Research Paper

Osteoglycin-induced VEGF Inhibition Enhances T Lymphocytes Infiltrating in Colorectal Cancer

Xiang Hu, Ya-Qi Li, Qing-Guo Li, Yan-Lei Ma, Jun-Jie Peng, San-Jun Cai *

Department of Colorectal Surgery, Fudan University Shanghai Cancer Center, 270 Dong'an Road, Shanghai 20032, China
Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China

ABSTRACT

Background: OGN could modify tissue inflammation and immune response via local and circulating innate immune cells, which was suggestive of a reciprocal relationship between OGN and T cell infiltration in cancer. Hence, we aim to measure the OGN expression patterns and immune cells response in colorectal cancer (CRC).
Methods: This study enrolled three independent sets of patients from TCGA and the Fudan University Shanghai Cancer Center(FUSCC). The effect of OGN on T cell infiltration and the mechanism were examined in vitro and in vivo.
Findings: Tumor OGN expression levels were positively associated with CD3, CD8, and PTPRC expressions in the training and testing sets from TCGA, respectively. In validation set from FUSCC, OGN expression level also paralleled positively with CD8+ cell density in colorectal cancer tissue ($p < .001$). For a unit decrease in outcome quartile categories, multivariable OR in the lowest (vs highest) OGN expression was 0.17 (95% CI 0.08–0.33). Consistently, immunofluorescence validated that OGN was preferentially expressed with CD8+ cells in both normal epithelium and cancer tissue. Xenograft tumors arising from MC38 cells with OGN-over-expression displayed a significant increase in CD8+ cells recruitment. Hence, high expression of OGN was associated with a profound longer survival ($P = .009$). In mechanism, elevated OGN expression inhibited the activation of the transcriptional genes HIF-1α in CRC cells, then significantly impeded the expression of VEGF. As a result of this, T cell tumor infiltration was reduced.
Interpretation: OGN expression is positively associated with CD8+ cell density in colorectal cancer tissue, suggesting a possible influence of OGN expression on tumor reactive T cells in the tumor niche.
Fund: No

1. Introduction

Colorectal cancers(CRC) are a heterogeneous group of neoplasms and are frequently affected by tumor-host interactions [1, 2]. T-cell-mediated adaptive immunity are involved in tumor initiation and progression, emerging as a novel strategy to treat various cancers [3]. High density of CD3+, CD8+, PTPRC+ or FOXP3+ T cells were supposed to be recruited, when strong immune response was manifested in colorectal cancer with better clinical outcome [4, 5]. Although some certain tumor molecular status, just as high-level microsatellite instability, has been identified to associate with enhanced infiltration of T cells [6], while the determinant of immune response or immune cells recruits in CRC is under unraveled. Osteoglycin (OGN) has been observed down-expression in a variety of

malignances including gastric cancer [7], squamous cervical, vaginal cancer [9], colorectal adenoma [8], invasive ductal breast carcinoma [10], and laryngeal carcinoma [11]. However, it is still undetermined according to the significance of OGN in CRC. OGN has been found in local and circulating innate immune cells [12], as OGN clearly co-stained with markers of both neutrophil and monocyte/macrophage in the myocardium. What is more, great distinguished phenotypic characteristics were indicated by significantly altered phosphorylation of c-jun in the innate immune cells with OGN high expression. In addition, OGN over-expressed cells were able to result in altered cell death and autophagy by leading to mTOR pathway activation [13]. Given the crucial role of tumor autophagy activity in modifying T cells and FOXP3+ Treg cells, there was suggestive of a reciprocal relationship between OGN and T cell infiltration. Based on these previous findings, we will measure oncologic outcomes for CRC based upon OGN expression patterns, further test the effect of OGN on immune cells.

* Corresponding author at: Department of Colorectal Surgery, Fudan University Shanghai Cancer Center, 270 Dong'an Road, Shanghai 20032, China.
E-mail address: caisanjun_sh@163.com (S.-J. Cai).

Research in Context

Evidence Before This Study

High density of CD3 + , CD8 + , PTPRC + or FOXP3 T cells were supposed to be recruited, when strong immune response was manifested in colorectal cancer with better clinical outcome. But the determinant of immune response or immune cells recruits in CRC is under unraveled and it is a crucial role in understanding their dynamics as to tumor invasion, immune-surveillance and evasion. A wide range of findings were suggestive of a reciprocal relationship between OGN and T cell infiltration.Added Value of this study

In TCGA and FUSCC cohorts, tumor OGN expression had a positive association with CD8 + cell density in colorectal cancer, suggesting a possible mechanism of a profound longer survival in the CRC with OGN expression. In mechanism, elevated OGN expression inhibited the activation of the transcriptional genes HIF-1α in CRC cells, then significantly impeded the expression of VEGF. As a result of this, T cell tumor infiltration was reduced.Implications of all available evidence

Our consolidated data draws a possible effect of OGN level on CD8 T cells in colorectal cancer microenvironment, and can promote further translational research on the associations of OGN with host immunity in colorectal cancer.

