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Title Page

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Abstract:

Chemoresistance to 5-fluorouracil (5-FU) remains a critical barrier in colorectal cancer (CRC) management. This study integrates multi-omics data from 26,192 human samples to elucidate the RNA methylation-mediated crosstalk between tumor-associated macrophages (TAMs) and tumor cells. Machine learning models were able to effectively stratify patients by risk and identified a core signature of six genes (including SCG2), whose expression patterns were associated with poor prognosis

and chemotherapy resistance-related phenotypes. Mechanistically, 5-FU elevates m6A modification in TAMs, polarizing them toward the M2 phenotype. SCG2 mRNA methylation promotes TNF- α ubiquitination, reducing its levels and thereby sustaining NF- κ B activation in tumor cells to drive PCD resistance. The core candidate genes (CCGs) model effectively predicts survival outcomes. Targeting SCG2 represents a novel strategy to reverse chemoresistance by disrupting TAM-tumor crosstalk, offering actionable targets for personalized therapy optimization.

Key Words:

5-FU; Immunocellular Therapy; SCG2 ; PCD; Colorectal Cancer

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors of the digestive system, ranking third in global cancer incidence and second in cancer-related mortality^{1,2}. Epidemiological data indicate that with changes in dietary habits and lifestyle, the incidence and mortality rates of CRC are on the rise, with a growing trend toward younger patients². Currently, the main treatment strategy for CRC consists of surgery combined with chemotherapy and/or radiotherapy³. However, despite comprehensive treatment, one-third of patients with locally advanced CRC still succumb to recurrence and metastasis within five years⁴. As an integral component of CRC treatment, chemotherapy plays a pivotal role in reducing recurrence risk and improving survival outcomes in patients with advanced-stage disease⁵. The first-line chemotherapy regimens for CRC primarily include FOLFOX and CAPOX (also known as XELOX), both of which are based on 5-fluorouracil (5-FU), thus remaining 5-FU the cornerstone of CRC chemotherapy. Nevertheless, statistics show that approximately 20%-30% of patients exhibit primary resistance to 5-FU, and among those who initially respond, the resistance rate can increase to 40%-50% over the course of treatment^{6,7}. The emergence of chemoresistance leads to missed optimal treatment windows and significantly

worsens prognosis in patients⁸. Therefore, the early identification of CRC patients at high risk of chemotherapy resistance, coupled with the discovery of potential biomarkers to enable timely interventions for improving survival outcomes, remains an urgent unmet clinical need.

A growing body of literature suggests that the primary mechanism of chemotherapeutic agents is to induce programmed cell death (PCD) in tumor cells⁹. This implies that when tumor cells develop adaptive changes to overcome or resist PCD, they acquire a resistant phenotype. Studies have demonstrated a strong correlation between CRC chemoresistance and various forms of PCD, including apoptosis, autophagy, ferroptosis, cuproptosis, disulfidptosis, pyroptosis, and panoptosis⁹. However, the intrinsic regulatory mechanisms governing this process remain incompletely understood.

Numerous studies have explored different mechanisms underlying 5-FU resistance in CRC, such as the "drug efflux pump" hypothesis, "signaling pathway remodeling" hypothesis and "enhanced DNA damage repair" hypothesis^{10,11,12}. Notably, all these mechanisms focus on tumor cell-intrinsic factors. In contrast, emerging evidence suggests that the tumor microenvironment (TME) plays a dominant role in mediating drug resistance, especially in solid tumors, where its influence may surpass that of intrinsic tumor cell alterations^{13,14}. After all, in CRC tissues, tumor stem cells, which possess self-renewal potential, constitute only 1-3% of the total tumor cell population, while differentiated tumor cells account for approximately 50%¹⁵. This implies that non-tumor components predominate in the CRC microenvironment. Several studies have indicated that the TME can actively remodel tumor cell drug resistance^{16,17}; however, the precise mechanisms remain elusive.

Particularly, tumor-associated macrophages (TAMs) in the TME play a crucial role in regulating tumor cell resistance to 5-FU-induced PCD by secreting various immunomodulatory factors. Dysfunctional TAMs can reduce tumor cell sensitivity to PCD signals, leading to impaired apoptosis and enhanced tumor cell survival^{18,19}. Targeting this process has emerged as a promising approach to overcoming chemoresistance, yet the underlying molecular mechanisms remain unclear.

Among the numerous cytokines secreted by TAMs, tumor necrosis factor-alpha (TNF- α) is of particular importance. TNF-

α is a multifunctional cytokine involved in inflammation, immune regulation, and cell survival²⁰. Recent studies suggest that TNF- α plays a dual role in TME-mediated regulation of tumor cells. On the one hand, it collaborates with TAM-derived factors such as CSF-1 and IL-10 to promote immune suppression in the tumor. On the other hand, it can initiate tumor cell apoptosis via the caspase system⁸. However, the exact mechanism by which it confers resistance to 5-FU-induced programmed cell death (PCD) in tumor cells remains to be further elucidated.

Experimental evidence indicates that chemotherapeutic agents, including 5-FU, can act directly on tumor cells or the TME via the microcirculation, influencing tumor RNA methylation levels, which are closely linked to PCD resistance²¹. RNA methylation is a dynamic and reversible epigenetic modification that includes N6-methyladenosine (m6A), N1-methyladenosine (m1A), 5-methylcytosine (m5C), N7-methylguanosine (m7G), and N6,2'-O-dimethyladenosine (m6Am)²². These modifications play a crucial role in enhancing cancer cell adaptability within the chemotherapy-induced microenvironment²³. Investigating the interplay between RNA methylation and the chemotherapy-induced TME may facilitate the identification of novel therapeutic targets to improve chemotherapy efficacy and overcome PCD resistance. Although both TAMs and RNA methylation have been confirmed to be involved in CRC chemotherapy resistance to 5-FU, whether there is a direct molecular link between them remains unclear. Specifically, it is unknown which specific RNA methylation modification can drive the functional phenotypic transformation of TAMs and further suppress tumor cell programmed death through a clearly defined signaling axis. Moreover, although targeting key molecules within TAMs to reverse chemotherapy resistance shows potential therapeutic value, the specific mechanisms of their action in vivo still need to be elucidated, and effectively validated intervention strategies are also lacking.

In this study, we integrated transcriptome sequencing, single-cell RNA sequencing, spatial transcriptomics, and Mendelian randomization analysis from 26,192 colorectal cancer samples. Through validation via radiomics and experimental multi-omics approaches, we elucidated the role of RNA methylation in tumor-associated macrophages in mediating resistance to

programmed cell death (PCD). Based on these findings, we developed a novel risk-stratification model for predicting survival outcomes in patients with colorectal cancer. Significantly, we identified Secretogranin II (*SCG2*) as a pivotal therapeutic target to overcome PCD resistance. Mechanistic studies revealed that *SCG2* orchestrates TAM-driven immunosuppression via RNA methylation-dependent pathways, ultimately promoting 5-FU chemoresistance. This work provides robust experimental evidence and theoretical foundations for early identification of CRC populations at high risk of 5-FU resistance and targeted reversal of chemoresistance through *SCG2* modulation, advancing precision oncology in CRC management.

Results

Multi-Omics analysis identified five DRRMAGs as critical intermediate variables

In the DR-CRC expression matrix with the threshold in 2.1.2 (Methods section) between DR and control groups to get the final 12,795 differentially expressed genes, of which 6,885 are up-regulated genes and 5,910 are down-regulated genes (**Supplementary Fig. 1a**), and then we carried out WGCNA-weighted co-expression network analysis of meta-GEO, and after eliminating the outlier samples, we introduced the RNA methylation sample traits (Writer, Reader, and Eraser correlation ssGSEA scores) into the Euclidean clustering, and found that the clustering effect was significant (**Supplementary Fig. 2a**). Further, we found that the optimal scale-free network evaluation coefficient R^2 is 0.85 and soft thresholding(β) is 6, the network best fits the scale-free topology (**Supplementary Fig. 2b**).

We classified meta-GEO into 15 expression modules according to the above thresholds, and selected the three modules with the darkest colors (a total of 3119 genes) for further analysis according to the RNA methylase ssGSEA score as a phenotypic feature (**Supplementary Fig. 1b**). Then 2284 differential genes (including 902 up-regulated and 1382 down-regulated, **Supplementary Fig. 1c**) were obtained from all the included resistant and sensitive samples of meta-GEO. The WGCNA core modules genes were intersected with the differential genes from the RNA-MRGs and the drug-resistant and sensitive samples

in 2.1.1, and five DRRMAGs were obtained, namely *HNRNPC*, *TRMT10C*, *TRMT61A*, *WDR4*, and *YTHDF1* (**Supplementary Fig. 1d**).

Optimal models fitted with 101 machine learning algorithms through DRRMAGs-derived CDRCGs can successfully predict survival risk of colorectal cancer patients

In the DR-CRC dataset, according to the OS time and status of the samples, the best score of DRRMAGs was obtained as a cutoff value and grouped in this way. It was found that the survival rate of samples in the high score group was significantly higher than that in the low score group, and the difference was statistically significant (**Supplementary Fig. 1e, left part**), and using this as the cutoff value, we obtained 2643 up-regulated genes and 1123 down-regulated genes between the two groups in DR-CRC (**Supplementary Fig. 1e, middle and right parts**). In meta-GEO we similarly found that DRRMAGs could distinguish between resistant and sensitive samples (**Supplementary Fig. 1f, upper part**). 2033 up-regulated genes and 1071 down-regulated genes were obtained between high and low scoring groups in meta-GEO (**Supplementary Fig. 1f, lower part**). Taking the intersection of all the above differential genes with the previous CDRGs (**Supplementary Fig. 1g**), 61 candidate genes (i.e., CDRCGs) were obtained.

In order to determine the role of CDRCGs, enrichment analysis was further applied. Among them, apoptosis signaling pathways (extrinsic and intrinsic signaling regulation) were significantly enriched (**Supplementary Fig. 1h**), which also provided clues for the later mechanism exploration; furthermore, based on the expression of CDRCGs, the samples in DR-CRC were used to Further, based on the expression of CDRCGs, a risk model was constructed with the survival data from DR-CRC samples to observe the diagnostic efficacy of CDRCGs for CRC chemoresistance and further screening: univariate Cox regression showed that *SFRP2*, *PTGIS*, *DAPK1*, *CRYAB*, *SRPX*, *SCG2*, and *IGF1* were the high-risk genes, and *BRCA1* and *TRAP1* were the low-risk genes (**Supplementary Fig. 1i**). In order to make the model more robust, we used 10 machine

learning means, totaling 101 machine learning algorithms (**Supplementary Data. 1**), and finally generated a model with the consensus of all algorithms (the model variables are CCGs): Risk score = $-1.1207192 \times BRCAL + 1.3759456 \times CRYAB + 1.2781438 \times DAPKI + 1.42673334 \times SCG2 + 1.2985338 \times SRPX - 0.9944488 \times TRAP1$.

First we tested the model using the DR-CRC training set: we found that there was a significant difference in survival between high and low risk groups (**Supplementary Fig. 1j**, $P < 0.001$), and the AUC value was > 0.5 , indicating that the model could predict the survival of patients at 1, 3, and 5 years (**Supplementary Fig. 1k**). If the median value of the Risk score was used as the cutoff point, the sample was divided into high and low risk groups and ranked by the risk score of each patient, it was found that the probability of patient's death was higher as the risk score increased (**Supplementary Fig. 1l**). Then the CCGs in the above model were substituted into the DR-CRC training set, and it was found that the expression of *CRYAB*, *DAPKI*, *SCG2*, and *SRPX* was relatively higher in the high-risk group, and the expression of *BRCAL*, and *TRAP1* was higher in the low-risk group (**Supplementary Fig. 1m**).

CCGs have demonstrated good survival prediction performance across multiple external datasets and can be further utilized as a prognostic model for evaluating long-term patient survival

In validation set 1 (GSE17537), this validation set was categorized into high and low risk groups according to the median value of the Risk Score of the CCGs model, and it was found that the survival rate of the high-risk group was significantly decreased and the AUC value of 1, 3, and 5 years was > 0.6 , which indicated that the performance of the model was solidly constructed, and further, all the Further, all patients were sorted by risk score, and it was found that patients were more likely to die as the risk score increased, and the expression of *CRYAB*, *DAPKI*, *SCG2*, and *SRPX* in CCGs was significantly higher in the high-risk group ($P < 0.05$, **Fig. 3a**).

To further ensure the efficacy of the model, the same validation was performed in validation set 2 (GSE17538), and found

that the survival rate was significantly decreased in the high-risk group, with an AUC value of >0.5 at 5 years, and the risk of patient death was significantly increased with the rise in risk score. (**Fig. 3b**).

