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Clinical relevance of CD70-CD27 axis in tumor microenvironment of patients with colorectal cancer

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Immune checkpoint molecules like CD70 provide a better knowledge of the tumor microenvironment. Some of the B and T lymphocytes express CD70 and it has a co-stimulatory factor on immune cells. When CD27 binds to its ligand (CD70), tumor cells can avoid detection by the immune system. We aimed to analyze the expression profiles of CD70, CD27, CD3, and FOXP3 molecules in the tumor microenvironment of colorectal cancer patients and the recruitment of tumor-infiltrating lymphocytes. We also investigated if soluble CD27 has any predictive diagnostic value for tracking cancer. A western blot wet transfer technique was used to examine the expression profile. ELISA was used to determine the amount of soluble CD27 protein in the patient's sera. CD70 expression was found to be low (15.15%) in tumoral tissue, whereas CD27 was abundant (84.80%). Tumoral tissues had high recruitment of CD3+ lymphocytes (81,80%) and FOXP3+ Tregs (48.50%). According to our findings, the level of sCD27 in patient's serum was high (P<0.0001), and there clear correlations between high sCD27 serum levels with CD70 positive and CD27 negativity in tumoral tissues. Distant organ metastases were found to be significantly correlated with high sCD27 serum levels (P=0.05). Dysregulation of the CD70-CD27 axis within the tumor and its microenvironment is associated with tumor progression and immunosuppression. Tightly controlled expression of CD70 and CD27 plays a role in co-stimulation in immune responses.

Keywords: Cancer immunology, ELISA assay, Immune checkpoints, Immunosuppressive mechanisms, Tumor-infiltrating lymphocytes, Western blot analysis

Colorectal cancer (CRC) is among the deadliest malignancies, with rising incidence and mortality rates each year[1]. A significant proportion of cases are metastatic at diagnosis, and nearly one-third of patients experience relapse after surgical resection[2]. Tumor development is influenced by both genetic and environmental factors, and the characterization of the tumor microenvironment (TME) is crucial for understanding disease progression. In particular, immune evasion and inflammatory states in the TME play a key role in CRC pathogenesis[3,4]. Recent discoveries of immunological biomarkers regulating the TME have improved tumor diagnosis and therapeutic strategies. By assessing immune cell infiltration in tumor and peritumoral regions, these biomarkers aid in predicting disease progression. Targeting immune checkpoint molecules has emerged as a promising approach to activate antitumor immunity[5,6]. These molecules, expressed on immune and tumor cells, modulate immune responses[7,8]. High regulatory T cell (Treg) ratios in the TME are linked to poor prognosis, and therapies targeting CTLA-4 and PD-1 pathways represent a next-generation approach. However, the effectiveness of these treatments remains limited to microsatellite instability (MSI) tumors, necessitating more effective immunotherapies for most CRC cases[9,10].

The tumor necrosis factor receptor superfamily (TNFRSF) member CD27 is a key co-stimulatory receptor in T-cell activation. Its ligand, CD70, is transiently expressed on activated lymphocytes but is aberrantly overexpressed in many malignancies[11-13]. The CD70-CD27 axis promotes T-cell activation and proliferation but, when excessively activated, contributes to tumor survival and immune evasion[14]. In hematological malignancies, co-expression of

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CD70 and CD27 supports cancer cell proliferation, while in solid tumors, CD70 expression facilitates immune evasion *via* CD27 interactions[15]. CD27 is widely present on CD4+ T cells and Tregs, with the latter implicated in suppressing antitumor immunity[12,16].

Therapeutic strategies targeting the CD70-CD27 axis, including monoclonal antibodies, are under clinical investigation and have demonstrated promising safety profiles[13,17]. In this study, we quantitatively analyzed CD70 and CD27 protein expression in CRC. We employed western blotting to assess CD3 and FOXP3 expression in the TME and used ELISA to measure soluble CD27 (sCD27) in CRC patients and healthy controls. Additionally, we examined the associations between these protein expressions, clinicopathological features, and clinical outcomes.

