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Role and Mechanism of S100A6 in Promoting Colorectal Cancer Cell Proliferation via Macrophages (Post-print)

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Abstract

Objective: To investigate whether S100A6 in the microenvironment promotes colorectal cancer (CRC) cell proliferation by affecting macrophages (M) and the underlying mechanism. Methods: Recombinant human S100A6 protein with GST (glutathione S-transferase) tag (recombinant GST-hS100A6, rS100A6) and control protein GST were prepared (via prokaryotic expression) and identified; trypan blue counting, CCK8 assay, and crystal violet staining were used to detect the proliferation capacity of CRC cell line HCT116; quantitative real-time polymerase chain reaction was employed to detect IL-6 mRNA levels in M ; Western blot was utilized to detect IL-6 protein levels in M and JAK2 and STAT3 as well as their phosphorylation levels in HCT116 cells. Results: (1) rS100A6 and GST proteins were successfully prepared. (2) After co-culture with rS100A6-treated M (A6-M), the proliferation capacity of HCT116 cells was enhanced (P<0.05); meanwhile, JAK2 and STAT3 levels in HCT116 cells showed no significant change, but their phosphorylation levels were increased (P<0.05). (3) In A6-M , both IL-6 mRNA and protein levels were elevated (P<0.05). (4) After adding IL-6R blocking peptide to the co-culture system of HCT116 and A6-M , the promoting effect of A6-M on HCT116 cell viability and proliferation capacity was partially reversed (P<0.05). Conclusion: S100A6 in the microenvironment can promote CRC cell proliferation by upregulating IL-6 expression in macrophages and subsequently activating the IL-6/JAK2/STAT3 signaling pathway in HCT116 cells.

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Full Text

Preamble

S100A6 Promotes Colorectal Cancer Cell Proliferation Through Macrophages: Role and Mechanisms

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Abstract

Objective: To investigate whether Calcyclin S100A6 in the tumor microenvironment promotes colorectal cancer (CRC) cell proliferation by affecting macrophages (M) and to elucidate the underlying mechanism.

Methods: Recombinant human S100A6 protein tagged with glutathione S-transferase (recombinant GST-hS100A6, rS100A6) and control protein GST were prepared and identified using prokaryotic expression. The proliferation capacity of the CRC cell line HCT116 was assessed by trypan blue counting, CCK8 assay, and crystal violet staining. IL-6 mRNA levels in macrophages were measured by quantitative real-time polymerase chain reaction, while IL-6 protein levels were determined by Western blot. The protein levels and phosphorylation status of JAK2 and STAT3 in HCT116 cells were also evaluated by Western blot.

Results: (1) rS100A6 and GST proteins were successfully prepared. (2) Coculture with rS100A6-treated macrophages (A6-M) enhanced the proliferative capacity of HCT116 cells ($P<0.05$). Concurrently, total JAK2 and STAT3 levels in HCT116 cells remained unchanged, but their phosphorylation levels increased significantly ($P<0.05$). (3) In A6-M , both IL-6 mRNA and protein levels were elevated ($P<0.05$). (4) Addition of an IL-6 receptor-blocking peptide to the co-culture system partially reversed the pro-proliferative effects of A6-M on HCT116 cells ($P<0.05$).

Conclusion: S100A6 in the tumor microenvironment promotes CRC cell proliferation by upregulating IL-6 expression in macrophages and subsequently activating the IL-6/JAK2/STAT3 signaling pathway in HCT116 cells.

Keywords: S100A6; macrophages; colorectal cancer; IL-6/JAK2/STAT3 signaling pathway

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors of the digestive tract, ranking third in incidence and fourth in mortality worldwide [1].

In China, its incidence continues to rise annually with a trend toward younger patients, and the five-year mortality rate for advanced-stage CRC remains high. While combined targeted therapy and chemoradiotherapy represent the current standard of care, outcomes remain unsatisfactory with poor prognosis. Therefore, investigating the pathogenesis of CRC and identifying novel therapeutic targets is of great significance.

The tumor microenvironment (TME) comprises tumor cells, cancer stem cells, stromal cells, immune cells, and other components. These cells influence each other, particularly between tumor cells and immune cells, collectively regulating tumor progression. Macrophages (M) are the most abundant immune cells infiltrating the TME, accounting for 30%-50% of tumor stromal cells. They promote tumor development through multiple mechanisms, including immune evasion, cell activation and proliferation, and tumor migration and invasion [2]. In CRC, macrophage infiltration is significantly increased and contributes to tumor growth and invasion [3,4], correlating closely with treatment failure and poor prognosis. However, the interactions between macrophages and cancer cells in the CRC microenvironment and their molecular mechanisms remain incompletely understood.