To assess the predictive ability of CCGs for patient survival, we constructed a prediction model using univariate Cox regression and found that risk score, age, and pathologic stages (T, M and N) were significantly associated with the prognosis of survival in all patients with TCGA-CRC (**Fig. 3c**, $P < 0.05$). We then retained the above four variables and performed multivariate Cox regression (**Fig. 3d, left part**) after passing the PH hypothesis test (**Supplementary Fig. 2C**), and found that risk score, age, and pathologic stages (M and N) were significantly associated with prognosis in this model (**Fig. 3d, right part**). Further, to visualize the utilization of the model, we plotted the nomogram (**Fig. 3e**).

We further evaluated the efficacy of this prognostic model and found that the above Nomogram was more predictive of 1-, 3-, and 5-year survival in CRC patients in the training set ($AUC > 0.7$, **Fig. 3f**) than individual variables—the expression of CCGs was measured in the patients' CRC tissues, and risk score was calculated according to the model, and then the postoperative pathological staging was added, which could be substituted into this model. Subsequently, we stratified the CRC cohort samples into groups based on their Rskscore from the CCGs model. After dividing them into high-risk and low-risk groups, we compared the survival rates between patients who received chemotherapy and those who did not within each group. The results showed no statistically significant difference in prognostic survival between the chemotherapy and non-chemotherapy patients in the high-risk group (**Fig. 3g, upper part**). However, a statistically significant difference in survival was observed between these two subgroups within the low-risk group (**Fig. 3g, lower part**). In other words, low-risk patients can benefit from chemotherapy. In contrast, among high-risk patients, there is no significant difference in survival rates between those who received chemotherapy and those who did not, indicating that chemotherapy does not improve the survival of high-risk patients, i.e., the high-risk group demonstrates a risk of chemotherapy resistance. Hence, by utilizing CCGs, we can not only assess a patient's potential risk of resistance to 5-FU chemotherapy but also evaluate the long-term survival outcomes.

Moreover, we found that among the genes constituting the CCGs model, *SCG2* and *BRCA1* were significantly upregulated in DR-CRC samples (**Fig. 3h**). In contrast, in non-chemotherapy-resistant CRC samples (GSE41258), *BRCA1* and *TRAP1* were significantly upregulated, while *SCG2* was significantly downregulated (**Fig. 3i**). However, from the preoperative neoadjuvant (NACT) chemotherapy cohort of our CRC patients, only *SCG2* expression was found to be significantly upregulated in NACT-resistant patient samples compared to NACT-sensitive patient tissue samples, and the difference was statistically significant (**Fig. 3j**).

In addition, among all TCGA- (DR) CRC and meta-GEO samples, only *BRCA1*, *CRYAB*, and *SCG2* were significantly associated with prognosis when the resistant samples were categorized into high- and low-expression groups based on the optimal truncation value of CCGs, and only *SCG2* played a positive role in both cohorts—high expression of *SCG2* significantly shortened the survival of the patients (**Supplementary Fig. 2d**). It is noteworthy that when patients were stratified by the median expression level of *SCG2*, the survival rate of those receiving chemotherapy was significantly better than those without chemotherapy in the low *SCG2* expression group ($p < 0.05$). In contrast, no significant difference in survival was observed between chemotherapy and non-chemotherapy patients in the high *SCG2* expression group ($p > 0.05$). These findings suggest that *SCG2* may play a potential role in mediating chemoresistance. (**Supplementary Fig. 2e**).

***SCG2* has potential to shape the TME and influences the infiltration of macrophage**

We tested the effect of CCGs on prognosis in the whole TCGA-CRC cohort and found that *CRYAB*, *BRCA1*, and *SCG2* had the smallest P-value (**Supplementary Fig. 3a**), and combined with the **Supplementary Fig. 2d** results, *SCG2* was selected as the key gene in CCGs, and *CRYAB* and *BRCA1* were the second selected genes. Then we categorized the enrichment scores of immune cell infiltration of all samples in the TCGA-CRC data into high- and low-risk groups according to the optimal cutoff value of CCGs expression, and found that the highest correlation among immune cells was MDSC-Macrophage ($R^2=0.93$,

Supplementary Fig. 3b), and among the 28 immune cells, the value of enrichment difference of MDSC and Macrophage was most significant between high- and low-risk groups. Risk groups with the most significant difference in enrichment between MDSC and Macrophage (**Supplementary Fig. 3c and d**).

Further, we analyzed the relationship between the key gene *SCG2*, the secondary genes *CRYAB* and *BRCA1* and the immune cells infiltrated in the tumor microenvironment, and found that MDSC (precursor cells belonging to macrophages or granulocytes) and Macrophage were significantly positively correlated with the expression of *CRYAB* and *SCG2* (**Supplementary Fig. 3e**). However, the neutrophil infiltration showed no significant association with the expression of these three genes ($P < 0.05$, **Supplementary Fig. 3e**), suggesting that Macrophage plays a central role in the CCGs-mediated tumor microenvironment.

To determine through which immune molecules Macrophage exerts its effects in the above microenvironment, we further analyzed the differences in ICB expression between high and low risk groups and found that CD276, CD11B, and CD24 on the surface of Macrophage differed most significantly between the two groups (**Supplementary Fig. 3f and g**), further illustrating the importance of Macrophage in this environment. Then to identify the roles played by *SCG2*, *BRCA1* and *CRYAB*, we again performed GSEA using training set 1 and found that *SCG2* was significantly enriched for intercellular interactions, indirectly suggesting the potential of *SCG2* in participating in the regulation of microenvironmental remodeling.

Next, analysis of 2 different scRNA-seq single-cell datasets also showed that the most significant difference in the distribution of SPP1+ Macrophage (**Supplementary Fig. 3h**) and myeloid (precursor cell of macrophage) was found between CRC tissues and paracancerous tissues (**Supplementary Fig. 3i and 3j**), suggesting that in the CRC tumor microenvironment, suggesting that macrophage may play an important role.

***SCG2* is the key gene in CCGs that is closely associated with the spatial distribution of macrophage and its precursor**

cells (Myeloid), and mediates the interaction between immune cells and tumor cells

First, we observed abnormalities in cell subpopulations in the single-cell sequencing results of tumor and adjacent normal tissues from two different colorectal cancer (CRC) datasets—the alterations in macrophages were exceptionally significant in both datasets (**Supplementary Fig. 4a, upper and lower left part**). Subsequently, in single-cell dataset 1, further analysis of key genes in the CCGs model revealed that compared to the macrophage subpopulation in adjacent normal tissue, the expression of *SCG2* in this subpopulation was significantly elevated in tumor tissue, demonstrating the most statistically significant difference among other genes in the CCGs ($P < 0.0001$ vs. $P = \text{ns}$ vs. $P < 0.01$, **Supplementary Fig. 4a, upper right part**). Moreover, in single-cell dataset 2, *SCG2* was found to be highly expressed in macrophages within tumor tissue (**Supplementary Fig. 4a, middle lower part**) and was also identified as one of the most significantly altered cell subpopulations (**Supplementary Fig. 4b**).

Next, to confirm the expression of *SCG2* in chemotherapy-resistant tumor tissues further, we extracted and reanalyzed 5-FU resistant/sensitive sample data from the chemotherapeutic tumor slice sequencing datasets of GSE178318 and Jabbari et al. (<https://doi.org/10.5061/dryad.pvmcxdngt>). The results demonstrated that in the former dataset, compared to chemotherapy-sensitive samples, the M2-type macrophage subpopulation was significantly increased in chemotherapy-resistant samples (**Fig. 4a**), and *SCG2* expression was markedly elevated within this M2 macrophage subpopulation (**Fig. 4b**). In the latter dataset, consistent with the former findings, the macrophage subpopulation was significantly increased in chemotherapy-resistant samples (**Fig. 4c**), and *SCG2* expression was also significantly higher compared to sensitive samples (**Fig. 4d**). Besides, following subpopulation clustering of SPPI+ macrophages, cells were classified into three subpopulations annotated as M1 and M2 macrophages. These cell types exhibited differential distribution ratios between colorectal cancer and adjacent non-cancerous tissue, with *SCG2* demonstrating higher expression in M2 macrophages (**Supplementary Fig. 4c**). Analysis of *SCG2* expression levels across various subpopulations within the tumor tissue revealed that *SCG2* exhibits relatively prominent

expression in TAMs (**Supplementary Fig. 4d**).

Further, in the spatial transcriptome sequencing data of 5-FU chemotherapy-associated CRC, to analyze the spatial distribution of *SCG2* with macrophages in tissues, as we were unable to annotate to macrophages, we annotated to the precursor cells of macrophages (myeloid), and found that the expression of *SCG2* was nearly consistent with its spatial distribution, and the expression was significantly up-regulated within this subpopulation (**Fig. 4e**), indicating that *SCG2* expression was positively correlated with the distribution of macrophage cell lineage and increased during 5-FU-based CRC chemotherapy, whereas the relationship between the expression of *BRCA1* and *CRYAB* and the distribution of myeloid was not clear (**Supplementary Fig. 5a and b**). Not only that, myeloid interacted closely with the tumor solid component (CMS2), in terms of the spatial interaction of cell subpopulations (**Fig. 4f**), suggesting that the interaction with macrophages could be an important part of drug resistance in this microenvironment.

In addition, bidirectional Mendelian randomization analysis found no causal relationship between CCGs as an exposure factor and resistance genes as an outcome, whereas reverse Mendelian showed a significant causal relationship between resistance genes (*ABCB1*) and CCGs (*BRCA1*) (**Supplementary Fig. 5c and Fig. 4g**), and the scatter plot of the exposure-outcome correlation indicated that the former is a protective factor, i.e., drug resistance genes inhibited RNA methylation-regulated cell death, while the IVW model also confirmed that the former was protective for the latter, and the funnel plot confirmed that the present MR conformed to Mendel's second law of randomized grouping, and the forest plot indicated that the MR was well stabilized (**Supplementary Fig. 6a**).

Immune cell-mediated Mendelian randomization was used to analyze key immune cells in RNA-methylation-mediated modulation of PCD resistance to CRC chemotherapy (5-FU), and 1 monocyte macrophage, 1 dendritic cell, 2 T cells, and 3 B cells were found to play key roles in 741 immune cell variables. Combined with the results of the aforementioned spatial transcriptome analysis, we focused on whether or not the mediating effect of monocyte macrophage was or was not present,

and scatter plots, forest plots, and funnel plots confirmed the presence of the mediating effect, suggesting that the mediating effect was stable (**Fig. 4h, Supplementary Fig. 6b and Fig. 7**), and also provided clues to the immune cells for the subsequent validation.

Finally, we used CTD to predict CCGs with related diseases, and the results showed that there was no association except for *SCG2* which could be accurately associated with CRC occurrence (**Fig. 4i**). Combined with the following experimental results, *SCG2* was screened as the key gene for this analysis for subsequent experimental validation.

***SCG2* is significantly upregulated in NACT-resistant CRC Tissues and significantly affects patients' prognostic survival of the primary chemotherapy outcome**

Six postoperative samples (3 in the NACT-resistant group and 3 in the NACT-sensitive group) were randomly selected from the 5-FU chemo-sensitive/resistant CRC cohort collected in our department, and all samples were obtained with informed consent from the patients and were reviewed by the Medical Ethics Committee of Second Affiliated Hospital of the Army Medical University (Ethics Number: 2019-YD103-01). We performed immunohistochemical staining for *SCG2* protein on the obtained samples.

In the samples from the Retreat Group: MRI and contrast-enhanced CT confirmed significant tumor regression after three cycles of chemotherapy (**as indicated by white arrows, Fig. 5a**). IHC staining revealed faint *SCG2* staining with minimal expression (**Fig. 5b**). In contrast, in the Non-retreat Group samples: MRI and contrast-enhanced CT showed no significant changes in the tumor after three cycles of chemotherapy, with even a tendency towards tumor progression, characterized by further tumor encirclement of the rectal lumen causing stenosis (**as indicated by white arrows, Fig. 5c**). Here, *SCG2* exhibited markedly intensified IHC staining in the tissue samples, indicating significantly upregulated *SCG2* expression (**Fig. 5d**).

Furthermore, we conducted an in-depth analysis of chemotherapy-treated samples in the TCGA-CRC database and found

that *SCG2* expression was significantly higher in PD (Progressive Disease) and SD (Stable Disease) samples (i.e., cases with no signs of tumor regression or even worsening conditions) compared to CR (Complete Response) and PR (Partial Response) samples (**Fig. 5e**). Next, we evaluated the prognostic impact of *SCG2* on survival outcomes following initial chemotherapy. The results demonstrated that, across all CRC chemotherapy subgroups, elevated *SCG2* expression was significantly associated with poorer patient prognosis, and these differences were statistically significant (**Fig. 5f**). Finally, semi-quantitative analysis of *SCG2*-stained regions using ImageJ revealed that the staining score in the sensitive group was markedly lower than that in the resistant group (**Fig. 5g**). This indicates that *SCG2* is predominantly expressed in the tumor microenvironment of NACT (neoadjuvant chemotherapy)-resistant cases, with statistically significant differences (**Fig. 5g**, *** $P < 0.001$).

