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ORIGINAL RESEARCH

REG4 is a Potential Biomarker for Radiochemotherapy Sensitivity in Colorectal Cancer

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Purpose: Colorectal cancer (CRC) is one of the most common types of malignancies, and radiochemotherapy (RCT) followed by surgery is the recommended approach for CRC treatment. However, some cases do not respond to first-line conventional chemotherapy or even progress further after treatment. Moreover, there is a risk of severe side effects, such as radiodermatitis. Therefore, identifying predictors for RCT sensitivity is an essential step toward predicting and eventually overcoming resistance.

Materials and Methods: We used integrative bioinformatics analysis and experimental validation to show that regenerating family member 4 (*REG4*) may be a potential biomarker for RCT sensitivity in CRC.

Results: *REG4*, whose expression is upregulated in some CRC tissues and downregulated in RCT-sensitive CRC cells, was identified as a potential genetic marker for RCT sensitivity in CRC. Immunohistochemistry-based tissue microarray of human CRC was used to experimentally validate *REG4* data obtained from the bioinformatics analysis.

Conclusion: Collectively, these results indicate that *REG4* may be a potential biomarker for RCT sensitivity in CRC.

Keywords: radiochemotherapy resistance, regenerating family member 4, immunohistochemistry microarray, colon cancer transcriptome

Introduction

Colorectal cancer (CRC), a challenging malignancy to treat, is responsible for approximately 10% of diagnosed cancers and cancer-related deaths annually worldwide.[1] Bimodal therapy consisting of surgical resection and adjuvant radiochemotherapy (RCT) has been the preferred option to optimize clinical outcomes for patients with CRC; approximately one-third of CRC patients respond favorably, whereas the rest respond poorly.[1,2] However, with respect to RCT, two undesirable adverse effects commonly arise in non-responders: (1) complications that threaten long-term quality of life, such as radiodermatitis, neuropathy, and liver toxicity; and (2) unpredictable tumor spread resulting from delayed surgical resection due to ineffective RCT.[2] This evidence highlights the lack of clinically useful biomarkers that can help forecast therapeutic responses in patients receiving RCT. Such biomarkers would enable effective personalized treatment instead of long and ineffective procedures.

Biomarker screening based on high-throughput platforms has been stressed as a promising approach for multiple diseases.[3–5] By adopting integrative bioinformatics and experimental analyses, this approach is helpful in identifying potential genetic

biomarkers to accurately predict the RCT response.[6,7] For instance, a non-invasive blood-based test, the ColonSentry® test, is commercially available to stratify the risk for patients with CRC.[3] Previously, we also identified TIMP1 as a molecule potentially highly correlated with the prognosis of ulcerative colitis-associated CRC using this strategy.[5]

In this study, by combining multiple bioinformatics methods and experimental validations, we identified regenerating family member 4 (*REG4*) as a potential biomarker for predicting the response to RCT in patients with CRC.

Materials and Methods

Clinical Tissue Specimens

From May 2012 to February 2017, 146 CRC patients were enrolled from the Department of Colorectal Surgery, Shanghai East Hospital. All procedures were approved by the hospital ethics committee in accordance with the Declaration of Helsinki. All patients agreed to the study procedure and signed consent forms. The average age of patients was 55.1 ± 12.5 years, with no other history of malignancies history. All patients underwent an imaging examination and clinical stages were defined based on the results of this examination. Before receiving preoperative neoadjuvant RCT, cancerous and normal tissue samples were collected from these patients through colonoscopy and developed into tissue microarrays. Sensitivity to RCT was assessed based on these tissues samples. The clinical response of tumors to RCT was evaluated according to National Comprehensive Cancer Network guidelines (2011 edition) with four-level evaluation criteria. Grade 0 indicated no cancer cell residue; Grade 1 indicated only a single cell or cellular cluster residue; Grade 2 indicated a fibrosis reaction that exceeds the cancer cell residue; Grade 3 indicated large tumor tissue residue with no fibrosis. Patients with Grade 0 to 1 were regarded as the RCT-sensitive group (n = 80 cases). Patients with Grade 2 to 3 were regarded as the RCT-resistant group (n = 66 cases).