Materials and Methods

Study groups

This study included 33 colorectal cancer (CRC) patients The sample consists of CRC patients, with demographic data such as gender and age recorded, early-stage and advanced-stage refer to the progression of the disease, and tumor characteristics classified by and tumor staging (T1 to T4) ,lymph node metastasis stages (n0 to n3) based on the number of affected lymph nodes and tumor size/invasion, and mucinous or not from the Department of General Surgery at Istanbul Training and Research Hospital. The study protocol was approved by the Clinical Research Ethics Committee of Istanbul University Medicine Faculty (Approval No: 2017/883), and all participants provided written informed consent for tissue and serum collection**.**

Protein extraction and western blotting

Total protein was extracted from surgically removed frozen tissue samples using TRIzol® Reagent (Ambion by Life Technologies, Cat. no. 15596-026). A total of 33 paired colorectal cancer tumor tissues and their corresponding histologically normal tissues, taken from areas distant from the tumor site, were retrieved from storage at -80°C, thawed on ice, and homogenized with 1 mL of TRIzol® Reagent per 50-100 mg of tissue. Following the manufacturer's protocol, total protein extracts were prepared for each sample.

Extracted protein samples were subjected to Western Blotting to identify CD70, CD27, CD3, and FOXP3 expression in tissues. Protein concentrations were measured using the SMART BCA Protein Assay Kit (Cat. No. 21071). Proteins were separated using the Mini-Protean® Tetra System (Bio-Rad) and transferred onto membranes with the Mini Trans-Blot® Cell System (Bio-Rad). A wet transfer system was used for blotting with Immun-Blot® PVDF Membrane Sandwiches (Bio-Rad, Cat. no. 162-0219).

Primary antibody incubation was conducted overnight with the following antibodies: Anti-beta-actin (Abcam, ab119716, 1:5000), Anti-CD27 (Abcam, ab70103, 1 µg/mL), Anti-CD70 (Abcam, ab175389, 1:2000), Anti-CD3 (Abcam, ab70103, 0.5 µg/mL), and Anti-FOXP3 (Abcam, ab70103, 1:500). Membranes were pre-incubated with secondary antibody solution using Goat polyclonal Secondary Antibody for Rabbit IgG-Fc (HRP) (Abcam, ab97069, 1:5000). Detection was performed using the Clarity™ Western ECL Substrate Kit (Bio-Rad, Cat. no. 1705060), and chemiluminescent signals were acquired using a CCD camera-based imager.

Band intensities of each blot were quantified using ImageJ 1.52a software. Blot images were converted to an 8 bit format, and the rolling ball radius method was applied for background subtraction. Each band was individually selected using a rectangular region of interest (ROI) with the “Gels” function. The peak area of each band was quantified from histograms, and data were collected as arbitrary area values. A well-described method[18]. for “background subtraction” was used to obtain images with clear bands. Each band was read three times independently, and relative band density was calculated by normalizing the data with beta-actin quantification. These adjusted densities were then used for statistical analyses.

sCD27analysis

Blood samples were collected, centrifuged to obtain serum, and stored at -80°C. Human sCD27 levels were measured using the Human sCD27 INSTANT ELISA™ Kit (ThermoFisher Scientific) according to the manufacturer's instructions. A standard curve was generated, and sCD27 concentrations in serum samples from 33 CRC patients and 32 healthy controls were calculated based on this curve.

Statistical analysis

Potential associations between clinicopathological characteristics, protein expression levels, and serum sCD27 levels were analyzed using Chi-square,

Student's *t*-test, Fisher's Exact test, Mann-Whitney U test, and Kruskal-Wallis test, using SPSS Version 17.0. A two-way ANOVA test was applied to determine the significance between tumor and standard tissue expressions, with $P<0.05$ considered statistically significant. Western blot data were quantified using Image J 1.52a software for Windows, and further analyzed using two-way ANOVA in GraphPad Prism Version 8.2.1 for Windows. Correlations between CD70 and CD27 expression in tumor and normal tissues with serum sCD27 levels in CRC patients were also examined by Spearman correlation test. Statistical significance was determined with $P<0.05$ using IBM SPSS (Version 17.0).