In TAMs, the expression of *SCG2* is not only regulated by m6A modification but also dependent on the 5-FU microenvironment, and it can enhance CRC resistance to 5-FU

Me-RIP results showed that the m6A modification level of *SCG2* transcripts was significantly upregulated in vitro culture system of TAMs treated with 5-FU (**Fig. 6a**, $P < 0.0001$). Compared with TAMs cultured with normal saline (NS), those cultured with 5-FU exhibited a significantly higher overall m6A methylation level (**Fig. 6b**, $P < 0.05$). After 5-FU treatment, the m6A level in TAMs also showed significant upregulation (**Fig. 6c**). We then plotted the m6A abundance (y) against OD value (x), deriving the formula for m6A calculation in this context as $y = 0.2172x - 0.0222$, $R^2 = 0.9972$ (**Fig. 6d**). Further experiments demonstrated that compared to saline control, the addition of 5-FU led to a significant upregulation in *SCG2* expression (**Supplementary Fig. 8a**). When TAMs were divided into three groups and cultured with 5-FU, DAA(-) (0.1% DMSO), or DAA (an RNA methylation inhibitor) respectively, no statistically significant difference in *SCG2* expression was observed between the first two groups, while *SCG2* expression was significantly decreased in the latter group (**Fig. 6e**). Combined with the results from Figures 4A-E, these findings indicate that 5-FU can upregulate the overall m6A modification

level in TAMs, and that the RNA content of the *SCG2* in TAMs is regulated by m6A modification. Specifically, the m6A modification induced by 5-FU upregulates *SCG2* expression levels. Furthermore, at the protein level of *SCG2*, western blot (WB) demonstrated that DAA significantly downregulated *SCG2* expression level (**Fig. 6f**, $P < 0.001$). Additionally, in tissues from early-stage CRC patients (who did not require 5-FU chemotherapy) (**Fig. 6g**), IHC staining for *SCG2* revealed no significant difference in expression between tumor tissues and adjacent non-cancerous tissues, indirectly suggesting that *SCG2* expression depends on the 5-FU chemotherapeutic microenvironment and remains low in the absence of 5-FU.

To validate the effect of 5-FU on *SCG2* expression, we conducted animal experiments using BALB/c-Nude mice. BALB/c-Nude mice were purchased from GemPharmatech Co., Ltd. (Nanjing, China). All animal studies were approved by the Institutional Animal Care and Use Committee of Army Medical University, and all mice were treated according to the committee's guidelines. The CDX model was constructed as described in Methods Part and **Fig. 6h**, with timed 5-FU administration. At the endpoint, all tumors were harvested (**Fig. 6i**). The immunohistochemistry (IHC) experiments performed on the two groups of tumor tissue sections revealed that the staining intensity of *SCG2* was significantly higher in Group I compared to Group II (**Supplementary Fig. 8b**). The group implanted with 5-FU-resistant cell lines exhibited significantly faster tumor growth, larger volumes (**Fig. 6j**), and higher tumor masses compared to the control group (**Fig. 6k**), all with statistical significance. Furthermore, *SCG2* expression was significantly upregulated in the resistant group (**Fig. 6l**). To investigate the relationship between *SCG2* and macrophages in the 5-FU chemotherapeutic microenvironment, we performed immunofluorescence co-localization of *SCG2* and the macrophage marker CD11b in tumor tissues. Results showed that in 5-FU-resistant tumors, both *SCG2* and CD11b fluorescence intensities were markedly enhanced, with stronger co-localization signals compared to control tumors (**Fig. 6m and n**). This confirms that under 5-FU treatment, *SCG2* expression in TAMs is significantly elevated.

SCG2 regulates TNF- α expression in TAMs by promoting UBQLN1-dependent ubiquitination and degradation

Given that our prior experiments confirmed the predominant localization of SCG2 in tumor-associated macrophages (TAMs), we sought to elucidate how SCG2 modulates tumor cell resistance to programmed cell death (PCD), thereby promoting chemoresistance. Considering that TAMs primarily regulate tumor cells via secretion of TNF- α (encoded by the *TNFA* gene), we hypothesized that SCG2 might influence this process by altering TNF- α expression. After verifying the knockdown efficiency of *SCG2* (**Supplementary Fig. 8c**), we knocked down *SCG2* in 5-FU-treated TAMs and found that the transcriptional level of *TNFA* showed no significant change (**Fig. 7a**). However, TNF- α protein levels were markedly reduced (**Fig. 7b**), suggesting that SCG2 regulates TNF- α expression at the post-transcriptional level. To identify the mechanistic link, we utilized the BioGrid database (<https://thebiogrid.org/>) and identified a potential interaction between SCG2 and UBQLN1, a protein known to interact with TNF receptor superfamily members. We thus speculated that SCG2 might mediate the interplay between UBQLN1 and TNF- α . Immunofluorescence co-localization analysis revealed that in TAMs cells, knockdown of SCG2 expression significantly enhanced the fluorescence intensity of TNF- α and reduced the co-localization efficacy between SCG2 and TNF- α (**Fig. 7c**). Similarly, knockdown of *UBQLN1* expression also markedly increased TNF- α fluorescence intensity and diminished the co-localization of UBQLN1 and TNF- α (**Fig. 7d**). Notably, compared with wild-type cells, *SCG2* knockdown did not alter UBQLN1 expression levels, but significantly reduced the co-localization between TNF- α and UBQLN1 (**Fig. 7e**), suggesting a regulatory role of SCG2 in mediating TNF- α and UBQLN1 spatial interactions. Molecular docking simulations using PyMOL further explored potential binding interfaces. For the SCG2-TNF- α interaction (**Fig. 7f, left part**), a hydrogen bond was predicted between Val253 of SCG2 and Asp121 of TNF- α . Similarly, for SCG2-UBQLN1 binding (**Fig. 7f, right part**), a hydrogen bond was identified between Ser200 of SCG2 and Asn244 of UBQLN1. To validate these findings, we assessed TNF- α ubiquitination in TAMs and observed a significant reduction in ubiquitinated TNF- α upon *SCG2* knockdown (**Fig. 7g**), accompanied by elevated TNF- α protein levels. Input controls confirmed these differences. Furthermore, *SCG2*

depletion in TAMs substantially weakened the binding affinity between TNF- α and UBQLN1 in both forward and reverse co-immunoprecipitation assays (**Fig. 7h**).

SCG2 is a mediator of macrophage–tumor cell crosstalk in the 5-FU chemotherapy microenvironment and further activates the NF- κ B pathway in tumor cells

In order to further determine the role of SCG2 in the microenvironment of 5-FU chemotherapy on the interactions between TAMs and tumor cells, we performed tumor cell-macrophage co-culture experiments according to section 2.2.7 of Method (experimental method is shown in **Fig. 8a**). After reaching the time cut-off point, the TNF- α concentration-absorbance fitting curve was plotted according to the concentration of the standard solution as follows: $y=42.901x-4.3187$, $R^2=0.9915$ (**Fig. 8b**).

Accordingly, it was calculated that the TNF- α content in the culture medium of chambers I-IV increased sequentially and the difference between any two groups was statistically significant (**Fig. 8c**). Flow cytometry assay revealed (**Fig. 8d**) that the apoptosis rate of cells in chamber I was $>60\%$ (i.e., the killing rate of tumor cells was higher with 5-FU alone), the apoptosis rate of cells in chamber II was 10% (i.e., macrophages alone were ineffective in killing 5-FU-resistant tumor cells by secreting TNF- α), and the apoptosis rate of tumor cells in chamber III was about 20% (i.e., the combination of 5-FU and a little TNF- α produced by TAMs had a modest killing effect on 5-FU-resistant tumor cells), and the apoptosis rate of the cells in compartment IV was 40% (i.e., a large amount of TNF- α was produced by the TAMs after interfering with the expression of SCG2, and a large dose of TNF- α in combination with the chemotherapeutic agent, 5-FU, produced a better killing effect on the 5-FU-resistant tumor cells).

Next, we performed Transwell assay on the upper chamber cells after co-culture, and found that the cell invasion ability was the strongest in chamber II, which was higher than that in chamber III. In addition. Except for the cells within chamber I, which had the highest apoptosis, chamber IV had the weakest invasive ability (**Fig. 8e and f**). Subsequently, to further confirm the

role of TNF- α in Chamber IV, we added a new Chamber V. After adding 20 $\mu\text{g/mL}$ infliximab, we observed through Transwell assays that the cell invasion capacity in this chamber was similar to that in Chamber II (**Supplementary Fig. 8d**). Further, in order to detect the activity and malignancy of tumor cells in the upper compartment after co-culture, we first went to the upper compartment cells for CCK8 assay, and the results showed that the cells in compartment II with the lowest apoptosis rate had the strongest proliferative ability, and the cells in compartment I with the highest apoptosis rate had the lowest proliferative ability, and the cells in compartment IV had the proliferative ability between the two (**Fig. 8g**). Then we performed WB analysis on cells within the upper compartment and found that among the cells in the four upper compartments, IKB α protein expression was significantly up-regulated in compartment II, while the expression in compartments III and IV decreased sequentially, but was still higher than that in compartment I (**Fig. 8i**); however, for other NF- κB pathway proteins, such as P50 and P65, due to the reduced absolute cell numbers in specific compartments (particularly compartments I and IV), their total protein levels were significantly reduced. Concomitantly, the expression levels of anti-apoptotic genes (such as *BCL2* and *BCL-X*) were also markedly downregulated (**Fig. 8h, j, k, and i**). Further ELISA analysis of the cell lysates from the several chambers, conducted after plotting standard curves for quantification, revealed that the phosphorylation levels of P65 (Ser529) across the four chambers followed the order: Chamber I > Chamber IV > Chamber III > Chamber II. This result is consistent with the aforementioned Western blot data (**Supplementary Fig. 8e and Supplementary Data. 2**).

Animal experiments of PDX and shSCG2-TAMs intervention have confirmed that SCG2 can enhance the resistance of CRC to 5-FU, while interfering with the expression of SCG2 in TAMs can restore the sensitivity of drug-resistant tumor cells to 5-FU

To accurately simulate the regulatory role of *SCG2* in programmed cell death (PCD) within the human tumor microenvironment (TME), we implanted postoperative tumor tissues from 5-FU-sensitive and 5-FU-resistant CRC patients

into the cecal end of immune-deficient mice, as illustrated in **Fig. 9a**. Starting from day 7, mice were intraperitoneally injected with 5-FU three times a week until the designated endpoint, at which point the mice were euthanized. Compared with 5-FU-resistant tumor tissues, the volume of 5-FU-sensitive tumor tissues significantly reduced after the experiment (**Fig. 9b**). We then performed immunohistochemistry (IHC) dual staining for SCG2 and CD11b on tumor tissues from both groups. The results showed that, compared to the 5-FU-sensitive group, the 5-FU-resistant tumor tissues exhibited a more pronounced co-localization of SCG2 and CD11b (**Fig. 9c**). Furthermore, comparing the tumor weight of both groups, we found that both the tumor mass and volume in the 5-FU-resistant group were significantly higher than those in the 5-FU-sensitive group (**Fig. 9d**). In addition, we observed a significant upregulation of SCG2 expression in the 5-FU-resistant tumor tissues compared to the sensitive group (**Fig. 9e, right part**). Semi-quantitative analysis revealed a larger area of co-localization between SCG2 and CD11b in the resistant tumors, and the difference was statistically significant (**Fig. 9e, left part**). To further elucidate the mechanism by which *SCG2* regulates tumor PCD resistance, we examined the expression levels of TNF- α in both groups. The results showed that the TNF- α content was significantly reduced in the 5-FU-resistant group (**Fig. 9f, left part**). In contrast, NF- κ B signaling pathway genes, including *P50*, *BCL2*, and *BCLX*, were all upregulated within the tumor cells (**Fig. 9f, right part**).

To further elucidate the role of SCG2 in TAMs in mediating resistance to PCD aiming at 5-FU among tumor cells, we conducted animal intervention experiments as outlined in **Fig. 9g**. The results demonstrated that tumors in Group I and Group III exhibited comparable size and mass (**Fig. 9h, i and j**), both significantly smaller than those in Group II. Notably, tumor growth rate in Group I (shSCG2-TAMs implanted) was markedly slower than that in Group II (**Fig. 9i**). Immunofluorescence co-localization assays revealed that Group II displayed the lowest levels of IKB α and TNF- α , whereas Group I showed significantly higher TNF- α and IKB α expression than Group II, with slightly elevated levels compared to Group III (**Fig. 9k**). Subsequent analysis of tumor samples from **Fig. 9h** demonstrated that Group II exhibited minimal TNF- α expression but

upregulated *SCG2*, *BCL-2*, and *BCL-X* levels. Conversely, Group I displayed the highest TNF- α expression and the lowest *SCG2*, *BCL-2*, and *BCL-X* levels among all groups (**Fig. 9l**). RT-qPCR analysis further confirmed that both Group I and Group III had comparable expression levels of *P50*, *BCL-2*, and *BCL-X*, which were significantly lower than those in Group II ($p < 0.0001$, **Fig. 9m**). These findings indicate that *SCG2*-knockdown cell therapy effectively reduces anti-apoptotic protein expression in tumor cells and restores their sensitivity to 5-FU.