Data Processing

The following gene expression profiles were retrieved from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo): GSE8671, GSE110224, and GSE15781. The GSE8671 dataset contained 32 colorectal adenomas and paired normal mucosa from the same individuals.[8] The GSE110224 dataset consisted of 17 colorectal adenocarcinomas and paired normal mucosa from the same individuals.[9] The GSE15781 dataset contained 21 patients with CRC, of which 11 patients received only surgery, while the other 10 patients received RCT preoperatively followed by surgery.[10] Colorectal tissues and the corresponding normal tissues were collected from the same individuals. We compared the microarray data from tumor tissues of patients receiving RCT with those from patients receiving surgery only. These datasets were analyzed using GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) as introduced previously.[5] $P < 0.05$ and $|\log_2 FC| \geq 1$ were considered significant.

Expression Analyses

The mRNA and protein levels of REG4 were validated using Gene Expression Profiling Interactive Analysis (http://gepia.cancer-pku.cn/) and The Human Protein Atlas (https://www.proteinatlas.org/), respectively.[11] Exosomal expression of *REG4* was downloaded from exoRBase (http://www.exorbase.org/).[12]

Functional Enrichment Analyses

Database for Annotation, Visualization and Integrated Discovery (https://david-d.ncifcrf.gov/) was used to perform Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses.[13]

Protein-Protein Interaction (PPI) Analysis

To generate a PPI network, the genes were mapped to Search Tool for the Retrieval of Interacting Genes (http://string-db.org) with a confidence score > 0.4. Two modules were then obtained using loaded Molecular Complex Detection in Cytoscape.[14]

Receiver Operating Characteristic (ROC) Curve Analysis

To evaluate the association between *REG4* expression and RCT sensitivity, MedCalc statistical software (version 19.2.6, https://www.medcalc.org/) was employed to calculate the ROC curve. Additionally, the 95% confidence intervals and log rank p values were calculated.

Western Blotting

Collected tissues were lysed in lysis buffer (Cell Signaling Technology, USA) supplemented with protease inhibitors (Calbiochem, USA) at 4 °C for at least 30 min. Protein samples were quantified using a bicinchoninic acid assay kit (Pierce, USA). Western

blotting was performed as described previously.[15] Bands were captured using the LI-COR system (Odyssey, USA). Primary antibodies against β-actin (cat#ab8227, 1:1000) and REG4 (cat#ab255820, 1:1000) were purchased from Abcam (Cambridge, UK).

Tissue Microarray Analysis

Clinical tumor tissues were fixed with 4% paraformaldehyde for at least 24 h. Dehydration and paraffin embedding included following treatments: 75% ethanol (4 h), 85% ethanol (2 h), 90% ethanol (2 h), 95% ethanol (1 h), 100% ethanol (0.5 h), 100% ethanol (0.5 h), ethanol and xylene mix (5–10 min), xylene I (5–10 min), xylene II (5–10 min), paraffin I (65 °C, 1 h), paraffin II (65 °C, 1 h), and paraffin III (65 °C, 1 h). Tissues microarray slides were then generated based on these paraffin-embedded samples. Deparaffinization included following treatments: xylene I (3 min), xylene II (3 min), xylene and ethanol (100%) mix (3 min), 100% ethanol I (3 min), 100% ethanol II (3 min), 95% ethanol (3 min), 70% ethanol (3 min), and 50% ethanol (3 min). Antigen retrieval and blocking of endogenous peroxidase with 3% H_2O_2 were then conducted. After incubation with 10% normal serum in phosphate-buffered saline for 1 h, slides were incubated with the primary antibody against REG4 (1:500) overnight at 4 °C, followed by incubated with the secondary antibody. Slides were then developed with a 3,3-diaminobenzidine tetrahydrochloride solution and counterstained with hematoxylin. Dehydration included following treatments: 75% ethanol (3 min), 85% ethanol (3 min), 100% ethanol I (5 min), 100% ethanol II (5 min), xylene I (10 min), and xylene II (10 min). Slides were then mounted; images were scanned by the Pannoramic MIDI scanner (3D HISTECH) and analyzed with the Quant center by calculating the H-Score.[16]

Statistical Analyses

Data are expressed as means ± standard deviation. Paired groups were compared using a two-tailed Student's *t*-test. $P < 0.05$ was considered significant. Statistical analyses were performed using the Prism 7 software (GraphPad, USA).

