Formyl peptide receptor 2 mediated chemotherapeutics drug resistance in colon cancer cells

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Abstract. – **OBJECTIVE:** To determine the expression of formyl peptide receptor 2 (FPRL2) and its drug resistance role in cancer colon cells, and its underlying mechanisms.

PATIENTS AND METHODS: The expression of FPRL2 and its legend (F2L) in colon cancer tissues or cancer cells was determined by immunohistochemistry assay and Real-time polymerase chain reaction (PCR), respectively. Chemosensitivity of 5-Fu and MMC in colon cancer cells were tested by cell counting kit-8 (CCK-8) method. Expression of p-ERK was determined by Western blot assay.

RESULTS: The expression of FPRL2 and its legend was significantly higher in resistant colon cancer tissues than those in non-resistant colon cancer tissues. The FPRL2 positive cells were two-thirds in tested cell lines. All of cells were F2L positive. The IC50 (inhibitory concentration 50) by 5-Fu and MMC was significantly higher in FPRL2 positive cells than those negative cells. The expression of p-AKT was markedly increased in FPRL2 positive cells. Pretreatment with AKT inhibitor enhanced the drug-sensitivity of these cells to 5-Fu and MMC.

CONCLUSIONS: The FPRL2 played a significant role in colon cancer drug resistance and this effect was through AKT pathway.

Key Words
Formyl peptide like receptor 2, Colon cancer, Drug resistance, ERK, 5-Fu, MMC.

Introduction

Colorectal cancer (CRC) is the most common cancer and the leading cause of cancer-related mortality worldwide[1]. Chemotherapy is routinely used for patients after surgical treatment with stage III and IV colon cancer. Although adjuvant surgical and chemotherapeutic treatment have yielded a good success rate, the failure of treatment in more than 90% of patients with metastatic cancer is due to drug resistance[2]. Blocking apoptosis was suggested to be responsible for drug resistance; hyper-activation of anti-apoptotic signaling pathway has frequently been observed in human cancers.

The formyl peptide receptors (FPRs) expressed by mammalian neutrophils are 7-transmembrane domain G-protein-coupled receptors (GPCRs) with important roles in innate immune defense reactions and regulation of inflammatory responses[3-5]. FPR has three isoforms: FPR1, FPR2, and the human-exclusive FPRL2[6,7]. The human FPRs were described to be in other primates as well as in rodents such as rabbits[6], rats, guinea pigs, and mice[7]. Compared to our knowledge on the FPRs, very little is known about the basic cell biology of these receptors. Recent evidence suggests that FPRL2 is a membrane mechanosensor that senses the mechanical fluid stress and signals intracellular cascades[8], including the protein kinase B (AKT/PKB), an essential regulator of apoptosis. In the present study, we described that FPRL2 is present in colon cancer cells, and then explored the relationship between the activation of FPRL2 and the resistance of colon cancer cells.

Patients and Methods

Patients' Tumor Samples

A total of 45 FFPE blocks with tumor samples were studied with II or III phase solitary colon carcinomas. Further exclusion criteria were: anamnestic or synchronous other malignant tumors, known familial adenomatous polyposis, colitis ulcerosa or Crohn's disease, neoadjuvant therapy, synchronous distant metastases, emergency operation, perioperative death and unknown tumor stage at the end of follow up. All carcinomas were classified according to the criteria of the World Health Organization and were recorded as invasive lymph node. Clinical data are summarized in Table I. All patients received at least one standard 5-Fu, mitomycin or combination of this two chemotherapy. There is no significant difference in the combination of 5-Fu, mitomycin or combined chemotherapy. Thus, the pathological specimens were divided into drug-resistant group and non-resistant group in this study. In 45 samples,

Table I. Clinical data of colon carcinoma patients.

Patient data	
Number of patients	n=45
Male	n=27 (60%)
Female	n=18 (40%)
Median age	60 y (range 32-79)
Median follow up	90 mo (range 2-180)
Tumor site	
Colon descendens	5 (11.1%)
Colon sigmoideum	40 (89%)
Tumor stage (UICC) or lymph node metastasis	
II or N0	22 (49%)
III or N+	23 (51%)
Depth of invasion	
pT2	6 (13%)
pT3	39 (87%)
Distant metastasis after 5 years follow up	n=8 (18%)

there are 34 cases of drug resistance and 11 cases without drug resistance. This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Signed written informed consents were obtained from all participants before the study.

Cell culture

HT-29, CaCo-2, LoVo, HCT-116 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Reagents

Fluorouracil was obtained from Haixin Pharmaceutical Company (Xi'an, China); mitomycin/C (MMC) was from Haizheng Pharmaceutical Company (Zhejiang, China); Dulbecco's Modified eagle medium (DMEM) and fetal bovine serum (FBS) were from Gibco Corporation (Rockville, MD, USA); RNeasy Miny Kit was from the Qiagen Company (Hilden, Germany). PrimeScript RTMaster Mix and SYBR Premix Ex TaqII were from TaKaRa (Otsu, Shiga, Japan). CCK-8 (Cell Count Kit 8) kit was from Dojindo Laboratories (Kumamoto, Japan). Rabbit anti-F2L/HBP (1-21) antibody was from Phoenix Biotech (San Antonio, TX, USA); rabbit anti-FPRL2 antibody, horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG were from the Santa Cruz Biotechnology (Santa Cruz, CA, USA); SuperPictureTM3rd Gen IHC Detection kit was from the Invitrogen (Carlsbad, CA, USA); Bicinchoninic acid (BCA) Protein Assay Kit was from Thermo Fisher Scientific (Waltham,

MA, USA); polyvinylidene difluoride membrane was from Millipore (Billerica, MA, USA); Anti-AKT, anti-phospho-AKT and anti-GAPDH were from Cell Signaling Technology (CST, Danvers, MA, USA); AKT blocker triciribine was from Peprotech (Rocky Hill, NJ, USA).