In summary, our findings suggest that in CRC patients undergoing 5-FU chemotherapy, the concentration of 5-FU in the tumor microenvironment (TME) upregulates the m6A modification levels in tumor-associated macrophages (TAMs). This modification leads to the upregulation of *SCG2*, which, through m6A modification, facilitates the ubiquitination of TNF- α by UBQLN1. As a result, TNF- α secreted into the TME is maintained at a persistently low level. This prolonged low-level exposure to TNF- α activates the NF- κ B signaling pathway in tumor cells, upregulating the expression of anti-apoptotic genes. Consequently, the tumor cells develop resistance to programmed cell death (PCD). Conversely, interfering with the expression of *SCG2* in TAMs can effectively upregulate the concentration of TNF- α in the tumor microenvironment (TME), downregulate the anti-apoptotic ability of tumor cells, and restore the sensitivity of drug-resistant tumor cells to 5-FU (**Fig. 9n**).

Discussion

CRC has emerged as a global health crisis, now ranking as the third most common malignancy and the second leading cause of cancer-related mortality worldwide²⁴⁻²⁶. Its escalating incidence among younger populations—the incidence in people under the age of 30 has increased by 142% in the past decade²⁷—combined with dismal 5-year survival rates (<15% for advanced stages), underscores the urgent need for therapeutic innovation. While 5-FU-based chemotherapy remains the first-line standard²⁸, clinical outcomes are severely compromised by rapid resistance development, observed in around 70% of patients within 12 months of treatment initiation²⁹. This resistance manifests through both clinical hallmarks (metastatic progression, CEA

elevation >5 ng/mL in 70% cases)³⁰ and molecular mechanisms centered on PCD pathway activation—a survival signaling cascade that sustains tumor cell viability despite cytotoxic assault. Addressing this therapeutic bottleneck requires urgent translation of resistance mechanisms into actionable clinical strategies.

To address this clinical challenge, this study integrated ten machine learning algorithms to construct a robust consensus multi-omics prognostic model. Through large-scale data mining, we successfully identified six core genes regulated by RNA methylation and established a CCGs predictive model. The model showed strong discriminative ability in predicting prognostic survival of colorectal cancer (AUC = 0.8). Further mechanistic investigation revealed that secretogranin II (*SCG2*), a key gene in the model, plays a central regulatory role in the polarization of tumor-associated macrophages. This study is the first to demonstrate that high expression of *SCG2* in TAMs, driven by m6A modification, mediates 5-FU resistance in colorectal cancer through downregulation of TNF- α and activation of the NF- κ B signaling pathway.

As advances in epigenetics have revealed RNA methylation as a pivotal regulator of CRC chemoresistance, offering unprecedented clinical opportunities. This dynamic modification drives three key resistance mechanisms: 1) Upregulation of drug resistance related to ABCB1/P-gp multidrug transporters, 2) Activation of GST π -mediated detoxification systems, and 3) PCD pathway amplification that reduces apoptotic susceptibility^{31,32,33}. However, existing strategies targeting tumor cells—whether through modulating ABC transporter-mediated efflux activity, suppressing alternative signaling pathways, or inhibiting histone lactylation-mediated DNA repair^{10,11}—these approaches, solely focused on tumor cell-intrinsic mechanisms, overlook over 60% of resistance drivers, as compelling evidence indicates that the majority of drug resistance factors are mechanistically linked to TME-related processes³⁴. In contrast, TME-centric research provides a systemic framework to elucidate tumor-stroma interactions, immune evasion, metabolic reprogramming, and microbiota crosstalk, with mounting evidence underscoring its superior clinical relevance³⁵. Our study pioneers an investigation into TAMs, the most abundant immune population in TME³⁶, to dissect their dynamic interplay with tumor cells and delineate the signaling axis connecting

TAMs-TME-TCs (tumor cells)-PCD. Notably, PCD emerges as the pivotal node in this network, offering actionable targets for overcoming therapy resistance. As reported, PCD activation status directly correlates with treatment response rates, with PCD-high tumors showing 60% higher objective response rates compared to PCD-low counterparts³⁷.

Recent studies have revealed the critical role of *SCG2* in regulating tumor cell growth^{38, 39}. However, due to the mutational heterogeneity of tumor cells, conventional targeted therapies face challenges, necessitating the development of new therapeutic strategies. Although bioinformatic analyses suggest that *SCG2* may be involved in tumor microenvironment remodeling^{40, 41}, the specific mechanisms lack experimental support. Notably, only one study has computationally predicted the potential role of *SCG2* in 5-fluorouracil (5-FU) resistance in colorectal cancer (CRC)⁴², but it merely experimentally validated the tumor-promoting function of *SCG2* without delving into its chemoresistance mechanisms. This study found that in the low *SCG2* expression group, patients receiving chemotherapy had significantly prolonged overall survival compared to those without chemotherapy, whereas no significant difference was observed in the high expression group, suggesting that high *SCG2* expression may be associated with 5-FU resistance.

To further elucidate the mechanism by which *SCG2* drives 5-FU resistance in CRC, multi-omics analysis in this study identified specific high expression of *SCG2* in M2-type macrophages. This aligns with previous research showing an increased proportion of M2 macrophages in the high *SCG2* expression group, indicating *SCG2* as a key driver promoting M2 polarization of macrophages⁴³. Spatial transcriptomics demonstrated upregulation of *SCG2* in almost all chemotherapy-resistant CRC metastatic lesions, although the extent to which serum *SCG2* levels reflect patient resistance to 5-FU remains unclear. Further cell-derived xenograft (CDX) model results showed a 110% (See Fig. 4n column chart) increase in the co-localization rate of *SCG2* with tumor-associated macrophages (TAMs) in resistant tumors compared to the sensitive group, and this TAM-mediated sustained inflammatory response was significantly correlated with poor prognosis. Previous studies have shown that TAMs can regulate tumor cell tolerance to 5-FU by secreting various

immunomodulatory factors^{44,45}. Among them, TNF- α produced by TAMs has a dual role in the tumor microenvironment: short-term exposure to high concentrations of TNF- α can induce tumor cell apoptosis, exerting an anti-tumor effect; conversely, long-term exposure to low concentrations of TNF- α activates pro-survival pathways such as NF- κ B, thereby promoting tumor cell proliferation, angiogenesis, tumor progression, and immune evasion^{45,46,47,48}. However, the specific function of SCG2 in TAMs remains unclear. This study further investigated the mechanism through co-culture experiments of TAMs and tumor cells (TCs), revealing that SCG2 regulates TNF- α expression in TAMs by promoting UBQLN1-dependent ubiquitination and degradation, suppressing extracellular TNF- α levels to approximately half of the pre-resistance concentration (Fig. 6C). This mechanism, while sustaining chronic activation of the inflammatory pathway, concurrently inhibits the apoptosis pathway. On one hand, it maintains the chronic activation of the NF- κ B pathway; on the other hand, it upregulates the expression of anti-apoptotic proteins BCL-2/BCL-XL (by 1.1–3 fold). Although UBQLN1 has been reported to be associated with various cancers^{49,50}—for instance, knockdown of UBQLN1 can inhibit CRC development via the ERK-c-Myc pathway⁵¹—its involvement in regulating CRC tolerance to 5-FU chemotherapy through TNF- α ubiquitination is a novel mechanism identified in this study.

Finally, therapeutic experiments validated the targetability of this mechanism. Therapeutic inhibition of SCG2 reversed this immunosuppressive environment, elevated TNF- α to cytotoxic concentrations (approximately doubling the original level, 82 vs. 44. That is, their mean fluorescence intensities were 82 and 44, respectively. See the column chart of Fig. 7k), and restored 5-FU efficacy to 79% (See Fig. 7i, line graph, for tumor volumes in Group I and Group II) in a cell therapy model. Based on the preclinical results from PDX models and cell therapy experiments, we hypothesize that clinical translation experiments will validate that compared with standard chemotherapy, anti-SCG2 cell therapy holds promise for elevating the disease remission rate in patients. These results not only systematically elucidate the novel mechanism by which SCG2 drives chemoresistance via the "m6A–SCG2–UBQLN1–TNF- α –NF- κ B" axis but also

provide a solid experimental basis and promising translational strategy for reversing chemotherapy resistance by targeting SCG2 in TAMs.

Building on these findings, we propose a clinical integration framework positioning *SCG2* at the nexus of CCGs precision medicine. Current efforts focus on developing detection technology based on CCGs, especially targeting SCG2, for real-time resistance monitoring during 5-FU chemotherapy. Our preclinical experiment demonstrated 79% disease control rates with SCG2-targeted regimens, potentially establishing a new therapeutic strategy. By bridging molecular insights with clinical applications, this work transforms SCG2-mediated chemoresistance mechanisms into actionable solutions—offering probable hope for CRC chemoresistance where therapeutic options have remained stagnant for decades. The CCGs model and *SCG2*-directed strategies collectively represent a promising finding, moving CRC management from empirical chemotherapy toward biomarker-driven precision oncology with measurable survival benefits.

Moreover, based on the above analysis and experimental results, our findings advocate a refined approach strategy for managing locally advanced CRC, emphasizing precision risk stratification and innovative resistance reversal. For treatment-naïve patients with clinical staging $\geq$ T2N1M0 (Stage II/III), preoperative quantification of SCG2 and CCGs via liquid biopsy or endoscopic sampling enables dynamic risk modeling. Patients exhibiting CCGs risk scores above the median require early MRI reassessment after 1-2 neoadjuvant chemotherapy (NACT) cycles (FOLFOX/CAPOX). Non-responders (tumor shrinkage $<30\%$ or growth ≥ 5 mm) should promptly transition to cetuximab/bevacizumab-based targeted therapy²⁴. Postoperatively, those with pT2N1M0 (Stage II/III) and elevated CCGs scores in surgical specimens warrant CEA-driven surveillance after adjuvant FOLFOX/CAPOX. Per NCCN guidelines²⁴, a CEA ≥ 5 ng/mL with $\geq 20\%$ elevation from baseline mandates immediate integration of targeted agents or immunotherapy (PD-1 blockade for MSI-H subsets) to prevent therapeutic delays. Prognostic modeling further refines survival predictions. Critically, for 5-FU-refractory cases, preclinical evidence supports *SCG2* knockdown in TAMs via endoscopic intratumoral delivery of engineered autologous macrophages – a universal approach

leveraging innate immune modulation to bypass CRC's low immunogenicity.

It is also necessary to recognize the limitations inherent in our investigation. First, although we constructed and validated the CCGs prognostic model using multiple public datasets (including TCGA and GEO), In some external validation cohorts (e.g., GSE17537, GSE17538), detailed annotation information regarding the chemotherapy regimens of the samples is incomplete, lacking standardized chemotherapy regimens (e.g., specific 5-FU dosage, treatment cycles, combination agents) and a unified definition of resistance. This may introduce a degree of bias in sample classification. Future validation of the model's predictive efficacy should rely on prospective clinical cohorts with uniform standards. Second, this study used overall survival (OS) as the primary clinical endpoint for assessing chemoresistance. It must be acknowledged that OS is influenced by various confounding factors, including subsequent treatments (e.g., targeted therapy, immunotherapy), the patient's systemic condition (comorbidities, nutritional status), and non-cancer-related mortality. Therefore, OS does not fully equate to specific resistance to 5-FU. More direct indicators of resistance, such as progression-free survival, objective response rate, or pathological complete response rate, should be applied in future studies. Furthermore, regarding the mechanistic investigation, although we demonstrated that SCG2 regulates the ubiquitination level of TNF- α via UBQLN1 (Figs. 5G, 5H), it is important to clarify that UBQLN1 itself is not an E3 ubiquitin ligase but functions as a ubiquitin receptor/chaperone. The specific E3 ligase catalyzing TNF- α ubiquitination has not yet been identified; therefore, the complete molecular mechanism of the "SCG2-UBQLN1-TNF- α " axis remains to be fully elucidated. Plausible explanations include recruitment of an unknown E3 ligase (such as CHIP, Parkin, or other family members) by the SCG2/UBQLN1 complex, or alteration of TNF- α conformation rendering it more susceptible to ubiquitination. Subsequent studies should aim to identify this key E3 ligase and elucidate the precise molecular details of the SCG2-UBQLN1 interaction. Additionally, while GST pull-down and co-immunoprecipitation assays confirmed the physical interactions among SCG2, UBQLN1, and TNF- α , domain-specific mutant rescue experiments have not yet been performed to precisely map the key amino acid residues or functional domains on SCG2 or UBQLN1

responsible for these interactions and downstream functions (e.g., TNF- α ubiquitination). Such experiments would help establish a more definitive causal relationship for SCG2-mediated regulation of TNF- α stability via UBQLN1. Finally, the in vivo experiments were conducted primarily in immunodeficient mice (BALB/c-Nude) and focused largely on innate immune cells; thus, the role of the adaptive immune system in SCG2-mediated chemoresistance was not comprehensively evaluated. Validating our findings in a more immunocompetent model, such as a humanized mouse model, represents an important direction for future research.

