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Osteopontin Gene Expression is Inversely Regulate 5-Fluorouracil Drug Resistance in Colon Cancer

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Abstract: *Background:* Resistance to chemotherapeutic drugs complicates the treatment of cancer patients. The extracellular matrix protein *osteopontin* (*OPN*) plays multiple roles in the proliferation and metastasis of cancer cells. We therefore attempted to determine whether *OPN* expression correlated with drug resistance. *Methods: OPN* expression in the HCT 116 colon cancer cell line was inhibited by an *OPN*-short interfering RNA (siRNA). We determined the cytotoxic effect (IC_{50}) of 5-fluorouracil (5FU) on these cells. Patients with recurrence or colorectal cancer or harbored residual tumor cells were treated with S-1-based chemotherapy. The levels of *OPN* mRNA expression in the tumors were determined and compared with the patients' responses to chemotherapy. *Results:* The IC_{50} of 5FU for HCT 116 cells transfected with the *OPN*-siRNA was 32.9 μM. In contrast, the IC_{50} values for cells transfected with a negative control siRNA, mock-transfected cells, or untreated cells were 6.58 μM, 7.08 μM, and 6.76 μM, respectively (P<0.001). The level of *OPN* mRNA expression in the S-1 non-responder group was significantly lower than that of the responder group (P = 0.0387). Survival analysis revealed no significant difference between the responder and non-responder groups (P = 0.8737). *Conclusions:* The level of *OPN* expression plays a role in the resistance of colorectal tumor cells to 5FU.

Keywords: Osteopontin, Gene Expression, Colon Cancer, 5FU, Drug Resistance

1. Introduction

Colorectal cancer is the third and fourth leading cause of cancer-related deaths in Japan and worldwide[1, 2], respectively. Strategies for treating patients with colorectal cancer have changed markedly during this decade. Newly developed chemotherapeutic agents and combination treatment regimens have increased the overall survival of patients with unresectable colorectal cancer to more than 20 months[3]. For example, FOLFOX, FOLFIRI, CapeOX and SOX are used as first line chemotherapy for these patients[3-5]. Biotherapeutic monoclonal antibodies, including bevacizumab, cetuximab, and panitumumab are used in conjunction with these key regimens[6-8]. The metabolic antagonist 5FU, in use as an anticancer drug for over 50 years[9], is common to all of these regimens and is widely used to treat a variety of cancers.

Diverse cell types express the extracellular matrix protein *osteopontin* (*OPN*), including osteoblasts, osteoclasts, macrophages, activated T cells, and renal tubular cells[10, 11].

OPN is widely expressed by tumor cells, such as those derived from the lung, breast, gastrointestinal tract, and ovary[12-15]. *OPN* plays many important roles in apoptosis, bone regeneration, immune responses, and inflammation[16-18]. The binding of *OPN* to *integrins* and *CD44* indicates that it participates in signaling pathways that promote the proliferation and metastasis of cancer cells[19, 20]. Here we show, for the first time to our knowledge, that the level of transcription of *OPN* in the colon cancer is strongly associated with resistance to 5FU.

2. Methods

2.1. Patients and Tissue Samples

Twenty-nine patients who underwent surgery from 1996 to 2002 for colorectal tumor resection were including in the present study. If tumors recurred or residual tumor cells were detected, patients were enrolled in a clinical trial and treated with either S-1, 5FU-based oral therapy to improve efficacy and reduce side-effects, or S-1 plus cisplatin (CDDP). The

dose of S-1 was determined based on the patient's body surface area (BSA) as follows: <1.25 m^2, 80 mg/day; ≥1.25 and <1.5 m^2, 100 mg/day; and >1.5 m^2, 120 mg/day. The treatment regimens were as follows: S-1 alone was administered orally two times daily for 28 consecutive days and then discontinued for 2 weeks. S-1 was administered orally two times daily, discontinued for 2 weeks, and CDDP (30 mg/m^2) was injected intravenously on days 1 and 8.