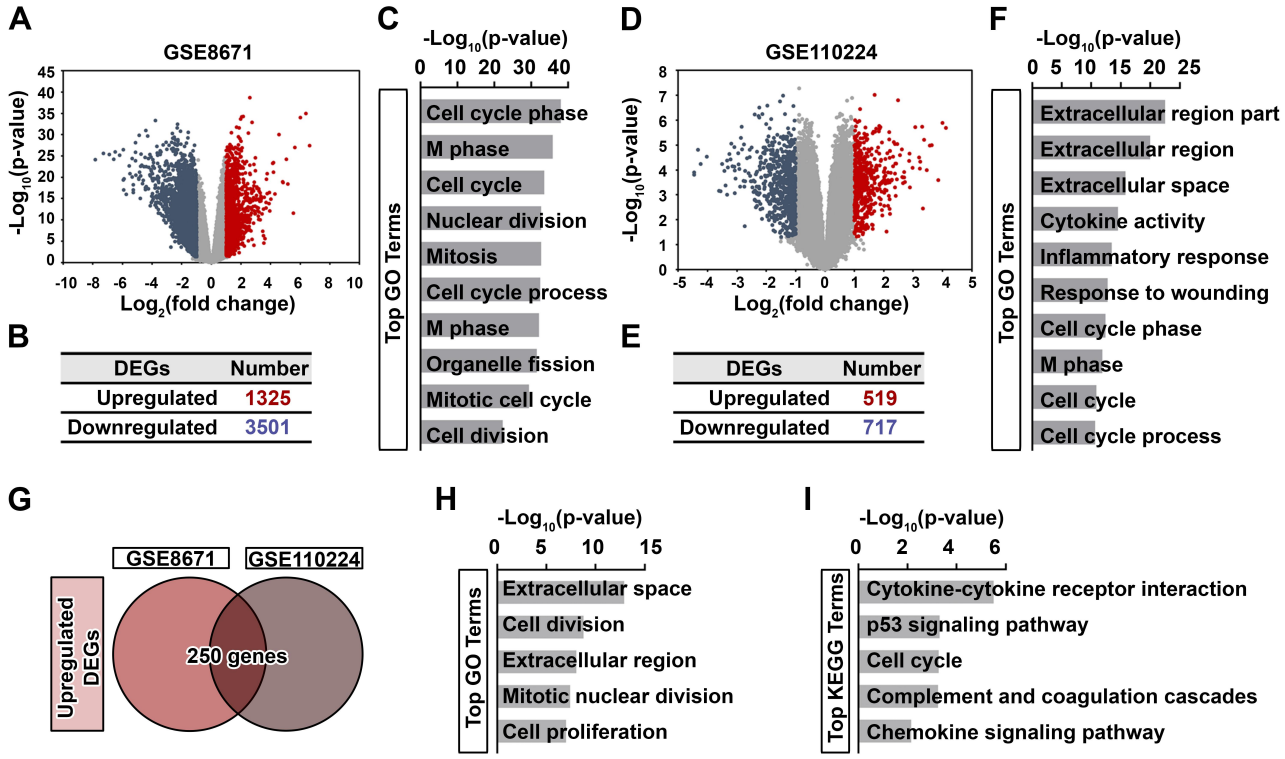

Figure 1 Generation and functional analysis of DEGs upregulated in CRC. Volcano plot (**A**) and the distribution (**B**) of DEGs in CRC tissues based on GSE8671 dataset. Red dots, significantly upregulated DEGs; blue dots, significantly downregulated DEGs; gray dots, no significant difference. $P < 0.05$ and fold change > 2 were considered as significant. (**C**) Top 10 enriched GO terms of significantly upregulated DEGs are indicated. Volcano plot (**D**) and the distribution (**E**) of DEGs in CRC tissues based on GSE110224 dataset. Red dots, significantly upregulated DEGs; blue dots, significantly downregulated DEGs; gray dots, no significant difference. $P < 0.05$ and fold change > 2 were considered as significant. (**F**) Top 10 enriched GO terms of significantly upregulated DEGs are indicated. (**G**) Venn diagram of significantly upregulated DEGs between GSE8671 and GSE110224 datasets. Top 5 enriched GO terms (**H**) and KEGG pathways (**I**) of these 250 genes are indicated.