In summary, this study integrated large-scale multi-omics profiling with clinical cohorts to systematically identify RNA methylation-driven cell death regulators critical for colorectal cancer (CRC) chemoresistance. We further established a robust risk stratification and prognostic prediction model validated. And the model performance was validated in multi-center internal and external cohorts, demonstrating its clinical applicability for pre-therapeutic risk assessment and personalized treatment decision-making. Since our experiments were conducted on patients after chemotherapy, the identified biomarkers correlated with the survival prognosis of patients following chemotherapy treatment (chemotherapy-sensitive and resistant cohorts were stratified with a 3-year time fence) . This evidence underscores the necessity of dynamic monitoring and early therapeutic intervention for high-risk patients identified by the model, which may substantially improve clinical outcomes by adjusting other substitute therapeutic regimen in time.

Furthermore, single-cell and spatial transcriptomic analyses delineated the tumor microenvironmental distribution of model-derived biomarkers, pinpointing targetable cellular subpopulations. Mechanistic investigations revealed that chemotherapy-induced RNA methylation modifications upregulate the core regulator SCG2 in TAMs, which orchestrates TNF- α ubiquitination via UBQLN1 to sustain TME immunosuppression (it also should be emphasized that although our results demonstrate UBQLN1 dependence for TNF- α ubiquitination in TAMs following 5-FU treatment, UBQLN1 itself possesses no E3 ubiquitin ligase activity. Instead, it most likely acts as an adaptor to recruit an E3 ligase to the vicinity of TNF- α , or stabilizes

the conformation of the TNF- α -SCG2 complex to maintain a microenvironment permissive for ubiquitination. Subsequent research will focus on identifying the specific E3 ligase participating in this process). Chronic TNF- α infiltration perpetuates pro-survival inflammatory signaling and anti-apoptotic pathways, driving programmed cell death resistance. Crucially, *SCG2* ablation reversed chemoresistance by restoring apoptotic sensitivity in preclinical models. These findings not only elucidate an RNA methylation-dependent axis governing CRC chemoresistance but also propose SCG2-targeted strategies as a precision therapeutic approach, providing actionable insights for refining combinatorial chemo-immunotherapy regimens.

Methods

Ethical statement

All the participants were over 18 years old. Formal informed consents were obtained from all participants for the relevant samples, and the study strictly adhered to the principles of the Declaration of Helsinki, maintaining all relevant ethical standards. All the procedures performed in studies involving human participants were in accordance with the ethical standards of the Medical Ethics Committee of Second Affiliated Hospital of Army Medical University (Ethics Number: 2019-YD103-01). All BALB/c-Nude mice were purchased from GemPharmatech Co., Ltd. (Nanjing, China). All animal studies were approved by the Institutional Animal Care and Use Committee of Army Medical University, and all procedures were conducted in accordance with the guidelines of the Army Medical University Institutional Animal Care (AMUWEC20210174). The animal study also accorded with the ARRIVE guidelines.

Identification and selection of core candidate genes for RNA methylation-mediated PCD in 5-FU-resistant CRC

Data preparation and gene retrieval

This study included a total of 26,192 colorectal cancer (CRC) chemoresistance-related samples: 686 CRC chemoresistance samples (DR-CRC) from UCSC-TCGA-COAD; metaGEO Dataset: 561 samples from GEO datasets (GSE28702, GSE19860, GSE62080, GSE69657, GSE72790, GSE35452, GSE39582, GSE29261) combined after batch effect removal, focusing on 5-FU resistance. Resistance criteria included one or more of the following: Lack of response to 5-FU treatment; Progressive disease (PD) or stable disease (SD) after treatment. The information on the resistance and sensitivity of patients in the CRC cohort, as well as their medication status, is shown in **(Supplementary Table 1 and 2)**. Survival falling short of three years post-chemotherapy. GEO Validation Cohort: 527 samples from datasets GSE17537, GSE17538, and GSE41258. Single-Cell Samples: 43 colon cancer samples from GSE132257 and GSE132465. Spatial Transcriptomics Samples: 12 samples from GSE225857 and scCRLM. Clinical Cohort: From our department (2019-2024), 60 CRC patients exhibiting resistance to adjuvant chemotherapy (ACT) or neoadjuvant chemotherapy (NACT) (defined as lack of significant tumor regression after four cycles of preoperative XELOX chemotherapy or recurrence within one year post-chemotherapy), and 60 chemotherapy-sensitive patients. IEU-OpenGWAS Mediator Variable Samples: 24,243 samples, including 2,765 eQTL-CAGE samples. Following the merging of six datasets, batch correction of gene expression data was performed using the ComBat method. The dataset identifiers were incorporated as batch variables, whilst the grouping information of samples was included as covariates within the model. This approach aimed to eliminate batch effects whilst preserving biological differences between groups.

From studies conducted between 2022 and 2024 by various research teams, such as Wang et al.^{52,53}, we compiled the latest cell death-related genes (CDRGs), encompassing apoptosis, autophagy, ferroptosis, cuproptosis, disulfidptosis, pyroptosis, and parthanatos. After integration and deduplication, we identified 1,352 unique CDRGs (Supplementary Data 1).

Additionally, from studies by Han et al.^{54,55} between 2021 and 2022, we obtained 72 RNA methylation-related genes (RNA-MRGs), including "Writer," "Reader," and "Eraser" genes (Supplementary Data 1). Furthermore, 301 extracellular matrix-related genes (ECM-RGs) were retrieved from the Reactome database (<https://reactome.org/>) (Supplementary Data 1). And the

overall analysis workflow is shown in **Fig. 1**.

TCGA-CRC drug resistance differential genes, RNA-MRGs signature module gene screening in meta-GEO, and establishment of drug resistance-associated RNA methylation genes

We first analyzed the TCGA-CRC expression matrix using the R package "DESeq2," applying thresholds of $\text{padj} < 0.05$ and $|\log_2\text{FoldChange}| > 0.25$ to identify differentially expressed genes associated with drug resistance. Subsequently, we employed the ssGSEA algorithm from the R package "GSVA" to calculate ssGSEA scores for "Writer," "Eraser," and "Reader" related genes in the expression profiles of resistant samples from the metaGEO dataset. These scores were then used as traits in Weighted Gene Co-Expression Network Analysis (WGCNA) to identify module genes highly correlated with these traits, referred to as Drug Resistance-Related RNA Methylation Module Genes (DRRMGs). Next, we performed differential expression analysis between "Resistant" and "Sensitive" groups in the metaGEO dataset using the "limma" package, with $\text{padj} < 0.05$. Finally, we intersected the results with the differentially expressed drug resistance-related genes from TCGA-CRC and the DRRMGs to obtain drug resistance-related RNA methylation-associated genes (DRRMAGs). This integrative approach facilitated the identification of key RNA methylation-associated genes involved in CRC drug resistance, providing a foundation for further functional studies.

Prognostic assessment of DRRMAGs in CRC resistance datasets

We performed ssGSEA scoring of all 5-FU-related chemotherapy samples in TCGA-CRC according to the expression profiles of DRRMAGs, and then calculated the best cut-off value of resistance-related RNA methylation-related ssGSEA scores based on the overall survival (OS) of the samples using the "survminer" package. The optimal cutoff value of ssGSEA score was then calculated using the "survminer" package based on the overall survival (OS) of the samples. The samples were then

divided into high and low scoring groups according to the optimal cut-off value, and after calculating the difference in survival between the two, the analysis of the difference between the high and low scoring groups was continued, while the resulting genes were further analyzed (denoted as differential expression genes 1, DEGs1).

Similarly, the samples were first scored by ssGSEA according to the expression profile of DRRMAGs in metaGEO to obtain the resistance-associated RNA methylation-associated ssGSEA score, and then to observe the ability of this score in distinguishing the difference between resistant and sensitive samples, and then finally to carry out the analysis of differences between high- and low-scoring components while the resulting genes were subjected to further analyses (denoted as differential expression genes 2, DEGs2).

Finally, all the differential genes ($\text{padj} < 0.05$ & $|\log_2\text{FoldChange}| > 0.25$) among the control group of drug resistance-associated tumor samples in the TCGA-CRC in 2.1.2 were recorded as DEGs. intersecting it with DEGs1, 2 and CDRGs collated earlier were taken to obtain cell death resistance candidate genes, denoted as CDRCGs.

Machine learning to build CDRCGs-based risk modeling and efficacy assessment

Based on the expression of CDRCGs, univariate COX regression analysis ($\text{HR} \neq 1$ & $p < 0.05$) was performed to obtain prognostically relevant CDRCGs in primary tumor samples of TCGA-CRC with drug treatment information. Further, to screen the best consensus model, 10 machine learning methods were integrated. The specific algorithms are as follows: Elastic Net (Enet), Least Absolute Shrinkage and Selection Operator (LASSO), and Ridge regression were implemented using the glmnet package, with regularisation parameter λ determined via 10-fold cross-validation ($\lambda = \text{cv.fit}\$lambda.min$). Enet employed $\alpha = 0.4$, LASSO fixed $\alpha = 1$, and Ridge regression fixed $\alpha = 0$. Stepwise Cox regression utilised the survival package for variable selection based on the AIC criterion ($\text{trace} = 0$); CoxBoost employed the CoxBoost package with 10-fold cross-validation to optimise penalty parameters ($\text{start.penalty} = 500$, $\text{maxstepno} = 500$, $\text{parallel} = F$); Partial Least Squares regression (PLSR)

fitted via the plsRcox package (sparse = T), with component number determined by 10-fold cross-validation (nt=10); Random Survival Forest (RSF) implemented via the randomForestSRC package (ntree = 1000, nodesize = 5, splitrule = “logrank”, importance = T, proximity = T, forest = T); Generalized Boosted Regression Modelling (GBM) fitted via the gbm package (distribution = “coxph”, n.trees = 10000, interaction.depth = 3, n.minobsinnode = 10, shrinkage = 0.001), with optimal tree number selected via 10-fold cross-validation (cv.folds = 10); Supervised Principal Components (SuperPC) analysis implemented via the superpc package, employing 10-fold cross-validation to determine feature threshold (s0.perc = 0.5, n.components = 3, min.features = 5, max.features set to sample size); Support Vector Machine-Recursive Feature Elimination (SVM-RFE) fitted using the survivalsvm package (gamma.mu = 1, opt.meth = “ipop”). A total of 101 algorithms were used for matching to derive the optimal risk model formulation. We further calculated the C-index (Concordance index) on the validation sets (GSE17537 and GSE17538). According to our experimental results, the top two performing models were: CoxBoost + SuperPC and Lasso + SuperPC. And the flowchart for machine learning is also provided (**Supplementary Fig. 9**).

The expression of prognostically relevant CDRCGs was used to calculate the Risk Score in TCGA-CRC based on the optimal risk modeling formula, and TCGA-CRC was categorized into high- and low-risk groups based on the median value (denoted as H1 vs L1).

Similarly, in the two validation cohorts GSE17537 and GSE17538, which are relevant to 5-FU chemotherapy for colon cancer, we calculated the Risk Score using the optimal risk model formula based on the expression levels of prognosis-related CDRCGs. The results were then divided into high- and low-risk groups according to the median value, resulting in two pairs of groups: H2 (High2) vs. L2 (Low2) and H3 vs. L3.

Kaplan-Meier survival analysis was used to compare the survival status of the three respective high- and low-risk groups of H1 vs L1, H2 vs L2 and H3 vs L3, and the accuracy and efficacy of the risk model was visualized using ROC curves. Additionally, to assess the predictive capability of risk scores for chemotherapy response, high-risk and low-risk cohorts were

stratified based on the 30th percentile. Survival rates were then compared between chemotherapy-treated and untreated patients within each risk group. Kaplan-Meier survival curves were generated using the “survminer” package in R. Concurrently, one-way and multifactorial Cox regression analyses based on the Risk Score and other clinical metrics were visualized using a Nomogram (at 1, 3 and 5 years). The genes (named Core Candidate Genes, CCGs) in the model that were most relevant to the prognosis of the resistant samples were also selected for further analysis and validation. Furthermore, to investigate the relationship between core candidate gene expression and chemotherapy efficacy, patients were divided into high- and low-expression groups based on the median expression level. Survival differences between chemotherapy and non-chemotherapy patients were then compared within each group, with results presented via Kaplan-Meier curves.