The formalin-fixed paraffin-embedded (FFPE) specimens taken from colorectal tumors were obtained from the Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Japan. Informed consent from all patients was obtained before administering chemotherapy or collecting tissues. FFPE tissues were prepared using the standard method of the Department of Pathology, Tokyo Women's Medical University, Japan. The paraffin blocks were cut into 10 μm-thick sections. The patients' characteristics are summarized in Table 1. Patients who responded completely (CR) or partially (PR) were assigned to the responder group (more than 30% tumor size shrinkage following chemotherapy), and patients with stable (SD) or progressive (PD) disease were assigned to the non-responder group.

2.2. Cell Culture and Transfection with Small Interfering RNA (siRNA)

The HCT 116 colorectal carcinoma cell line was obtained from the American Type Culture Collection (ATCC #CCL-247™, VA, USA) and maintained in McCoy's 5A medium supplemented with 10% heat-activated fetal bovine serum (FBS) (Life Technologies, NY, USA). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were added to the wells of a 96-well plate and transfected with 5 nM (final concentration) Silencer® Select siRNA (Life Technologies, NY, USA) targeting *OPN* mRNA (*OPN*-siRNA cells) or with Silencer® Negative Control #1 siRNA (NC#1 cells) in Lipofectamine® RNAiMAX (Life Technologies, NY, USA) according to the manufacture's protocol. Other controls included cells treated with transfection reagent only (TA cells) and untreated cells (NTC cells).

2.3. Cytotoxicity Assay

The control and siRNA-transfected cells were incubated as described above for 24 h and washed twice with PBS. The cells were either untreated or exposed to 5FU (Sigma-Aldrich, MO, USA) in 10-fold increments of increasing amounts (0–1000 μM, final concentration) and incubated for 48 h. Cell counting was performed using WST-8 (Dojindo, Kumamoto, JAPAN) according to the manufacturer's protocol. In brief, WST-8 was added to each well after removing the medium containing 5FU, incubated for 2 h, after which the absorbance at 450 nm was measured using a microplate reader (Bio-Rad laboratories, CA, USA).

2.4. RNA Isolation, cDNA Synthesis, and Real-time PCR

Cells (1 × 10^5) in 6-well plates were transfected with the siRNAs described above according to the manufacture's protocol, incubated for 24 h, and washed twice with PBS. TRIzol® reagent (Life Technologies, NY, USA) was added to each well, and total RNA was isolated according to the manufacture's protocol. For FFPE specimens, an RNeasy® FFPE Kit (QIAGEN, CA, USA) was used according to the manufacturer's protocol. Synthesis of complementary DNA (cDNA) was performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, NY, USA) according to the manufacturer's protocol. The reverse transcription reaction was performed at 25°C for 10 min and then at 37°C for 120 min. Quantitative real-time PCR was carried out using a StepOne™ Real-Time PCR System (Life Technologies, NY, USA). TaqMan® Gene Expression Assays (Life Technologies, NY, USA) were used according to the manufacturer's protocol as follows: *OPN* (Assay ID: Hs00960942_m1) and *GAPDH* (Assay ID: Hs99999905_m1). PCR reactions were conducted as follows: 95°C for 3 min, 40 cycles at 95°C for 15 s, and 60°C for 35 s.

2.5. Data Analysis

The threshold cycle (C$_T$) value for each target was determined using StepOne™ software v.2.2 RQ Study Results (Applied Biosystems Inc.). The $2^{-\Delta\Delta Ct}$ method was used to compare the levels of gene expression. The levels of expression of each mRNAs were normalized by calculating the delta-C$_T$ (ΔC$_T$) values by subtracting the C$_T$ value of target mRNA from the C$_T$ value of the endogenous control *GAPDH* mRNA. The delta-ΔC$_T$ ($\Delta\Delta$C$_T$) was calculated, and this value represents the difference in the ΔCT value for each sample and the highest ΔCT value as a calibrator. The $2^{-\Delta\Delta Ct}$ value was used for relative quantitation. Statistical analyses were performed using MedCalc® for Windows, version 12.3.0.0 (MedCalc Software, Mariakerke, Belgium). ANOVA was used for assessing OPN-siRNA efficiency, and for calculating the differences of IC$_{50}$. The statistical significance of differences between the level of expression of *OPN* in the responder and non-responder groups was calculated using the Mann–Whitney test. The log-rank test for generating Kaplan–Meier survival curves was used to assess the association between the level of *OPN* expression and the survival rate. Statistical significance was defined as P ≤0.05.

