RON and si-Src were transfected into the 5637 cells. Data indicated that the Ser473 phosphorylation levels on Akt and the phosphorylation of ERK1/2 and p38 were significantly decreased by RON knockdown in these cells (Fig. 6). Considering these results, it appears likely that si-RON induced the inhibition of Akt and MAPK phosphorylation; these processes are involved in phenotypic changes to 5637 cells, including inhibition of cell proliferation and motility, induction of G₁/S cell cycle arrest and increased apoptosis. Knockdown of RON by siRNA affected the phosphorylation level of Akt, ERK1/2 and p38; however, the more complicated mechanisms underlying the Akt and MAPK signaling pathways following RON knockdown require further investigation in future studies.

In summary, the data produced in the present study indicated that RON was overexpressed in BC and was associated with poor pathological features. Knockdown of RON regulates BC cell behaviors through the modulation of Akt and MAPK signaling in human BC cells. These results indicate that RON is a potential target for therapeutic intervention in BC and have provided an experimental basis for future investigations.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81272828 to Dr QM), Zhejiang Provincial Foundation for Medical and Health Sciences (grant nos. 2016KYB263 and 2014KYB355 to Dr QM, and grant no. 2017KY576 to Dr JFC) and Ningbo Natural Science Foundation (grant no. 2015A610224 to Dr JFC, and grant no. 2016A610159 to Dr XYL).

Availability of data and materials

The authors declare that the datasets generated are all included in the current study and are available from the corresponding author on reasonable request.

Authors’ contributions

JFC participated to the experimental design, interpreted the results and wrote the manuscript; BXY, LM and XYL performed experiments and analyzed the results; JHJ coordinated the experimental work, interpreted the results and contributed to the critical revision; QM designed the research plan, interpreted the results and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
References