Results

The study on colorectal cancer (CRC) patients reveals that (23 males, 10 females; mean age: 62.83 ± 12.49 years) and 32 healthy controls (25 males, 7 females; mean age: 60.65 ± 16.84 years) 69.70% of the patients are male, while females constitute 30.30%. and 57.58% of the patients have early-stage colon cancer, while 42.42% have advanced-stage colon cancer. The tumor staging values are distributed as follows: 3.03% of patients have a T1 tumor (small, localized), 9.09% have a T2 tumor (grown but still localized), 39.39% have a T3 tumor (spread to nearby tissues), and 48.48% have a T4 tumor (invaded other organs or structures), In terms of lymph node metastasis, 54.55% of the patients have no metastasis (n0), 18.18% have metastasis to 1-3 lymph nodes (n1), 24.24% have metastasis to 4-9 lymph nodes (n2), and 3.03% have metastasis to 10 or more lymph nodes (n3), and 27.27% of the colorectal cancer (CRC) patients in this study have mucinous adenocarcinoma, while the majority, 72.73%, do not have this subtype.

Western blot

This study analyzed 33 CRC primary tumor tissues and their corresponding normal tissues using Western blot to measure the protein expression levels of CD70, CD27, CD3, and FOXP3. Both positive and negative expression profiles were quantified using ImageJ and GraphPad Prism, as described in the methods. The expression results for CD70, CD27, CD3, and FOXP3 in tumor tissues from the 33 CRC patients were as follows: CD70 protein was expressed in 5 out of 33 tumor samples (15.15%). CD27 protein was detected in 28 out of 33 tumor samples (84.80%). CD3 protein was positive in 27 out of 33 tumor samples (81.80%). FOXP3 protein was positive in 16 out of 33 tumor samples (48.50%).

Protein expression in the tumor microenvironment (TME) was quantified and compared to corresponding adjacent healthy tissues. Each patient's samples were labeled as tumor (cancerous tissue) and normal (non-cancerous tissue), from P1 to P33. Among the 33 paired tissue samples: CD70 expression was detected in 5 primary tumor tissues, while none of the adjacent normal tissue samples showed CD70 expression (Fig. 1).

CD27 expression was absent in 28 normal tissues, with only 7 normal tissues showing positivity for CD27. CD3 expression was observed in 27 tumor tissues, while only 7 normal tissues were positive for CD3. FOXP3 expression was found in 16 tumor tissues, with 5 normal tissues showing FOXP3 positivity.

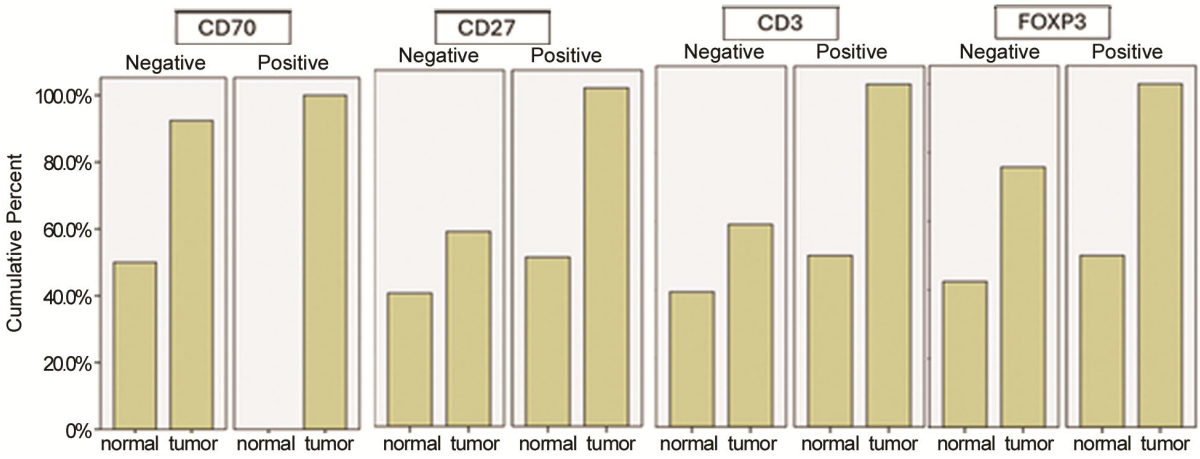

Fig. 1 — Quantification of CD70, CD27, CD3 and FOXP3 protein expressions on primary tumor tissues and normal tissues of CRC patients displayed in bar graphs. Band densities were quantified and represented in arbitrary units (a.u). ($P<0.0001$)

ELISA (sCD27)

The mean serum level of sCD27 in CRC patients was 101.8 ± 28.02 (Mean±SD) U/mL, indicating an average level of 101.8 U/mL with some variability. Serum sCD27 levels ranged from 19.90 to 139.43 U/mL, representing the lowest and highest values recorded in the study. ROC (Receiver Operating Characteristic) analysis established a cut-off value of 89.15 U/mL for sCD27, used to differentiate CRC patients from healthy controls. The diagnostic performance of sCD27 was characterized by: Sensitivity 0.74, meaning the test correctly identified 74% of CRC patients (true positives). Specificity 0.72, indicating that 72% of healthy controls were correctly identified as negative (true negatives) (Fig. 2).