3. Results

Cytotoxic effects of 5FU on HCT 116 cells

The knock down efficiency for *OPN* expression by *OPN*-siRNA was more than 99% (P<0.001) (Figure 1-A). The value of the IC$_{50}$ of 5FU for *OPN*-siRNA cells was 32.9 μM. The IC$_{50}$ values for cells transfected with a negative control siRNA, mock-transfected cells, and untreated cells were 6.58 μM, 7.08 μM, and 6.76 μM, respectively (P<0.001) (Figure 1-B).

The response of colorectal cancer patients to treatment with S-1 and analysis of their survival

The levels of *OPN* mRNA expression were significantly lower in the non-responder group compared with those of the

responder group (P = 0.0387) (Figure 2). There was no significant difference in the survival times between the responder and non-responder groups (P = 0.8737) (Figure 3).

Table 1. *Patient Characteristics.*

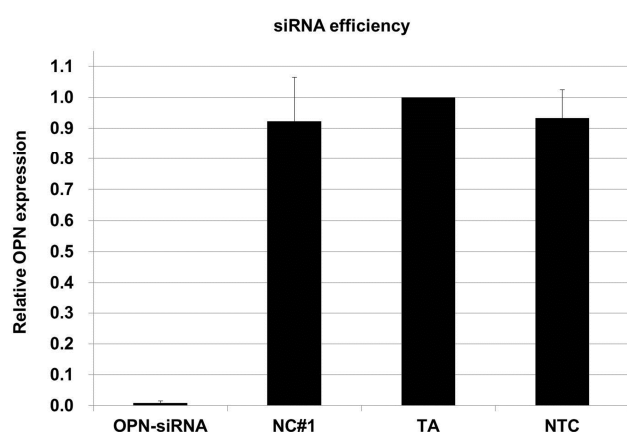| Characteristics	Distribution	Percentage (%)
Age		
mean (range)	58.8 (33–77)	
Gender		
male	16	55.2%
female	13	44.8%
Primary site		
cecum	2	6.9%
ascending	4	13.8%
transverse	1	3.4%
descending	2	6.9%
sigmoid	11	37.9%
rectum	9	31.0%
Histology		
well	18	62.1%
moderately	8	27.6%
mucinous	1	3.4%
well + mucinous	1	3.4%
unknown	1	3.4%
Treatment		
S-1 alone	18	62.1%
S-1 plus cisplatin	11	37.9%

Figure 1-A. *The knock down efficiency for OPN expression by OPN-siRNA was more than 99% (P<0.001).*
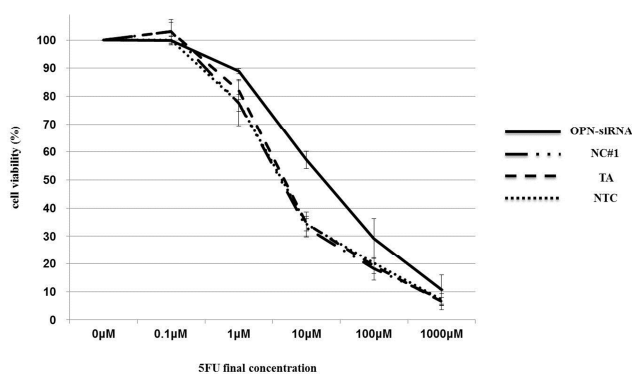

Figure 1-B. *The value of the IC_{50} of 5FU for OPN-siRNA cells was 32.9 μM. The IC_{50} values for cells transfected with a negative control siRNA, mock-transfected cells, and untreated cells were 6.58 μM, 7.08 μM, and 6.76 μM, respectively (P<0.001).*
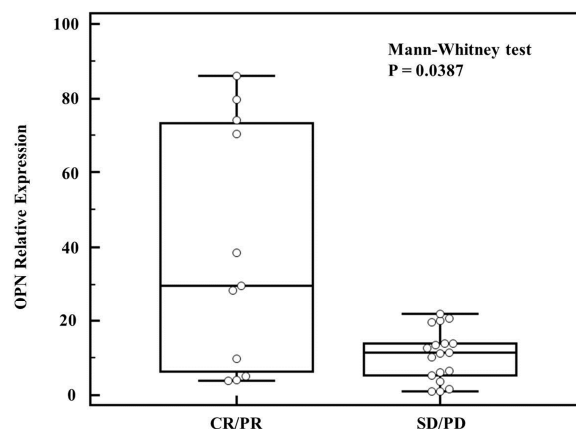