Association between CCG and Immune cells infiltration as well as identification of sensitive drugs

Firstly, the R package “GSVA” was used to calculate the enrichment scores of 28 types of immune infiltrating cells in the high and low risk groups of TCGA-CRC resistance-related samples based on the ssGSEA algorithm, and then the R package “psych” was used to analyze the correlation between differential immune infiltrating cells and prognostic genes based on the enrichment scores of the 24 differential immune infiltrating cells and the expression of the core candidate genes. The R package “psych” was then used to analyze the correlation between differentially infiltrating cells and prognostic genes based on the enrichment scores of the 24 differentially infiltrating cells and the expression of the core candidate genes, in order to search for the core immune cell subpopulations in the RNA methylation-mediated PCD regulatory model. Then we investigated the predictive ability of 138 chemotherapy/targeted therapy drugs using the R package “pRRophetic”. The IC50 value was used as an indicator to assess the antitumor activity of the drugs, and differences in IC50 values were compared between the high- and low-risk groups identified in section 2.1.4. Then we selected the drugs with the top 10 differences, and calculated the correlation between them and the core candidate genes correlations. Next we analyzed the commonalities between the antitumor drugs

with the highest correlation coefficients and 5-FU to find potential resistance mechanisms.

Identification of functions of CCGs in the TME using single-cell and spatial transcriptomics

Data quality control was performed on single-cell raw gene expression matrices of colon cancer chemotherapy-related samples with the criteria: $nFeature_RNA \in (200, 6000)$, $nCount_RNA < 50,000$, and $percent.mt < 10\%$. The genes that met the requirements were used to select highly variable genes with the `FindVariableFeatures` function. The data were normalized using the `ScaleData` function and then subjected to PCA dimensionality reduction analysis, clustering using UMAP/TSNE, and clustering based on the Biomarker database (<http://bio-bigdata.hrbmu.edu.cn/CellMarker/>) and obtained from the marker genes for subcluster annotation of cell populations, searching for significantly different cell populations in drug-resistant and sensitive samples, analyzing different intercellular communications, and analyzing the expression of core candidate genes in them. Further, based on the GSE132465 dataset, subpopulation clustering was performed on SPPI+ macrophages. Manual annotation was conducted using the CellMarker database (<http://117.50.127.228/CellMarker/>) to compare cellular differences between cancer and adjacent non-cancerous tissues. DotPlot visualisation was employed to illustrate the expression levels of key genes.

To investigate cellular differences between chemoresistant and sensitive colorectal cancer samples, we analysed data from the GSE178318 dataset and published literature by Jabbari et al. (<https://doi.org/10.5061/dryad.pvmcvdngt>). First, cells underwent standard quality control (criteria: $nFeature_RNA: 200 - 5000$; $nCount_RNA < 20000$; $percent.mt < 10\%$), followed by clustering and annotation of cell subpopulations based on known cellular markers. Thereupon, we compared differences in cell type proportions between the resistant and sensitive groups, assessed the overall expression patterns of core candidate genes, and investigated the expression of key genes within the macrophage population.

In order to further clarify the spatial heterogeneity of the core candidate genes in sensitive/drug-resistant colon cancer tissues, we used chemotherapy-related spatial transcriptome samples of colon cancer to statistically analyze the spatial transcriptome

data by the R package “Seurat”, and then performed a linear dimensionality reduction of the data after the batch was de-batched to classify the cell clusters according to the optimal dimensionality value. After batch removal, the data were linearly dimensionalized to classify the cell types according to the optimal dimensionality value, and the samples in the spatial transcriptome dataset were visualized using the SpatialFeaturePlot function, and then MIA (Model-based Integrated Analysis) was performed using the thresholds $\text{avg_log2FC} > 0.3$ & $\text{p_val_adj} < 0.05$, and subpopulation MIA was performed based on the characteristic genes of the different cell types. of characteristic genes were visualized after performing subpopulation MIA.

Bidirectional Mendelian Randomization (BMR) and Mediation Mendelian Randomization (MMR) validation of causal relationships between CCGs and RNA methylation-mediated PCD regulatory patterns and key immune cells

To further determine the causal relationship, i.e., whether RNA methylation-regulated PCD resistance triggers CRC chemotherapy resistance or CRC chemotherapy resistance triggers RNA methylation-regulated PCD resistance, we collected the key genes for drug resistance from the relevant literature^{56,57}: *ABCB1*, *ABCC1*, *MRP1*, *HPBP1*, *HS71A*, *HS71B*, *HSP90*, *NR1H2*, *GSTP1*, *CALCR*, *PRR13*, *LRP1*, and bidirectional Mendelian randomization analysis of the relationship between the above genes and the core candidate genes. The core candidate genes were first used as the endpoints, and the resistance genes were used as the exposure factors, and then the reverse attempts were made. eQTL data were obtained from GWAS data, Steiger test was used to determine the directionality, and five algorithms were used for MR analysis (MR Egger, Weighted median, Inverse variance weighted, Simple mode , Weighted mode), and MRsteiger analysis was performed on exposure factors to determine the direction of exposure and outcome.

Considering that immune cells play an important role in the tumor microenvironment, in order to further clarify what kind of immune cells are involved in the process of PCD mediated by core candidate genes and RNA methylation in the 5-FU resistance process and to assess the mediating effect played by immune cells in it, the exposure and outcome factors with

verified causality were then subjected to immune cell MMR again and the mediating effect was calculated.

Cell culture, acquisition and processing of pathological tissue and measurement of cellular RNA Methylation levels

Acquisition of cell lines and tissue samples: Human monocytic THP-1 cells with colorectal cancer cell line LOVO were purchased from ATCC (TIB-202 and CCL-229), and human 5-FU-resistant colorectal cancer cell line LOVO/5FU was purchased from Cellverse Co. In addition 10 postoperative samples that developed 5-FU resistance after 4 cycles of NACT and 10 matched control samples were randomly selected from within our clinical cohort in 2.1. Of these, 6 were fresh postoperative tissues and the rest were paraffin-embedded samples. All samples were obtained with patient informed consent and were reviewed by the Medical Ethics Committee of Second Affiliated Hospital of Army Medical University (Ethics Number: 2019-YD103-01).

Preparation and polarization of 5-FU-M2 macrophages: The experimental procedures are mainly followed by protocols published before^{58,59} (composed of 3 steps listed as follows).

Step 1 (generation of 5-FU-associated macrophages, 5-FU-M): THP-1 monocytes (Hainan Choitec Pharmaceutical Co., Ltd) were treated with 15 μ M 5-fluorouracil (5-FU, HY-18739, MedChemExpress) in complete culture medium. Subsequently, phorbol myristoyl acetate (PMA, 100 nM, HY-18739, MedChemExpress) was added to induce macrophage differentiation, followed by incubation for 12 hours under standard conditions (37°C, 5% CO₂).

Step 2 (tumor-conditioned medium, TCM, preparation): LOVO colorectal carcinoma cells were cultured to 70% confluency, washed twice with PBS, and maintained in serum-free medium for 24 hours. The supernatant was collected, centrifuged at 1,500 $\times$ g for 10 min, and filtered through a 0.22 μ m membrane to obtain sterile TCM.

Step 3 (polarization to 5-FU-M2 phenotype): 5-FU-M macrophages were incubated with TCM supplemented with fresh medium (1:1 ratio) and 20 ng/mL recombinant human IL-4 (Thermofisher, NO. 14-7048-81) for 48 hours. M2 polarization

was confirmed via flow cytometry using anti-CD206 (BioLegend, Clone 15-2) as a surface marker. CD206⁺ cells were sorted and expanded for downstream experiments (namely as 5-FU-M2).

The constructed 5-FU-M2 were assayed according to the standard procedure of RNA methylation assay kit (EpiQuik m6A RNA Methylation Quantification Kit, NO. P-9005-96). The RNA methylation level was then calculated from the standard curve.

Next, the preparation of TAMs consists of 2 steps. Firstly, generation of macrophages, M: THP-1 monocytes (Hainan Choitec Pharmaceutical Co., Ltd) were treated with phorbol myristoyl acetate (PMA, 100 nM, HY-18739, MedChemExpress) to induce THP-1 differentiation, followed by incubation for 12 hours under standard conditions (37°C, 5% CO₂); Secondly, establishment of tumor-associated-macrophages (TAMs): Macrophages were incubated with TCM (mentioned above) supplemented with fresh medium (1:1 ratio) and 20 ng/mL recombinant human IL-4 (Thermofisher, NO. 14-7048-81) for 48 hours. And then the TAMs were established.

Then the methods of establishment of stable SCG2-knockdown TAMs (shSCG2-TAMs) are listed as following: Independent short hairpin RNA (shRNAs) targeting human *SCG2* (*shSCG2*, Supplementary Data.1) was cloned into the doxycycline-inducible lentiviral Tet-pLKO-puro vector. For lentiviral production, the constructed plasmids were co-transfected into THP-1 cells with packaging plasmids psPAX2 and envelope plasmid pMD2.G using Lipofectamine 3000 (Invitrogen, L3000015), following the manufacturer's protocol. Viral supernatants were harvested 72 hours post-transfection, filtered through 0.45 µm PVDF membranes (Millipore, SLHVR33RB), and used to transduce target cells at a multiplicity of infection (MOI) of 10 in 6-well plates (10⁶ cells/well). Transduced cells underwent puromycin selection (2 µg/mL, Biofroxx, 1299) for 14 days to generate polyclonal populations. SCG2 knockdown was induced by treating cells with 5 µg/mL doxycycline (MedChemExpress, HY-N0565B) for 48 hours prior to functional assays.

Successful knockdown cells were validated by Western blotting and quantitative real-time PCR (qPCR) using β-actin and

GAPDH as internal controls, respectively. The qualified cells were collected and induced by PMA (25nM, 24h), then were cultured with IL-4 (20 ng/mL) and 50% TCM for 48 hours. Finally, the shSCG2-TAMs were established.

Moreover, the siRNA interference sequences were designed and synthesized by Guangzhou Ribobio Co. 5 μ L siSCG2-1, siSCG2-2, siSCG2-3 and negative control (si-NC) were mixed with 5 μ L Lipofectamine RNAiMAX (13778150, Invitrogen, USA) in 200 μ L Opti-MEM medium (31985070, Gibco, USA) and incubated at room temperature for 15 min. The human colorectal cancer cell line LOVO was trypsin-digested and inoculated in 6-well plates at a density of 5×10^5 cells/well. The plates were placed in a humidified incubator containing 5% CO₂ for 60 h at 37°C.

Following validation by quantitative PCR (qPCR), the siRNA sequence exhibiting the highest knockdown efficiency (namely si-SCG2) was selected for subsequent experimental procedures (Supplementary Data. 1).

Immunohistochemistry and immunofluorescence

Paraffin sections were dried in an oven at 65 °C for 30 minutes, followed by deparaffinization in xylene. After rehydration through a graded ethanol series, antigen retrieval was performed using EDTA antigen retrieval solution (Beyotime, P0081). Non-specific binding sites were blocked with goat serum (ZSGB-BIO, ZLI-9056). Primary antibodies used included SCG2 (rabbit polyclonal antibody, Proteintech, 20357-1-AP, 1:100 dilution; Novus, NBP1-85631, 1:100 dilution). Visualization was achieved using the Dako REAL™ EnVision™ Detection System. Sections were counterstained with hematoxylin for 1 minute. Images of representative areas were captured, and integrated optical density (IOD) was measured using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, MD, USA).

Cells were cultured on glass coverslips, fixed in 4% paraformaldehyde for 20 minutes, and blocked with 10% goat serum (ZSGB-BIO, ZLI-9021) at room temperature for 30 minutes. Coverslips were then incubated with primary antibodies: (SCG2, proteintech, 20357-1-AP, 1:100 dilution; UBQLN1, Novus, H00029979-M01, 1:100 dilution; TNFA, ThermoFisher, PA5-

46945, 1:100 dilution) and secondary antibodies (alternative secondary antibodies Alexa Fluor 488:1:500, Invitrogen, A-27034; Alexa Fluor 546, 1:500, Invitrogen, A-11010; Alexa Fluor 647, 1:500, Invitrogen, A-32733). Finally, slides were restained with DAPI (Beyotime, C1005) and visualized using a laser confocal scanning microscope (ZEISS, LSM980 and Olympus Corporation, FV3000). Image Pro Plus 6.0 software was used for quantitative analysis.

(Real-time, Rt)qPCR And Western Blot(WB)

qPCR—total RNA extraction: total RNA was extracted from cell samples, thus obtaining all mRNA and non-coding RNA in these samples; Real-time PCR: real-time PCR experiments were performed using SYBR PrimeScript PCR Kit II (TaKaRa) to detect the expression levels of specific genes. GAPDH was used as an internal reference gene. Primer sequences are shown in Supplementary Data. 1.