However, an interesting finding was that among patients with CD3 positivity in tumor tissues, sCD27 levels were higher, nearing significance, particularly in those with tumors containing mucinous components (P= 0.051) Patients with serum sCD27 levels exceeding the cut-off of 99.3 U/mL were considered positive, while those below this threshold were deemed negative.

Correlation analysis

No correlation was found between sCD27 levels and the expression of CD3 or FOXP3 in CRC patients. A significant negative correlation was observed between CD70 expression in tumor tissues and CD27 expression (r = -0.764, P< 0.001), suggesting an inverse relationship between these two molecules in the TME.A positive correlation is observed between sCD27and CD70 (r = 0.739, P< 0.01), indicating that higher sCD27 levels are associated with increased CD70 expression in tumors.

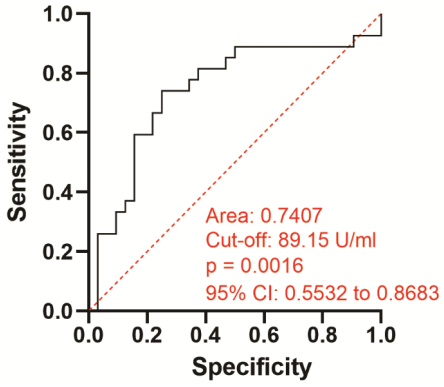

Fig. 2 — ROC curve measurement of sCD27 level in the serum of CRC patients and healthy controls. Above the value of 89.15 U/mL for sCD27, the sensitivity was determined as 74% and specificity was 72% in the patient group (P=0.0016).

In contrast, a negative correlation is found between sCD27and CD27 (r = -0.628, P< 0.01), as well as between CD70 and CD27 (r = -0.764, P< 0.01), suggesting an inverse relationship between sCD27 levels and CD27 expression in tumor tissue (Fig. 3). Spearman's correlation also investigates the relationships between CD27, CD70, CD3 and FOXP3 with tumor microenvironment factors like tumor staging values and lymph node metastasis. The key results are as follows: Tumor CD70 and lymphnode metastasis (r = 0.309, P < 0.05), normal FOXP3 and lymphnode metastasis (r = 0.309, P < 0.05), tumor FOXP3 and tumor staging values (r = 0.294, P < 0.05), tumor FOXP3 and lymphnode metastasis (r = 0.394, P< 0.05).

A moderate positive correlation is found, indicating that higher lymph node metastasis scores (n0, n1, n2, n3) are associated with increased CD70 expression in tumor tissue. A similar positive correlation is observed between FOXP3 expression in normal tissue and lymph node metastasis scores, suggesting that FOXP3 may be involved in immune regulation related to lymph node spread.FOXP3 expression in tumor tissue is positively correlated with tumor staging values (T1, T2, T3, T4), indicating its potential role in more advanced stages of tumor development. A stronger