Calcyclin S100A6, a member of the S100 protein family, regulates cell proliferation, apoptosis, cytoskeletal remodeling, and stress responses [5]. S100A6 is highly expressed in CRC tissues and cell lines, where it promotes CRC cell proliferation, migration, and invasion [6] and correlates with poor patient prognosis. S100A6 is also a secreted protein that functions through paracrine/autocrine mechanisms by binding to the receptor for advanced glycation end products (RAGE) and Toll-like receptors on target cell surfaces [7]. Macrophages express RAGE and possess corresponding signal transduction machinery [8,9,10]. Based on this, we hypothesized that S100A6 in the microenvironment may participate in macrophage regulation. Previous studies have shown that S100A12 and S100B activate macrophages through the RAGE signaling pathway [11], and activated macrophages can secrete cytokines such as IL-10 and IL-6 [8]. Therefore, this study aimed to investigate whether S100A6 influences CRC development by affecting IL-6 expression in macrophages.

1.1.1 Cell Lines

The human colorectal cancer cell line HCT116 was kindly provided by the Chongqing Key Laboratory of Molecular Oncology and Epigenetics at the First Affiliated Hospital of Chongqing Medical University. The human monocytic macrophage cell line THP-1 was maintained in our laboratory (Key Laboratory of Laboratory Medical Diagnostics of Ministry of Education, Chongqing Medical University).

1.1.2 Plasmids and Bacteria

Recombinant plasmids pGST-moluc-hS100A6 and pGST-moluc were gifts from the Molecular Oncology Laboratory at the University of Chicago Medical Center. Competent *E. coli* BL21 was maintained in our laboratory.

1.1.3 Transwell Chambers

Transwell chambers were purchased from Corning (diameter: 24 mm, pore size: 0.4 µm).

1.1.4 Reagents

DMEM/high glucose medium and RPMI 1640 medium were obtained from Hyclone. Fetal bovine serum was purchased from Gibco. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma-Aldrich (USA). Human IL-6 receptor-blocking peptide was purchased from Santa Cruz Biotechnology. TRIzol reagent was from Invitrogen (USA). Reverse transcription kits and SYBR Green II (Tli RNaseH Plus) real-time PCR kits were from TaKaRa (Japan). PCR primers for IL-6 and the internal reference GAPDH were synthesized by GenScript (Nanjing). Protein extraction and Western blot reagents were from Beyotime Institute of Biotechnology (Shanghai). Rabbit anti-human S100A6 monoclonal antibody and mouse anti-human GST monoclonal antibody were from Abcam (USA). Mouse anti-human IL-6 polyclonal antibody was from Bioss. Rabbit anti-human total JAK2 (t-JAK2) and phosphorylated JAK2 (p-JAK2) monoclonal antibodies were from Diagbio. Rabbit anti-human total STAT3 (t-STAT3) and phosphorylated STAT3 (p-STAT3) monoclonal antibodies were from Cell Signaling Technology (CST). Mouse anti-human -actin monoclonal antibody, goat anti-rabbit IgG, and goat anti-mouse IgG were from Zhongshan Golden Bridge Biotechnology (Beijing, China). ECL reagent kits were from Pierce (USA). All other reagents were analytical grade from domestic sources.

1.2.1 Cell Culture

Both HCT116 and THP-1 cells were cultured at 37°C in a humidified incubator with 5% CO . HCT116 cells, which grow adherently, were maintained in DMEM high-glucose complete medium (containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin) and passaged every 2-3 days using 0.25% trypsin. THP-1 cells, which grow in suspension with aggregation, were cultured in RPMI 1640 complete medium (containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin) and passaged every 2-3 days by direct dilution.

1.2.2 Preparation and Identification of Recombinant Proteins rS100A6 and GST

The preparation method followed that described in reference [12]. Unless otherwise specified, the final concentration of both recombinant proteins was 100 µg/mL in subsequent experiments.

1.2.3 Assessment of S100A6-Treated Macrophages on HCT116 Cell Viability and Proliferation

1) Preparation of S100A6-treated macrophages (A6-M): THP-1 cells in logarithmic growth phase were seeded into Transwell chambers (2×10 cells/well) and induced with 100 ng/mL PMA for 24 h. After induction, cells were washed and fresh serum-free medium was added, followed by treatment with rS100A6 or GST. After 24 h, the medium was replaced with fresh serum-free medium, yielding A6-M .