Results

Generation and Functional Analysis of Differentially Expressed Genes (DEGs) Upregulated in CRC

To obtain the functionally important genes in CRC, the GSE8671 dataset was retrieved (Figure 1A); 1325 significantly upregulated DEGs in CRC tissues were identified (Figure 1B). The potential functions of these genes were then predicted by performing Gene Ontology (Figure 1C) and Kyoto Encyclopedia of Genes and Genomes (Figure S1A) analyses. As expected, these upregulated DEGs were highly enriched in cell cycle-related biological processes.[2] Additionally, the GSE110224 dataset comprising 17 paired normal and CRC tissues, was downloaded (Figure 1D); 519 significantly upregulated DEGs were obtained (Figure 1E). These genes were also highly enriched in cell cycle-related biological processes (Figure 1F and S1B). To acquire more reliable data on functional genes in CRC, upregulated DEGs in both the GSE8671 and GSE110224 datasets were intersected to reduce the number of candidates (Figure 1G). The overlapped DEGs were highly enriched in cell proliferation- and cell cycle-related biological processes (Figure 1H and I). Taken together, these bioinformatics-based results highlighted 250 potential functional candidate genes in CRC.

Identification of REG4 as a Highly Susceptible Gene in Response to RCT in CRC

It is imperative to identify reliable biomarkers to identify patients that will benefit from RCT, avoiding long, costly, and ineffective procedures in favor of treatments with precision medicine. Accordingly, we retrieved the GSE15781 dataset consisting of CRC samples receiving RCT and paired control samples (Figure 2A); 1396 downregulated DEGs were obtained (Figure 2B). These downregulated DEGs were significantly enriched in the apical part of the cell (Figure 2C) and aldosterone-regulated sodium reabsorption (Figure S2). To decrease the number of potential candidates, the identified oncogenes (Figure 1G) were intersected with the 1396 downregulated DEGs, 26 genes were identified (Figure 2D). The top three RCT-responsive genes included *S100P, REG4*, and *SDR16C5* (Figure S3A), suggesting that *REG4* might be

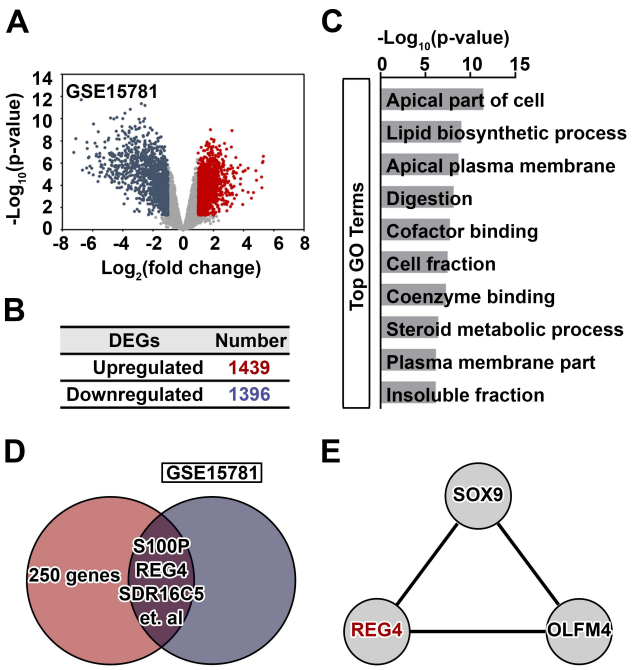

Figure 2 Identification of *REG4* as a highly susceptible gene in response to RCT treatment in CRC. Volcano plot (**A**) and the distribution (**B**) of DEGs in tissues collected from patients receiving RCT treatment based on GSE15781 dataset. Red dots, significantly upregulated DEGs; blue dots, significantly downregulated DEGs; gray dots, no significant difference. $P < 0.05$ and fold change > 2 were considered as significant. (**C**) Top 10 enriched GO terms of significantly downregulated DEGs are indicated. (**D**) Venn diagram showing the shared genes between 250 identified genes and significantly downregulated DEGs obtained from GSE15781 dataset. (**E**) Module#2 exported from PPI network generated based on the shared genes.

a predictive biomarker for RCT response in CRC patients. To identify the hub genes, a PPI network was constructed based on these 26 overlapping genes. Two modules with high scores were exported after using Molecular Complex Detection in Cytoscape (Figure 2E and S3B). We found that *REG4* was a highly RCT-responsive hub gene. Therefore, our results suggest that *REG4* can be regarded as a potentially essential gene for RCT sensitivity in CRC patients.