WB—suspension cells were lysed using RAPI (Beyotime, P0013B). After centrifugation to remove supernatant, protein concentration was determined using the BCA Protein Assay Kit (Beyotime, P0010). Proteins were denatured by heating in a water bath at 100°C for 10 min after addition of Protein Sampling Buffer. After the samples were loaded, the proteins were separated by electrophoresis. The proteins were then transferred to PVDF membrane. The antibodies involved were as follows: SCG2, proteintech, 20357-1-AP, 1:1000 dilution; TNFA, proteintech, 240702B7, 1:1000 dilution; GAPDH (Cell Signaling Technology, 2118L, 1:3000 dilution), peroxidase-coupled goat anti-rabbit antibody, and peroxidase-coupled goat anti-rabbit antibody. GAPDH (Cell Signaling Technology, 2118L, 1:3000 dilution), peroxidase-coupled goat anti-rabbit IgG (H+L) (ZSGB-BIO, ZB-2301, 1:2000 dilution). Target proteins were developed using SuperSignaling™ West Femto substrate (Thermo Scientific, 34095) and a Bio-Rad gel imager.

Ubiquitination assay

Cells were co-transfected with si-SCG2, si-NC and HA-ubiquitin for 48 hours. Cells were then collected by treatment with 10 μ M MG132 (MCE, HY-13259, a proteasome inhibitor) for 8 hours. After collection, cells were washed with PBS and lysed with IP lysis buffer (ABclonal, RM00022) containing protease inhibitors and phosphatase inhibitors (Thermo Fisher, 78446) for 30 min on ice. Subsequent pull-down experiments were performed using Protein A/G PLUS agarose (Santa Cruz, sc-2003), followed by Western blot analysis using an anti-ubiquitin antibody (BioLegend, 646302) to detect the level of ubiquitination of TNF- α .

Immunoprecipitation assay (IP) and RNA immunoprecipitation assay (RIP)

IP: Cell lysis was performed using IP Lysis Buffer (ABclonal, RM00022) containing protease and phosphatase inhibitors and protein concentration was determined using a BCA assay kit. After centrifugation at 4°C, 12,000 $\times$ g for 30 min, 1/10 volume of the protein supernatant was taken as input sample and 1/4 volume as IgG sample. Incubate the remaining volume of each supernatant sample with the indicated primary antibody at 4°C overnight. The following dilutions of primary antibodies were used: mouse IgG (Cell Signaling Technology, 5127S, 1:100); mouse monoclonal anti-UBQLN1 (ThermoFisher, A305-896A, 1:100); and rabbit monoclonal anti-SCG2 (ThermoFisher, A300-105A). 1:100. Pierce Protein A/G magnetic beads (Santa Cruz, sc-2003) were then added and the mixture was incubated for 8 hours. After washing with IP buffer containing protease and phosphatase inhibitors, the immunoprecipitated complexes were eluted, neutralized and denatured with SDS for further analysis by immunoblotting (IP experiment between TNF- α and UBQLN1 was conducted in a manner similar to the steps described above.).

RIP: Cells were fully lysed using lysis buffer (Thermo Scientific™ Pierce IP, 87788) and incubated on ice for 15 min, after completion of the ice incubation centrifuged at 16,000g for 10 min at 4 degrees Celsius and the supernatant was collected for use in subsequent experiments. Next, 0.02 ml of magnetic bead suspension was added to each sample's EP tube, followed by

washing the magnetic beads with 0.1 ml of RIP washing solution; the EP tubes were placed on a magnetic rack, the supernatant was discarded, and the washing procedure was repeated; after completion, 0.5 ml of RIP wash buffer was added to each tube, along with 5ug of m6A primary antibody (SAB5600251, Merck), and the tubes were rotated and incubated for 3hours at room temperature; the supernatant was then discarded through the magnetic rack and 0.5 ml of RIP Then add 0.5ml of RIP wash buffer to each tube and vortex. After discarding the supernatant by magnetic rack, put the antibody-conjugated magnetic beads on ice as follows: add the supernatant of lysate into the magnetic beads, rotate and incubate at 4 degrees overnight, then discard the supernatant, add 1ml of RIP wash buffer to the system and transfer it to a new EP tube, and then resuspend the magnetic beads in 200ul of RIP wash buffer after repeated washes. After repeated washing, the beads were resuspended in 200ul of RIP wash buffer, pending further RNA extraction for qPCR quantitative analysis in 2.2.4.

Co-culture of macrophages and tumor cells, Elisa and flow cytometry

In a humidified incubator at 37 °C with 5% CO₂, a Transwell chamber system was employed to culture LOVO and 5-FU-resistant LOVO (LOVO/5FU) cells in the upper chambers, each supplemented with their respective growth media: Group I: LOVO cells with 5-FU; Group II: LOVO/5FU cells without 5-FU; Group III: LOVO/5FU cells with 5-FU; Group IV: LOVO/5FU cells with 5-FU. The lower chambers were configured as follows: Group I: RPMI-1640 complete medium; Group II: RPMI-1640 complete medium with THP-1 monocytes; Group III: RPMI-1640 complete medium with pre-cultured 5-FU-treated tumor-associated macrophages (5-FU-TAMs); Group IV: RPMI-1640 complete medium with *SCG2*-silenced 5-FU-TAMs. After gentle agitation to ensure even cell distribution, the co-culture was maintained for 48 hours. Subsequently, the levels of *TNF-α* in the culture supernatants were quantified using enzyme-linked immunosorbent assay (ELISA). Activation of the NF-κB pathway in tumor cells from the upper chambers was assessed. Cell viability in the upper chambers was evaluated via Transwell and CCK8 assays, and apoptosis rates were determined using flow cytometry.

Animal experiment

BALB/c-Nude mice were purchased from GemPharmatech Co., Ltd. (Nanjing, China). All animal studies were approved by the Institutional Animal Care and Use Committee of Army Medical University and all mice were treated according to the guidelines of the animal committee. All mice were housed in a specific pathogen-free (SPF) animal facility at Army Medical University, where environmental conditions were strictly maintained at a temperature of 22 ± 2 °C, relative humidity of $50 \pm 10\%$, and a 12-hour light/dark cycle (lights on at 7:00 AM, off at 7:00 PM). The animals had ad libitum access to standard sterilized rodent feed and autoclaved drinking water; bedding was changed twice weekly, and cages were regularly sanitized. To minimize selection bias, all animals were randomly assigned to experimental groups using a complete randomization procedure following model establishment (e.g., after tumor formation in the CDX model). Furthermore, blinding was implemented during experimental outcome assessments, meaning personnel performing the measurements and analyses were unaware of the group allocations until all data collection was complete. For procedures that could cause brief discomfort, such as tumor cell inoculation and tumor volume measurement, which typically lasted less than two minutes, mice were physically restrained without general anesthesia to enable rapid completion. For terminal tissue collection, euthanasia was performed by carbon dioxide (CO₂) inhalation anesthesia: mice were placed in a pre-filled euthanasia chamber containing 100% CO₂, which was then infused at a flow rate displacing 10–30% of the chamber volume per minute; death was confirmed by the absence of movement, respiration, and pupillary dilation, followed by an additional 2–3 minutes of observation before tissue harvesting. The detailed experimental methodology is described below.

First, the construction method of cancer cell-derived animal xenografts (CDX) was described: Cell suspensions were prepared and 2×10^6 LOVO cells or 2×10^6 LOVO/5FU cells were injected into the abdominal subcutaneous tissue of two groups of nude mice (3 mice per group) to generate primary tumors. Then 5-FU at a concentration of 25 mg/kg was injected

into the two groups of mice at the second and third weeks after tumor formation, respectively (administered on the first three days of each week). The body weight and tumor volume of the mice were monitored, and the mice were killed when the maximum length of the tumors reached 1.5 cm or at the fourth week of observation; the tumor tissues of the transplanted tumor-bearing mice in each group were taken, and one part was used for RNA extraction to measure the expression of *SCG2* and *TNF- α* in the tissues, while the other part was fixed with 4% paraformaldehyde, and then sliced into 3 μ m-thick slices transversely by using a sectioning machine, and then stained with immunofluorescent staining after blocking the slices.

Following the cell preparation protocol outlined in Section 2.2, nine BALB/c-Nude mice were randomly divided into three groups. Mice in Groups I and II received subcutaneous inoculation of 2×10^6 LOVO/5-FU cells into one flank, while Group III was inoculated with an equivalent volume of LOVO cells at the same site to establish primary tumors. One week post-inoculation, intratumoral injections were administered at four distinct sites within each tumor using a 27-gauge needle. Specifically, Group I received sh*SCG2*-TAMs, whereas Groups II and III were injected with shNC-TAMs (dose: 1×10^6 cells in 50 μ L PBS per mouse). Tumor dimensions were monitored every three days using vernier calipers. Beginning in the third week post-inoculation, all mice underwent intraperitoneal 5-FU administration (25 mg/kg) for three consecutive days per week. The experiment was terminated at 4 weeks post-inoculation (or earlier if any tumor exceeded 1 cm in maximum diameter), followed by euthanasia. Primary tumors were excised, weighed, and measured for volume quantification to generate growth curves. Tissue sections were prepared for immunofluorescence staining to assess *TNF- α* and *IKB α* fluorescence intensity. Western blot analysis was performed to evaluate protein expression levels of *SCG2*, *TNF- α* , *BCL-2*, and *BCL-XL* across experimental groups.

Second, the construction protocol for patient-derived tumor (PDX) models is described in detail as follows: In order to further determine the effect of TME on 5-FU resistance and the relationship between the two, we cut the fresh tissues mentioned in Methods section 2.2.1 into small pieces measuring 2–3 mm³ and implanted them into the incision at the distal end of the

mouse cecum. The incision was then sutured, followed by disinfection. After the tumors were formed for 1 week, 5-FU at the same dose as CDX was given on the first three days of each week from the second week until the maximum length of the tumors reached 1.5 cm, or when the observation time was up to the fourth week, the mice were put to death, and some part of the tissues was fixed and sectioned and stained with immunohistochemistry, while the other parts were extracted with RNA, to measure the levels of *TNF- α* in the tumor microenvironment, the expression of *SCG2* and the activation of the NF- κ B pathway.

Statistical analysis

Experimental data were analyzed using SPSS 25.0 and GraphPad Prism 9.0 software. All quantitative data were expressed as standard deviation (SD) $\pm$ mean. Comparisons between more than two groups were made using one-way analysis of variance (ANOVA) with Holm-Sidak multiple comparisons test (post hoc), and comparisons between two groups were made using two-tailed independent t-tests. No animals or data points were excluded from the analysis. Data were assumed to have a normal distribution and hypothesis testing was performed. Mann-Whitney U test and Student's t test were used to analyze the data of qRT-PCR, immunofluorescence staining and other assays. For the mouse studies, the culture conditions were as identical as possible to exclude interference from environmental factors, and the tumor volumes were compared between different groups using analysis of variance (ANOVA) after necropsy. For immunohistochemical analysis, one-way ANOVA was used for statistics. P values less than 0.05 were considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Moreover, the data processing and experimental framework structure is shown in **Fig. 2** (Graphic abstract).

Authors' Disclosures

No disclosures were reported.

Data and code availability

Data including GSE28702, GSE19860, GSE62080, GSE69657, GSE72790, GSE35452, GSE39582, GSE29261, GSE17537, GSE17538, GSE41258, GSE132257 and GSE132465 are available from: <https://www.ncbi.nlm.nih.gov/geo/>, and can be obtained after retrieving corresponding dataset. TCGA-COAD and TCGA-READ are from: <https://xena.ucsc.edu/>. sc-CRLM: <http://www.cancerdiversity.asia/scCRLM/>.

The clinical data of the CRC cohort in our hospital: Due to ethical and private restrictions, we cannot publicly share individual-level raw data of our clinical cohort. However, subject to data use agreements and collaborator approval, we can provide regulated access to bona fide researchers on our hospital secure servers after official approval.

ECM-RGs are from Reactome database: <https://reactome.org/>. IEU-OpenGWAS data are from: <https://gwas.mrcieu.ac.uk/>. microRNA data are from Starbase: starbase.sysu.edu.cn/. Hub genes and diseases interaction network are built upon CTD database: <https://ctdbase.org/>.

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Author contributions

Y. Sun: Conceptualization, Methodology, Software, Formal analysis, Writing-Original Draft; Experiment, Visualization. K. Peng: Software, Validation, Investigation. Y. Li: Software, Formal analysis, Writing-Original Draft, Visualization, Software, Investigation. D. Zheng: Software, Validation, Investigation. L. Liu: Conceptualization, Methodology. J. Yin: Writing-Original Draft, Supervision, Investigation. K. Yu: Methodology, Writing-Review Supervision, Funding acquisition. C. Xu: Conceptualization, Methodology, Writing-Review Supervision, Funding acquisition. C. João: Writing-Review Supervision. Z. Li: Conceptualization, Methodology, Visualization, Supervision, Experiment. C. Yin: Writing-Original Draft, Supervision, Investigation. W. Wang: Conceptualization, Methodology. W. Xiao: Writing-Original Draft, Supervision. All authors reviewed the manuscript and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Patient consent for publication

Consent obtained directly from patient(s).