Figure 2. *The levels of OPN mRNA expression were significantly higher in the S-1 responder group compared with those of the non-responder group (P = 0.0387).*
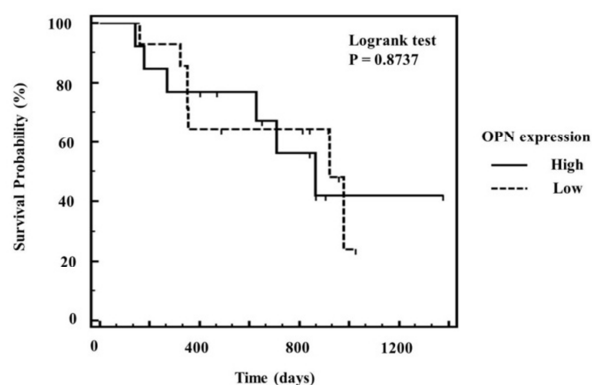

Figure 3. *There was no significant difference in the overall survival times between the S-1 responder and non-responder groups (P = 0.8737).*

4. Discussion

Numerous studies have failed to define the mechanism of resistance to 5FU, although they have indicated that several factors may be involved. Nevertheless, they have contributed to the improvement of cancer treatment by revealing that cancer cells can acquire resistance to chemotherapy by expressing drug transporters, such as the ABC transporter that removes cytotoxic drugs from cells[21]. Other mechanisms of drug resistance include changes in drug metabolism, resistance to or inhibition of drug-induced apoptosis, damage to the DNA repair machinery, and overexpression of the proteins that promote the survival of tumor cells[22, 23].

The details of the mechanisms by which cells metabolize 5FU have been revealed by research conducted over many years. When 5FU is incorporated by tumor cells, most of it is degraded by dihydropyrimidine dehydrogenase (DPD). Undegraded 5FU is phosphorylated, and the product, 5-fluoro-deoxyuridine monophosphate (FdUMP), prevents de novo DNA synthesis by forming complexes with thymidylate synthase (TS) with folate as a co-factor[24]. The expression by tumors of higher than normal levels of TS or DPD results in shorter survival of patients[25].

OPN forms complexes with integrins and *CD44*[19, 20],

which promote the proliferation, migration, and metastases of tumor cells[26-28]. Furthermore, its level of expression reflects the malignant potential of a tumor[29]. We show here that a human colorectal cell line acquires resistance to 5FU when *OPN* mRNA expression is reduced by an *OPN*-specific siRNA. Because the expression of O*PN* accelerates the proliferation of tumor cells[26], inhibiting its expression reduces the rates of DNA synthesis and cell proliferation. Further studies will be required to determine the pathways through which *OPN* influences the response of tumor cells to 5FU.

The sensitivity of HCT 116 cells to 5FU as a function of *OPN* mRNA expression is reflected by our analysis of *OPN* expression in patients that did or did not respond to therapy with 5FU. Interestingly, the levels of *OPN* expression in tumors derived from the non-responder group were significantly lower compared with those of the responder group. This result is consistent with the aforementioned results of the siRNA experiments. However, the survival times of the responder and non-responder groups were not significantly different. Currently, several drugs are used sequentially to treat colon cancer, and the survival data reported here may reflect treatment with a single drug. This result suggests possibilities for developing new therapeutic strategies development or new agents, and may help elucidate the mechanism of resistance to 5FU.

In conclusion, we demonstrate that a colon cancer cell line becomes more resistant to the cytotoxic effects of 5FU when *OPN* expression is inhibited. These findings are consistent with the low levels of *OPN* expression in patients with colon cancer that responded poorly to 5FU based chemotherapy.

Acknowledgement

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