2) Co-culture and assessment of cell activity and proliferation: HCT116 cells in logarithmic growth phase were seeded into six-well plates (2×10 cells/well) and co-cultured with A6-M in Transwell chambers for 24 h. Control HCT116 cells were treated directly with rS100A6 or GST for 24 h. Cell activity and proliferation were assessed by trypan blue counting, CCK8 assay, and crystal violet staining.

3) Rescue experiments: IL-6 receptor-blocking peptide (1 µg/mL) was added to the co-culture system of HCT116 cells and A6-M , and cells were cultured for 24 h. HCT116 cell viability and proliferation were then assessed using the three methods described above.

1.2.4 SDS-PAGE and Western Blot Detection of Proteins and Phosphorylated Proteins

1) Protein sample preparation: Total protein from A6-M and HCT116 cells was extracted using lysis buffer, and concentrations were determined by BCA assay. Prepared rS100A6 and GST proteins were also collected. All samples were boiled for 10 min before use.

2) SDS-PAGE: Protein samples were separated by SDS-PAGE. Gels were either stained with Coomassie brilliant blue for molecular weight verification or processed for Western blot analysis.

3) Western blot and image analysis: After electrophoresis, proteins were transferred to PVDF membranes using wet transfer. Membranes were blocked with 5% BSA at 37°C for 2 h, then incubated overnight at 4°C with primary antibodies: rabbit anti-human S100A6 monoclonal antibody, mouse anti-human GST monoclonal antibody, mouse anti-human IL-6 polyclonal antibody, rabbit anti-human p-JAK2 monoclonal antibody, rabbit anti-human t-JAK2 monoclonal antibody, rabbit anti-human p-STAT3 monoclonal antibody, rabbit anti-human t-STAT3 monoclonal antibody, and mouse anti-human -actin mono-

clonal antibody (diluted 1:1000, except -actin at 1:500). After washing with 1% TBST, membranes were incubated with corresponding secondary antibodies (diluted 1:5000) at 37°C for 1 h. Following additional washes, bands were visualized using ECL reagent and captured with a gel imaging system. Band intensities were analyzed using Image Lab 5.1 software.

1.2.5 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

PMA-induced macrophages were seeded in six-well plates at 2×10 cells/well and treated with rS100A6 (30, 60, or 100 µg/mL) or GST (100 µg/mL) for 24 h. Cells were then collected, total RNA was extracted, and 1 µg was reverse-transcribed into cDNA. After 4-fold dilution, 1 µL of cDNA was used as template for qPCR with GAPDH as the internal reference gene (primer sequences: forward 5' -CAGCGACACCCACTCCTC-3' , reverse 5' -TGAGGTCCACCACCCT-3') to detect IL-6 expression levels (primer sequences: forward 5' -GGCCCTTGCTTTCTCTTCG-3' , reverse 5' -ATAATAAGTTTTGATTATGT-3').

The qPCR reaction mixture (10 µL) contained: SYBR enzyme 5 µL, mixed forward and reverse primers 1 µL, ddH O 3 µL, and cDNA template 1 µL. Cycling conditions were: 94°C for 3 min; 40 cycles of 94°C for 40 s, 56°C for 30 s, and 72°C for 40 s; final extension at 65°C for 5 s. Data were analyzed using Bio-Rad software, and relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method.

1.3 Statistical Analysis

All experimental data were analyzed using SPSS 17.0 and GraphPad Prism 5 software. All experiments were independently repeated three times, and results are presented as mean ± standard deviation ($\bar{x} \pm s$). Comparisons among multiple groups were performed using one-way ANOVA, followed by LSD-t test for pairwise comparisons. $P<0.05$ was considered statistically significant.

Results

2.1 Preparation and Identification of Recombinant Proteins rS100A6 and GST

SDS-PAGE analysis [Figure 1: see original paper] revealed that the prepared rS100A6 and GST proteins had molecular weights of approximately 36 kDa and 26 kDa, respectively, consistent with expected sizes. Western blot [Figure 1: see original paper] confirmed that rS100A6 was recognized by rabbit anti-human S100A6 monoclonal antibody and GST by mouse anti-human GST monoclonal antibody, indicating successful protein preparation for subsequent experiments.

2.2 Differentiation of Human Monocytic THP-1 Cells into Macrophages

After 24 h of PMA induction, the human monocytic cell line THP-1 transitioned from suspension to adherent growth and developed pseudopodia, characteristic of macrophage differentiation [Figure 2: see original paper].