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Fig. 1 Overall workflow for the data analysis section. This study integrates differentially expressed genes between chemosensitive and chemoresistant samples from multiple Gene Expression Omnibus (meta-GEO) datasets and RNA methylation- and cell death-related genes extracted from the literature. Resistance-associated RNA methylation genes were identified via weighted gene co-expression network analysis (WGCNA) and single-sample gene set enrichment analysis

(ssGSEA). Combined with differentially expressed genes from The cancer genome atlas-drug resistant colorectal cancer (TCGA-DR-CRC) cohort, a candidate gene set was obtained through intersection. Subsequently, the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and extracellular matrix (ECM) enrichment analyses were performed on the candidate genes. A prognostic model was constructed using Cox regression and 101 machine learning algorithms, and key genes were screened via Kaplan-Meier (KM) curves. External dataset validation, independent prognostic analysis, and nomogram construction were completed, along with immune infiltration and immune checkpoint analyses. Further analyses of two independent single-cell datasets and spatial transcriptome data validated the cellular expression distribution and spatial localization of key genes at single-cell resolution and spatial dimensions. Finally, bidirectional Mendelian randomization and mediation analysis confirmed the causal relationship, identifying SCG2 as the core regulatory gene and key target for 5-fluorouracil (5-FU) chemoresistance in colorectal cancer.

Fig. 2 Graphic abstract. Population screening was first performed using cohorts including the Gene Expression Omnibus (meta-GEO) and The cancer genome atlas-drug resistant colorectal cancer (TCGA-DR-CRC) cohort. Subsequent integration of multi-omics approaches encompassing single-cell RNA sequencing, spatial transcriptomics, Mendelian genetics, and machine learning led to the identification of SCG2 as the core target mediating 5-fluorouracil (5-FU) chemoresistance. Subsequent cellular experiments confirmed that SCG2 mediates 5-FU resistance in colon cancer through tumor-associated macrophages (TAMs)-dependent modulation of NF- κ B pathway signaling.

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Fig. 3 Validation demonstrated that the CCG model exhibits robust stability across multiple external datasets, consistently demonstrating risk prediction capability and serving as a prognostic model for assessing long-term survival in patients. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) a) In external validation cohort 1, the CCG model stratified patients into high- and low-risk groups based on the median value, with a significantly reduced survival rate in the high-risk group; Ac and d. The risk scores derived from the CCG model in Validation Cohort 1 effectively predicted mortality probabilities, with significantly elevated CCG expression levels. b) In external validation cohort 2, the CCG model stratified

patients into high- and low-risk groups using the median value, showing a significantly lower survival rate in the high-risk group; Bc and d. The risk scores from the CCG model in Validation Cohort 2 effectively distinguished mortality probabilities, with significantly higher CCG expression levels. c) Univariate cox regression analysis of the CCG model identified prognostic variables in the TCGA-CRC full cohort. d) Variable selection and multivariate Cox regression analysis were performed using the CCG model in the TCGA-CRC full cohort. Among them, risk score, metastatic extent and high-grade lymph node stage were statistically significant. e) A nomogram was developed to visualize risk scores derived from the CCG model for predicting prognosis and survival outcomes. f) Evaluation of the nomogram confirmed its prognostic predictive efficacy. g) Kaplan-Meier curves for patients with and without chemotherapy in the high- and low-risk groups based on CCGs model. h) Within the CCG model, SCG2 and BRCA1 were significantly upregulated in DR-CRC samples. i) In non-chemotherapy CRC samples, BRCA1 and TRAP1 were significantly upregulated, while SCG2 was significantly downregulated. j) In our institutional NACT cohort, it was confirmed that in 5-FU-resistant samples, only SCG2 was significantly upregulated, with the difference being statistically significant.

Fig. 4 SCG2 is the most critical gene within the CCGs, closely associated with the spatial distribution of macrophages and their precursor cells (myeloid) and mediating interactions between immune cells and tumor cells. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) a) Single-cell sequencing data from multiple CRC chemotherapy-related studies show significant differences in M2 macrophage subpopulations between resistant tumor tissues and sensitive tumor tissues (upper and lower left part, black dashed rectangle). b) SCG2 expression was markedly elevated within this M2 macrophage subpopulation. c) Macrophage subpopulation was significantly increased in chemotherapy-resistant samples (black dashed rectangle). d) SCG2 expression was markedly elevated within chemotherapy-resistant tumors; e) In spatial transcriptomic data from 5-FU chemotherapy-related CRC tissues, SCG2 demonstrates spatial co-localization with macrophage precursor cells

(myeloid) and is significantly upregulated within this subpopulation. f) There is significant communication between myeloid cells and CRC tumor cells. g) Bidirectional Mendelian randomization confirms the causal relationship between CCGs and chemoresistance. h) Immune cell-mediated Mendelian randomization identifies monocyte-derived macrophages as the key immune cells in RNA methylation-mediated regulation of PCD, contributing to CRC chemotherapy (5-FU) resistance (yellow and red rectangles). In other words, macrophages are identified as critical immune cells in this context. i) CTD predictions reveal associations between CCGs and diseases, with SCG2 being the only gene specifically linked to CRC.

Fig. 5 SCG2 is significantly upregulated in NACT-resistant CRC Tissues and significantly affects patients' prognostic survival of the primary chemotherapy outcome. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) a) Several rectal cancer patients initially diagnosed in our department underwent three cycles of NACT (CAPOX regimen). Enhanced CT and MRI showed significant tumor regression, indicating a response to 5-FU treatment (white arrows indicate the cross-sectional view of the tumor, and the scale is shown in the lower right corner). b) Immunohistochemical staining of SCG2 was performed on tumor tissues from patients in group A following surgery in our department (Upper parts, blue arrows). c) Several rectal cancer patients initially diagnosed in our department underwent three cycles of NACT (CAPOX regimen). Enhanced CT and MRI revealed no significant tumor regression or even progression, indicating non-responsiveness or resistance to 5-FU treatment (white arrows indicate the cross-sectional view of the tumor, and the scale is shown in the lower right corner). (d) Immunohistochemical staining of SCG2 was performed on tumor tissues from patients in group C following surgery in our

department (Lower parts, orange arrows). e) Analysis of differences in SCG2 expression between the chemotherapy-responsive and non-responsive groups within the CRC cohort. f) Kaplan-Meier survival curves for SCG2 expression and patient prognosis in the CRC cohort, including patients who received initial chemotherapy (regardless of its efficacy). g) Semi-quantitative analysis of immunohistochemical staining results from groups B and D using ImageJ.

Fig. 6 SCG2 expression in TAMs is regulated by m6A modification and dependent on the 5-FU microenvironment, enhancing CRC resistance to 5-FU. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) a) m6A modification levels and expression of SCG2 in TAMs within 5-FU or NS. b) Overall m6A levels in TAMs cultured. c) m6A concentration measurements in TAMs with and without 5-FU treatment. d) m6A abundance-absorbance curve in 5-FU-TAMs under 5-FU treatment. e) Analysis of the impact of m6A modification on SCG2 mRNA expression levels. f) Regulation of SCG2 protein expression by m6A modification in TAMs. g) Immunohistochemical staining to compare SCG2 expression levels between tumor tissues and adjacent normal tissues in CRC patients not treated with 5-FU chemotherapy or not requiring 5-FU treatment. h) Schematic diagram illustrating the construction of the CDX animal model and drug administration methods. i) Mice and tumor tissues after the completion of the experiment. j) Tumor growth curves of CDX models under different treatment conditions. k) Comparison of tumor mass between the drug-resistant group and the normal group of mice. l) Differences in SCG2 expression between the drug-resistant and normal groups in tumor tissues after the completion of the CDX experiment. m) Immunofluorescence colocalization of SCG2 and macrophage markers in CDX tumor tissues. n) Fluorescence intensity of

SCG2 in CDX tumors among different groups.

Fig. 7 SCG2 acts as a scaffold protein to mediate the ubiquitination of TNF- α by UBQLN1, and its expression level correlates with chemotherapy. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) a) Regulation of TNFA mRNA levels by SCG2 expression in TAMs. b) Regulation of TNF- α protein expression levels by SCG2 content in TAMs. c) In TAM cells, SCG2 and TNF- α were co-localized, and knockdown of SCG2 significantly upregulated TNF- α expression. d) Similarly, UBQLN1 and TNF- α also exhibited co-localization. Knockdown of UBQLN1 led to elevation in TNF- α protein levels. e) While SCG2 knockdown upregulated TNF- α expression, it did not affect UBQLN1 protein levels. However, the co-localization efficacy between TNF- α and UBQLN1 was markedly reduced indicating that SCG2 modulates their spatial interactions. f) Machine learning-based molecular docking to identify the binding sites of SCG2 with UBQLN1 and TNF- α . g) Knockdown of SCG2 significantly reduced the ubiquitination level of TNF- α , indicating that SCG2 promotes TNF- α ubiquitination in a UBQLN1-dependent manner (left part). (Right and lower part) semi-quantitative measurement of the ubiquitination levels of TNF- α mediated by SCG2 in TAMs; Changes in TNF- α protein levels after ubiquitination; Variations in SCG2 content as an internal reference; The effect of SCG2 content on TNF- α protein expression. h) Knockdown of SCG2 markedly attenuated the binding between TNF- α and UBQLN1, suggesting that SCG2 may act as a scaffold protein to promote the interaction between

UBQLN1 and TNF- α , which is required for efficient TNF- α ubiquitination (left part). (Right and lower part) semi-quantitative analysis of the binding ability of TNF- α to UBQLN1 with or without SCG2 knockdown; Semi-quantitative analysis of TNF- α protein levels under the same conditions; Semi-quantitative analysis of UBQLN1 binding to TNF- α with or without SCG2 knockdown; Semi-quantitative analysis of TNF- α protein levels.

Fig. 8 SCG2 is a mediator of macrophage–tumor cell crosstalk in the 5-FU chemotherapy microenvironment and further activates the NF- κ B pathway in tumor cells. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) a) Schematic diagram of the tumor cell-macrophage co-culture model under a 5-FU microenvironment. b) TNF- α abundance-absorbance curve determined by ELISA in the co-culture system. c) Calculation of TNF- α concentrations in each group of the co-culture system based on the curve. d) Flow cytometry analysis of tumor cell apoptosis across four groups. e) and f) After the experiment, cells from the upper chambers of each group were collected for a Transwell assay to evaluate cell invasion ability. g) After the experiment, cells from the upper chambers of each group were collected for a CCK assay to evaluate cell viability. h), i)- l). After the experiment, proteins from the upper chamber cells of each group were extracted to assess intracellular NF- κ B pathway activation.

Fig. 9 Animal experiments using PDX (patient-derived xenograft) and cell therapy models demonstrates that upregulation of SCG2 in TAMs confers tumor resistance to 5-FU, while interference with SCG2 expression restores tumor sensitivity to PCD mediated by 5-FU. (*, $P<0.05$; **, $P<0.01$; *, $P<0.001$; ****, $P<0.0001$)** a) Schematic diagram illustrating the construction of the PDX animal model and drug administration methods. b) Mice and tumor tissues after the completion of the experiment. c) Immunohistochemical colocalization of SCG2 and CD11b in transplanted tumors derived from NACT-sensitive/resistant patients after the mice were sacrificed (colocalized regions indicated by a small square in the bottom-right corner). d) Changes in tumor weight between the two groups after the completion of the PDX experiment (left part); Measurement of tumor volume of NACT-sensitive and NACT-resistant groups (right part). e) Differences in SCG2 expression between NACT-sensitive and resistant groups (right part); Comparison of the area of SCG2 and TAMs colocalization regions in NACT-sensitive and resistant groups (left part). f) Differences in TNF- α expression between NACT-sensitive and resistant samples (left part); Expression levels of key molecules in the NF- κ B pathway and anti-apoptotic genes in tumor tissues from both groups after the PDX model mice were sacrificed (right part). g) Schematic of SCG2 in TAMs mediating 5-FU resistance in colorectal cancer: in vivo therapeutic evaluation. h) Experimental results of three groups in murine tumor xenograft models. i) Tumor growth curves across three experimental groups. j) Weight measurement of tumor xenografts in three experimental groups. k) Expression profiles of IKB α and TNF- α (NF- κ B pathway-associated markers) in tumor xenografts across three groups. l) Immunoblot analysis of SCG2, TNF- α , and anti-apoptotic proteins (Bcl-2, BCL-X) in tumor

tissues from three experimental cohorts. m) Transcriptional profiling of NF- κ B pathway core genes in xenograft tumors across three groups. n) Integrated mechanistic schema summarizing SCG2-mediated regulation of 5-FU resistance in colon cancer via TAMs-dependent modulation of NF- κ B pathway signaling (overall mechanism diagram).

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