---

2. Patients and Methods

2.1. Antibodies and Reagents

We utilized these antibodies for research: human Osteoglycin (OGN) antibody for Western blotting(WB)from R&D Systems, Vascular endothelial growth factor A (VEGF-A) antibody, hypoxia inducible factor-1α (HIF-1α) from Abcam; human and mouse Osteoglycin (OGN) for immunohistochemistry (IHC) from Sigma-Aldrich and Santa Cruz Biotechnology; Akt, phospho-Akt (Ser473), epidermal growth factor receptor (EGFR), phospho-epidermal growth factor receptor (EGFR, Y1068), Erk1/2 antibody, CD3, CD8, PTPRC, and FOXP3 from Cell Signaling Technology; beta-actin antibody from proteintech; AKT activator: sc79 from Selleck.

2.2. Cell Culture

We originally purchased SW620, HT29 cell lines of human colon cancer and MC38 murine colon cancer cell line, which will be utilized in following experiments, from the American Type Culture Collection (Manassas, VA), and the cells were cultivated in DMEM medium according to the Defense Technical Information Center recommendation (DTIC) in addition of 10% fetal bovine serum (FBS, Gibico, Life Technology, Austria), 1% penicillin/streptomycin (PS) in a humidified 5% (v/v) atmosphere of CO2 at 37 °C.

2.3. Study Population

2.3.1. Patients

This study enrolled three independent sets of patients with colorectal cancer from the cancer genome atlas (TCGA) and the Fudan University Shanghai Cancer Center (FUSCC). The training set and testing set including 170 consecutive patients and 434 consecutive colorectal cancer patients with radical surgery were obtained from TCGA database

available from Cancer Genomics Browser of University of California Santa Cruz (https://genome-cancer.ucsc.edu/). The validation set comprised 276 consecutive colorectal cancer patients after surgery at the Department of colorectal Surgery, FUSCC, Shanghai, China. All patient data were prospectively entered in the FUSCC database since 2006, such as: age at the diagnosis, race, tumor localization, diagnostic year, tumor diameter, histological grade, number of lymph nodes retrieved, post-operative multimodal treatment (adjuvant chemotherapy or radiation), details of surgical procedures, complications rate, postoperative histopathology, and follow-up information (date of last visit, tumor relapse, tumor-related or unrelated death, overall survival, OS and disease-free survival, DFS). The research protocol was reviewed and approved by the institutional review board of the FUSCC. Informed consent was obtained from all patients.

2.3.2. Tissue Microarray(TMA) Construction and Immunohistochemistry (IHC) Staining

In all, 276 unselected, non-consecutive, primary colorectal cancers were enrolled form January 2007 to November 2009 in FUSCC to construct the tissue microarray(TMA). Li et al. has previously described the construction in detail for TMA [14]. There were two independent pathologists scoring the TMA section utilizing a semiquantitative scoring system. This scoring system included staining intensity score defined as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong), and extent score defined as 0 (<5%), 1(range from 5% to 25%), 2 (range from 26% to 50%), 3 (range from 51 to 75%), 4 (>75%) according to the ratio of the positive staining areas in the whole carcinoma area. The immunoreactivity score (IRS) was generated as the results of the percentage score multiplied by the staining intensity score. OGN expression with High, Intermediate and Low pllevel was defined as detectable immunoreactions in cytoplasm and stoma with IS>6, 6 ≥ IS>2 and IS ≤2, respectively. And the densities of CD3+, CD8+, PTPRC+, and FOXP3+ cells in tumor tissue were measured as the average density per microarray cores.

2.3.3. Plasmids Construction and Viral Transduction

For protein over-expression, gene-specific overexpression vectors (pCDH-CMV-MCSEF1-Puro or CMV-MCS-3FLAG-SV40) were used. The lentiviral expression vector with OGN over-expression or the control vector were used to transfect the human or murine colon cancer cell lines (SW620, HT29, MC38).

2.3.4. Immunofluorescence

Cells reaching 80% confluent on a chamber slide or xenografts were fixed with paraformaldehyde (PFA), and we permeate the cells at room temperature for 15 min with 0.5% Triton X-100. Thereafter, sufficient and diluted primary antibodies for OGN, CD3+, CD8+, PTPRC+, and FOXP3+ were added to be incubated at 4 °C overnight. At last, the secondary antibody of the alexa-flours 488, 594 with anti-rabbit or mouse (1:200, Invitrogen) was added for an hour at room temperature. In addition, DAPI was utilized to stain nuclei when necessary. Fluorescence images were photographed with a fluorescence microscope.

2.3.5. Western Blot

Whole-cell lysates were collected in RIPA buffer for total protein extraction. Protein were separated by SDS-PAGE (10% gel) and then transferred to PVDF membranes. The primary antibodies against specific protein were incubated with PVDF membranes for overnight. HRP conjugated secondary antibodies were used at a 1:5000 dilution for 1-h incubation at room temperature. At last, specific proteins were detected using ECL (Pierce, Thermo Scientific) in a Bio-Imaging System.

2.3.6. Xenotransplant Murine Models

MC38 cells ($5 * 10^6$ cells/mouse) which transfected with OGN overexpression or empty vector control were injected subcutaneously into the right flank of C57 mice ($n = 5$, male; 4-week C57 mouse), and