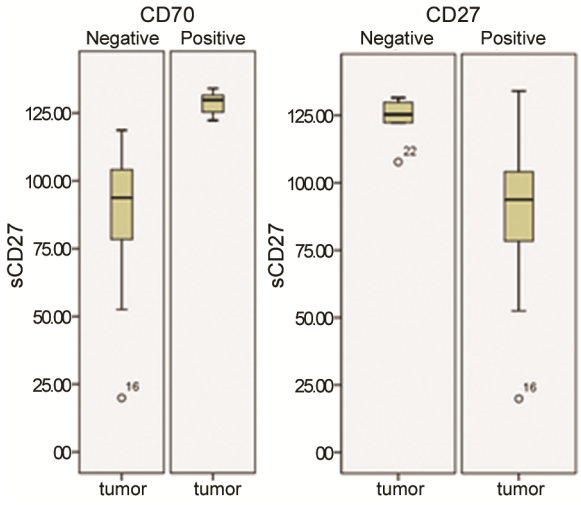

Fig. 3 — Spearman's correlation between sCD27, CD70, and CD27 in tumor Samples. Boxplot shows higher sCD27 levels in CD70 positive tumors compared to CD70 negative ones, with a broader range in CD70 negative tumors. Spearman's correlation shows significant positive correlation between sCD27 and CD70 (r = 0.739, P< 0.01) and negative correlations between sCD27 and CD27 (r = -0.628, P< 0.01), and between CD70 and CD27 (r = -0.764, P< 0.01).CD27 (r = -0.628, P< 0.01), as well as between CD70 and CD27 (r = -0.764, P< 0.01), suggesting an inverse relationship between sCD27 levels and CD27 expression in tumor tissue.

positive correlation is found between FOXP3 expression in tumor tissue and lymph node metastasis, suggesting that FOXP3 may play a key role in tumor aggressiveness or the immune response to tumor spread.

Discussion

The CD70-CD27 axis holds great potential as a target for cancer immunotherapy. Host immunity and the tumor microenvironment (TME) are critical factors to consider when developing therapies for malignancies. Treatments should aim to kill tumor cells while boosting the host's immune system. The TME plays a key role in regulating antitumor immune responses and the activity of tumor-infiltrating lymphocytes (TILs)[19,20]. The expression of CD70 regulates the CD70-CD27 signaling axis, and abnormal expression of CD70 can result in the increased production of inflammatory cytokines and immune dysfunction. Aberrant CD70 expression is present in some lymphomas and solid tumors, and its presence is associated with a worse prognosis. Although CD70 is usually restricted to activated lymphocytes and some tumor cells, immunoconjugates and antibodies that enhance antibody-dependent cellular cytotoxicity (ADCC) are being developed to target CD70-expressing cells. CD70 expression on tumor cells has been linked to immune evasion and tumor growth through mechanisms such as Treg expansion, reduced tumor-specific T-cell responses, and increased angiogenesis[19].

In normal tissues, CD70 expression is limited, but its overexpression in solid tumors such as renal cell carcinoma (RCC) has been associated with poor clinical outcomes[21]. Immunotherapies for colorectal cancer (CRC) currently benefit a small subset of patients, particularly those with microsatellite instability (MSI). There is an urgent need for therapies targeting other CRC subtypes. The CD70–CD27 pathway influences T-cell function, B-cell activation, and NK-cell survival. In the TME, persistent CD27 signaling can occur through CD27 expression on tumor-infiltrating lymphocytes (TILs), which interact with CD70+ carcinoma cells[22]. Abnormal activation of the CD70-CD27 axis leads to tumor growth, survival, and immune evasion, making it a promising therapeutic target[9,23,24]. TAFs are a significant component of the TME, contributing to tumor development by promoting proliferation, angiogenesis, and immune modulation[6]. The interaction between CD70 and CD27 also regulates T-cell fibroblast associations[24,25], but their specific role in CRC remains unclear. Jacobs *et al*. demonstrated that while CD70 is absent in most tumor cells, it is prevalent in certain TAFs, particularly under inflammatory conditions such as chronic viral infections, cancer, or autoimmune diseases[26,27]. CD70-positive TAFs contribute to malignant cell migration and Treg recruitment, offering a potential target for anticancer therapies. Investigating the role of CD70-positive TAFs in the tumor-associated microenvironment (TMA) is critical for developing new therapeutic strategies[10].