REG4 is a Potential Predictor for RCT Sensitivity in CRC Patients

Several bioinformatics and experimental methods were adopted to verify the expression of *REG4* in CRC tissues as well as its prognostic value for RCT. As shown in Figure 3A, the mRNA expression of *REG4* was significantly increased in CRC tissues based on the results from Gene Expression Profiling Interactive Analysis. By using the Human Protein Atlas, we found that *REG4* was highly expressed in CRC tissues and rarely detected in non-

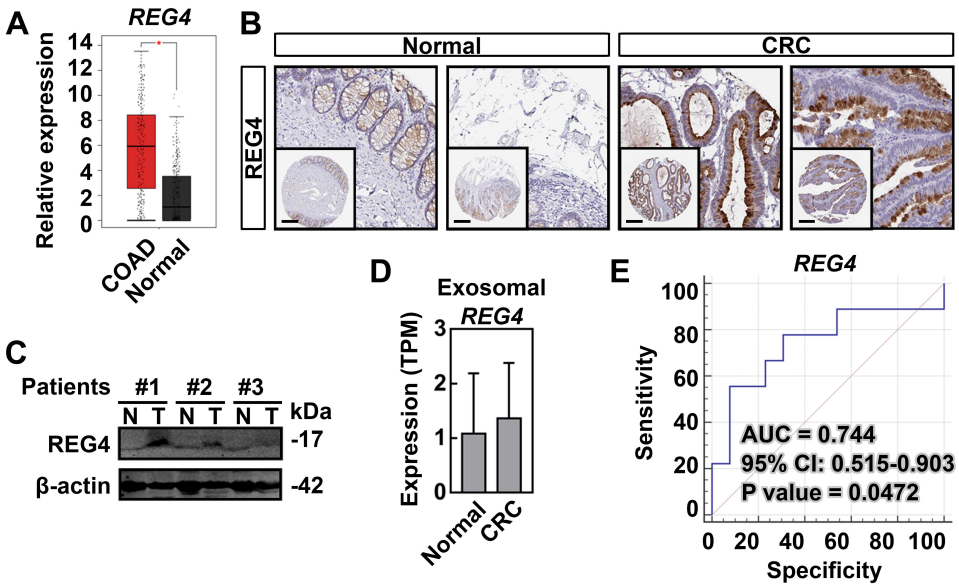

Figure 3 Experimental validation of the expression of REG4 in CRC. (**A**) REG4 is significantly upregulated in CRC tissues compared with normal colorectal tissues based on GEPIA. *, significantly different from normal tissues ($p < 0.05$). (**B**) The protein expression of *REG4* in normal and CRC tissues, respectively. Representative immunohistochemistry staining images are obtained from the Human Protein Atlas online database (magnification, x40). (**C**) Western blot analysis of REG4 in tumor (T) and non-tumor (N) tissues collected from CRC patients. (**D**) The relative expression of *REG4* in serum exosomes downloaded from exoRBase. (**E**) ROC curve analysis of *REG4* based on GSE15781 dataset.

malignant tissues (Figure 3B). Three patients with CRC were enrolled, and malignant and adjacent non-malignant tissues were collected; levels of REG4 were also significantly increased in CRC tissues (Figure 3C and S5). There was no significant difference in the expression of *REG4* in serum exosomes collected from control and CRC patients (Figure 3D). To evaluate the potential diagnostic value of *REG4*, an ROC curve analysis was performed based on the GSE15781 dataset. The area under the curve value was 0.744 ($p = 0.0472$), highlighting the sensitivity and specificity of *REG4* in distinguishing RCT-responsive samples from the control (Figure 3E). Altogether, these results suggest that *REG4* is a potential RCT-responsive biomarker in CRC patients.

Immunohistochemistry-Based Validation of REG4 as a Potential Biomarker for RCT Sensitivity in CRC

To verify the role of *REG4* in the response to RCT, a clinical specimen-based histological analysis of patients with CRC was conducted (Figure S4). These CRC patients with lower *REG4* expression responded efficiently to RCT (Figure 4A and B). To quantify the potential value of *REG4* for RCT sensitivity in CRC, an ROC curve analysis was conducted. As shown in Figure 4C, the area under the curve value was 0.675 ($p < 0.0001$), highlighting the sensitivity and specificity of *REG4* expression in distinguishing RCT-sensitive from resistant samples (Table S1). Collectively, our data verify that CRC patients with an reduce expression of *REG4* is more sensitive to RCT in the clinic.

Discussion

In this study, *REG4* was initially identified as a highly expressed gene in CRC. Moreover, a significant decrease in the expression of *REG4* was found in CRC patients receiving RCT. This reduction has shown favorable diagnostic value regarding whether the patient has received effective RCT. Furthermore, RCT-resistant and -sensitive clinical specimen-based immunohistochemical validation of *REG4* indicated that it was a potential biomarker for RCT sensitivity in CRC.