2.3 S100A6 Enhances HCT116 Proliferation Through Macrophages

Co-culture of HCT116 cells with rS100A6-treated macrophages (A6-M) or GST-treated macrophages (GST-M) demonstrated that the A6-M group exhibited significantly higher numbers of viable HCT116 cells, increased OD values, and enhanced cell viability as measured by trypan blue counting [Figure 3: see original paper], CCK8 assay [Figure 3: see original paper], and crystal violet staining [Figure 3: see original paper] ($P<0.05$). These results collectively indicate that S100A6 can promote HCT116 cell proliferation not only through direct effects but also indirectly via macrophages.

2.4 S100A6 Upregulates IL-6 Expression in Macrophages

Based on literature reports, we hypothesized that IL-6 might be a key mediator through which A6-M promote HCT116 proliferation. qPCR and Western blot analyses revealed that IL-6 mRNA levels increased in a dose-dependent manner in A6-M [Figure 4: see original paper] ($P<0.01$), and IL-6 protein levels were also elevated [Figure 4: see original paper]. These findings demonstrate that S100A6 upregulates IL-6 expression in macrophages.

2.5 S100A6 Promotes HCT116 Proliferation Through Activation of the IL-6/JAK2/STAT3 Signaling Pathway

To determine whether IL-6 signaling is involved in A6-M -mediated promotion of HCT116 proliferation, we added an IL-6 receptor-blocking peptide to the co-culture system. Trypan blue counting [Figure 3: see original paper], CCK8 assay [Figure 3: see original paper], and crystal violet staining [Figure 3: see original paper] all showed that IL-6R blockade significantly reduced HCT116 viable cell numbers, OD values, and cell viability compared to controls ($P<0.05$), indicating that IL-6 signaling activation is an important mechanism for S100A6-mediated, macrophage-dependent promotion of HCT116 proliferation.

Western blot analysis [Figure 5: see original paper] further demonstrated that co-culture with A6-M increased p-JAK2 and p-STAT3 protein levels in HCT116 cells ($P<0.05$) without affecting total t-JAK2 and t-STAT3 levels. These results confirm that activation of the IL-6/JAK2/STAT3 signaling pathway is involved in S100A6-mediated promotion of HCT116 proliferation through macrophages.

Discussion

CRC is among the most common malignant tumors, ranking high globally and in China for both incidence and mortality [14]. CRC development is a complex, multi-factorial, multi-stage pathological process. Although novel targeted therapies have been widely applied clinically, low five-year survival rates and high recurrence rates remain major challenges.

The presence of S100A6 in the CRC microenvironment suggests its involvement in CRC progression through TME modulation, with various stromal cells potentially serving as S100A6 targets. Macrophages are a major and important component of tumor stromal cells. Literature reports indicate that myeloid cells, including macrophages, promote pro-tumorigenic microenvironment formation through RAGE and TLR4 signaling pathways [8]. The S100A7/RAGE axis promotes macrophage chemotaxis to breast cancer tissues [9] and regulates macrophage differentiation toward M2 tumor-associated macrophages while upregulating MMP9 expression, thereby facilitating breast cancer growth and metastasis [10]. However, no studies have reported on S100A6 effects on CRC progression via macrophages.

Our study demonstrates that S100A6 upregulates IL-6 expression in macrophages. IL-6 is a crucial pro-tumorigenic factor that plays key roles in various cancers, including melanoma, breast cancer, and CRC [15]. In glioma, S100B recruits and modulates macrophages while upregulating IL-6 expression [16]. IL-6 also promotes macrophage differentiation toward M2 tumor-associated macrophages, thereby facilitating CRC growth [17]. Elevated IL-6 levels are detectable in both serum and tumor tissues of CRC patients, and its expression correlates with tumor stage, size, and poor prognosis [18]. IL-6 binds to soluble IL-6 receptor (sIL-6R) and activates downstream JAKs and transcription factor STAT3 through gp130, promoting tumor cell proliferation and inhibiting apoptosis [18]. Phosphorylated STAT3 promotes transcription of downstream target genes involved in cell survival (Bcl-2, Mcl-1) and proliferation (c-Myc, Cyclin D1). We confirmed elevated IL-6 expression in A6-M [Figure 4: see original paper].

To verify IL-6 involvement in A6-M -mediated promotion of HCT116 proliferation, we added IL-6R-blocking peptide to the co-culture system and observed partial reversal of the pro-proliferative effects [Figure 3: see original paper], confirming our hypothesis that IL-6 and its downstream signaling pathway activation represent one mechanism by which A6-M enhance HCT116 viability and proliferation. Increased IL-6 secretion from A6-M leads to enhanced phosphorylation of JAK2 and STAT3 in HCT116 cells [Figure 5: see original paper], thereby boosting their viability and proliferative capacity. Thus, S100A6 promotes CRC development through both direct effects on CRC cells and indirect effects mediated by macrophages.