CD27 is a co-stimulatory receptor expressed on a small proportion of NK cells, memory B cells, and resting CD8+ and CD4+ T cells, including CD4+FOXP3+Tregs[28]. The transcription factor FOXP3 is crucial for Treg development and function[29]. Yang *et al*. showed that intratumoral Tregs increase in the presence of CD70-expressing tumor cells in patients with non-hodgkin lymphoma (NHL). Blocking the CD70-CD27 pathway reduced FOXP3 expression in intratumoral CD4+CD25 T cells, highlighting its role in immunosuppression[30]. Our study found a positive correlation between FOXP3 and CD70 expression in CRC tumor tissues. Furthermore, in three patients with CD70(+) and FOXP3(+) tumors, sCD27 levels were higher than the determined cut-off value. These results suggest that sCD27 has some role in CD70(+) and FOXP3(+) tumors. CD27 is cleaved from the cell surface following its interaction with CD70, releasing soluble CD27 (sCD27) into the serum[31-33]. Elevated sCD27 levels have been detected in hematological malignancies and autoimmune diseases[34,35]. In this study, we observed elevated sCD27 levels in CRC patients compared to healthy controls. However, studies in prostate cancer have reported that elevated sCD27 levels do not correlate with improved survival[36]. Additionally, matrix metalloproteinase-8 (MMP-8) has been implicated in sCD27 release, as MMP-8 inhibitors blocked sCD27 release in vitro[37]. We found no significant correlation between sCD27 levels and histopathological findings, but sCD27 levels were higher in patients with CD3+ tumor tissues, particularly those with mucinous components. CD27 expression on CD4+ T-cells differentiates Tregs from effector T-cells, and CD27 correlates with FOXP3 expression and Treg activity[38]. In non-small cell lung cancer (NSCLC), CD70 acts as an inducer of Tregs, resulting in increased FOXP3 expression and higher CD4/CD8 ratios in CD70+ tumor regions[39]. CD70

expression on tumor cells is associated with Treg expansion, lymphocyte apoptosis, and immune evasion in RCC, glioma, and glioblastoma[40,32]. Thus, targeting CD70 while enhancing T-cell activation could improve cancer immunotherapy outcomes[10].

CD70 is highly expressed in RCC and glioblastoma, where it serves as a clinicopathological biomarker[1,14]. Even in tumors with limited CD70 expression, CD70 can promote tumor progression through its interaction with other components of the TME, such as cancer-associated fibroblasts (CAFs). In cancers like colorectal and pancreatic carcinoma, CAFs contribute to oncogenesis and therapy resistance[43,44]. Serum sCD27 concentrations correlate with CD70-CD27 interaction in the TME[45], with high sCD27 levels linked to advanced disease and poor prognosis in lung cancers[46,47].

Studies have shown that CD70-high CAFs mediate immune suppression by increasing CD4+FOXP3+CD25+Tregs and promoting colorectal carcinoma cell migration[39]. However, CD70 expression is absent in several cancers, including Kaposi sarcoma, prostate carcinoma, and CRC[48,49].

The role of the CD70-CD27 axis in promoting tumor progression has been observed in both hematological and solid tumors, including NSCLC. Elevated sCD27 levels have been found in patients with hematological and solid malignancies[39]. However, the relationship between CD70 expression and patient prognosis is unclear in CRC. Only 5 out of 33 CRC patients in our study exhibited CD70 positivity. CD70 expression was absent in normal tissues adjacent to the tumors, with significant differences observed between tumor and normal tissues. CD27 expression in tumor tissues was negatively correlated with CD70 expression, and patients with high CD70 expression had elevated sCD27 levels.

Surprisingly, high sCD27 levels were associated with CD27-negative tumors, suggesting a complex regulatory mechanism in the TME. The dual role of the CD70-CD27 axis, promoting both immune activation and suppression, highlights its importance in CRC progression.

Conclusion

Our findings highlight the significant role of the CD70-CD27 axis in colorectal cancer (CRC) progression and immune suppression within the tumor microenvironment (TME). The elevated serum sCD27 levels, particularly in patients with CD70-positive and CD27-negative tumors, suggest its potential as a biomarker for CRC progression. The inverse correlation between CD70 and CD27 expression and the positive association between sCD27 and CD70 levels indicate a complex regulatory mechanism that may contribute to immune evasion and tumor aggressiveness. These findings underscore the therapeutic potential of targeting the CD70-CD27 axis in CRC. Strategies aimed at modulating this pathway could help restore antitumor immunity and improve clinical outcomes, particularly for patients who do not benefit from current immune checkpoint inhibitors. However, given the limited sample size of our study, further research with larger patient cohorts is necessary to validate these observations and explore the mechanistic underpinnings of CD70-CD27 signaling in CRC. Future studies should also investigate whether sCD27 can serve as a reliable prognostic or predictive biomarker in clinical settings. By deepening our understanding of the immunosuppressive mechanisms within the TME, this study contributes to the ongoing search for more effective immunotherapeutic approaches in CRC management.

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Conflict of interest

The authors declare that they have no conflict of interest.

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