CRC is one of the most common malignancies worldwide; genetic alterations and pathological characteristics collaboratively mark the malignant transformation from early to invasive neoplasia.[1,17,18] In this study, by combining multiple bioinformatics methods and experimental validation, *REG4* was identified as a marker to assess RCT sensitivity in CRC patients; patients with low *REG4* expression were more likely to benefit from RCT. A previous study also suggested that high expression of *REG4* was associated with a poor therapeutic response,

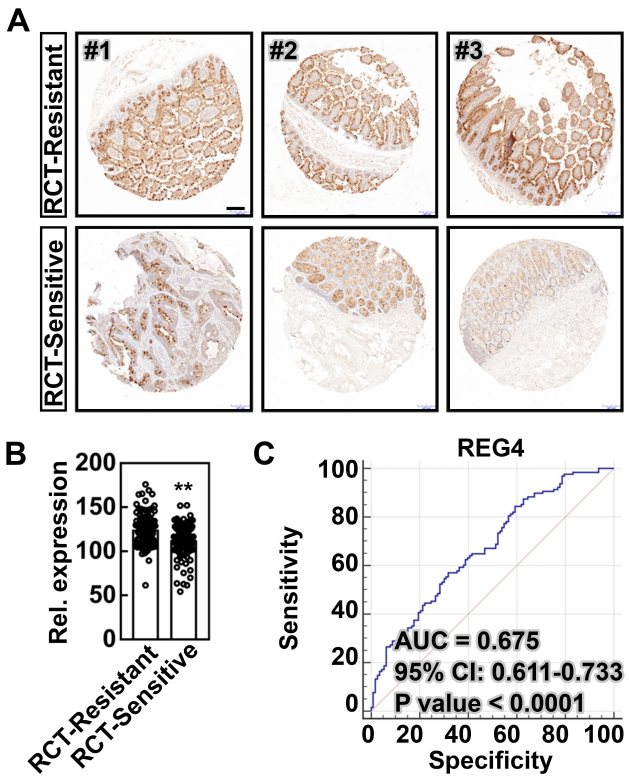

Figure 4 Immunohistochemistry-based validation of REG4 as a potential biomarker for RCT sensitivity in CRC. (**A**) Representative immunohistochemical images of REG4 in RCT-resistant and RCT-sensitive CRC tissues collected from the clinic. Scale bar, 200 μm. (**B**) Quantification of REG4. **, significantly different from RCT-resistant CRC samples ($p < 0.01$). (**C**) ROC curve analysis of REG4 based on the microarray.

adverse outcome, and an aggressive phenotype in rectal cancer patients treated with adjuvant RCT, justifying *REG4* as a surrogate marker to predict RCT resistance.[19]

REGs are a group of small secretory proteins that perform important functions during cell regeneration and proliferation. Within the REG family, *REG4*, the most recently discovered member, is involved in cell proliferation and regeneration and has been identified as being critical in the pathogenesis of gastroenterological cancers.[20] Consistently, two previous reports revealed that *REG4* expression was markedly increased in CRC tissues.[21,22] Collectively, findings from the current and previous studies suggest that *REG4* can be a predictor of treatment response, and CRC patients with downregulated *REG4* expression may benefit from RCT. Specifically, several previous studies highlighted that *REG4* might be a double-edged sword during the occurrence and/or progression of CRC. Survival data retrieved from the Human Protein Atlas showed that CRC patients with high REG4 expression exhibit increased survival (Figure S3C), whereas targeting REG4 resulted in remarkably decreased tumor growth in experimental CRC models.[23,24] However, the underlying mechanism has not yet been determined, making this a worthwhile topic for future studies. Targeting *REG4* in CRC patients with high levels of *REG4* may reinforce the clinical efficacy of RCT.

Conclusions

In summary, we demonstrated that *REG4* is a potential biomarker for RCT sensitivity in CRC using integrative bioinformatics and experimental analyses. However, further investigations designed to elucidate the RCT resistance mechanisms of *REG4* in RCT-resistant CRC models are still urgently required.

Data Sharing Statement

The datasets used and/or analyzed during the present study are available from Dr. Kaijing Wang upon reasonable request.

Ethics Approval and Informed Consent

All procedures were approved by the ethics committee of Shanghai East Hospital in accordance with the Declaration of Helsinki. All patients agreed to the study procedure and signed consent forms.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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