In summary, S100A6 in the tumor microenvironment promotes proliferation of CRC HCT116 cells by stimulating IL-6 secretion from macrophages and sub-

sequently activating the IL-6/JAK2/STAT3 signaling pathway. These findings provide new experimental evidence for elucidating S100A6 mechanisms in CRC development and offer novel insights for improving CRC diagnosis and treatment.

References

[1] Weitz J, Koch M, Debus J, et al. Colorectal cancer. *Lancet*, 2005, 365(9454):153-165.

[2] Komohara Y, Fujiwara Y, Ohnishi K, et al. Tumor-associated macrophages: Potential therapeutic targets for anti-cancer therapy. *Advanced Drug Delivery Reviews*, 2016, 99(Pt B):180-185.

[3] Erreni M, Mantovani A, Allavena P. Tumor-associated Macrophages (TAM) and Inflammation in Colorectal Cancer. *Cancer Microenvironment*, 2011, 4(2):141-154.

[4] Nakayama Y, Nagashima N, Minagawa N, et al. Relationships between tumor-associated macrophages and clinicopathological factors in patients with colorectal cancer. *Anticancer Research*, 2002, 22(6C):4291-4296.

[5] Donato R, Sorci G, Giambanco I. S100A6 protein: functional roles. *Cellular & Molecular Life Sciences*, 2017, 74(4):2749-2760.

[6] Duan L, Wu R, Zou Z, et al. S100A6 stimulates proliferation and migration of colorectal carcinoma cells through activation of the MAPK pathways. *International Journal of Oncology*, 2014, 44(3):781-90.

[7] Donato R, Cannon B R, Sorci G, et al. Functions of S100 Proteins. *Current Molecular Medicine*, 2013, 13(1):24-57.

[8] Byun K, Yoo Y, Son M, et al. Advanced glycation end-products produced systemically and by macrophages: A common contributor to inflammation and degenerative diseases. *Pharmacology & Therapeutics*, 2017,177:44-55.

[9] Nasser M W, Qamri Z, Deol Y S, et al. S100A7 enhances mammary tumorigenesis through upregulation of inflammatory pathways. *Cancer Research*, 2012, 72(3):604-615.

[10] Nasser M W, Wani N, Ahirwar D K, et al. RAGE mediates S100A7-induced breast cancer growth and metastasis by modulating the tumor microenvironment. *Cancer Research*, 2015, 75(6):974-985.

[11] Chavakis T, Bierhaus A, Nawroth P P. RAGE (receptor for advanced glycation end products): a central player in the inflammatory response. *Microbes Infection*, 2004, 6(13):1219-1225.

[12] Duan L, Wu R, Zou Z, et al. S100A6 stimulates proliferation and migration of colorectal carcinoma cells through activation of the MAPK pathways. *International Journal of Oncology*, 2014, 44(3):781-90.

[13] Zha H, Sun H, Li X, et al. S100A8 facilitates the migration of colorectal cancer cells through regulating macrophages in the inflammatory microenvironment. *Oncology Reports*, 2016, 36(1):279-290.

[14] Forman D, Ferlay J, Jemal A, et al. Global cancer statistics. *Ca A Cancer Journal for Clinicians*, 2015, 65(2):87-108.

[15] Kampan N C, Xiang S D, Mcnally O M, et al. Immunotherapeutic Interleukin-6 or Interleukin-6 receptor blockade in cancer: challenges and opportunities. *Current Medicinal Chemistry*, 2017, 24(00):1-22.

[16] Wang H, Zhang L, Zhang I Y, et al. S100B promotes glioma growth through chemoattraction of myeloid-derived macrophages. *Clinical Cancer Research An Official Journal of the American Association for Cancer Research*, 2013, 19(14):3764-3775.

[17] CM Wunderlich, PJ Ackermann, AL Ostermann, et al. Obesity exacerbates colitis-associated cancer via IL-6-regulated macrophage polarisation and CCL-20/CCR-6-mediated lymphocyte recruitment. *Nature Communication*, 2018, 9(1):1646.

[18] Waldner M J, Sebastian F, Neurath M F. Interleukin-6 - A Key Regulator of Colorectal Cancer Development. *International Journal of Biological Sciences*, 2012, 8(9):1248-1253.

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