Immunohistochemical Analysis

After deparaffinization and rehydration, antigen retrieval was performed with the citrate buffer (pH 6.0) by heating the slides for 15 min. After blocking, sections were incubated overnight at 4ºC with the primary antibodies. Two-step technique was used for visualization, with diaminobenzidine (DAB) as a chromogen. Finally, sections were counterstained with hematoxylin and mounted. Imaging was acquired on an Olympus BX51 microscope using an Olympus DP70 digital camera (Tokyo, Japan); photographs of the tissue specimens were taken at ×200.

Evaluation of immunohistochemistry

The number of positively stained cells was counted in each of the 5 randomly selected consecutive fields under 400-fold magnification. With regard to staining diffuseness, the stained areas of sections were graded as follows: 0- no staining; 1- < 25% of the area stained; 2- 25-50% of the area stained; 3- 50-75% of the area stained; 4- > 75% of the area stained. With regards to staining intensity, the sections were graded as follows: 0- no staining; 1- weak but detectable staining above the control level; 2- distinct staining; 3- intense staining. Total IHC scores were obtained by adding diffuseness and staining intensity scores. In the patient samples, scores <1.5 in cancer tissue were considered negative staining, whereas scores >1.5 were considered positive staining.

Real-time PCR

Total RNA was extracted from colon CC cell lines using the RNeasy Miny Kit and Real-time quantitative PCR (qRT-PCR) analysis was carried out using a BioRad iCycler iQ Real-time PCR System (Bio-Rad, Hercules, CA, USA). Results were calculated relative to GAPDH expression and expressed as mean ± SD or SEM. The primers used were listed in Table II.

LC50 determination

Colon cancer cell line HCT116 (PRRL2 positive) SW620 cells (PRRL2 negative) were grown in 96-well plates (100 μL, 2×10^3/well) and treated with 5-Fu in the dose of 100, 50, 25, 12.5 and 6.25 mg/L, or MMC in the dose of 10, 5, 2.5, 12.5 and 0.25

Table II. Primers utilized in quantitative Real-time PCR assays.

Gene	Forward Primer	Reverse Primer
FPRL2	5'-ACTACTACGCCAAGGAGGTCAC-3'	5'-GAGCAACACGGGGTTCAGGT-3'
heme binding protein (HBP)	5'-AGACGGCTCCCTAAACACTAC-3	5'-GAATGCTCTATGTCACCCTCTC-3'
GAPDH	5'- TGCACCACCAACTGCTTAGC-3'	5'- GGCATGGACTGTGGTCATGAG-3'

mg/L for 72 h. Cell viability was determined using Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions. After treatment, the CCK-8 solution (10 μL) was added to each well and the cells were incubated for another 3 h at 37°C; the optical density was measured at 450 nm using an absorbance microplate reader (Bio-Tek, Elx800, Winooski, VT, USA). Cells that stained positively with CCK-8 were considered viable cells and expressed as a percentage compared with control cells. The cells in the control group were treated without treatment and only the medium was used as a blank control. The half-dose inhibition rate (LC50) was calculated based on the absorbance report. The FPRL2 positive HCT116 cells were pre-treated with AKT blocker triciribine (130 nm) and added to the above concentrations of 5-Fu and MMC for 72 h to calculate LC50.

Western Blot Analysis

HCT-116 and SW620 cells were treated with LC50 dose of 5-Fu and MMC for 24 h and then lysed on ice in lysis buffer. After centrifuged at 14000 g at 4°C for 15 min, the protein concentration of lysates was determined by bicinchoninic acid (BCA) Protein Assay Kit. Equal amounts (25 μg/lane) of total proteins were subjected to electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, proteins were electro-transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% skim milk in Tris buffered saline-Tween 20 (TBST) at room temperature for 2 h and subsequently incubated with the primary antibodies (diluted 1:500-1:1000) 4°C overnight. The membranes were washed three times in TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (diluted 1:5000) for 1 h. The immune complexes were visualized by fluorography using enhanced chemiluminescence (ECL) system (Millipore, Billerica, MA, USA).

Statistical Analysis

Data are presented as mean ± standard deviation (SD) of triplicate experiments. Statistical differences between treatment groups were analyzed by Student's *t*-test using Statistic Package for Social Science (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was defined at p-value < 0.05.

Results

Expression of FPRL2 and F2L/HBP (1-21) in colon cancer tissues

In immunohistochemistry analysis, 94% (32/34) drug resistance specimen showed FPRL2 positive staining, while only 9% (1/11) in the non-resistant specimen showed FPRL2 positive staining. The immunohistochemical score in drug resistance specimen was significantly higher than that in the non-resistant group (2.8VS0.5) (Figure 1A, B, C). The F2L/HBP (1-21) was expressed both in drug-resistant and non-resistant groups. There was no significant difference in score (Figure 2A, B).
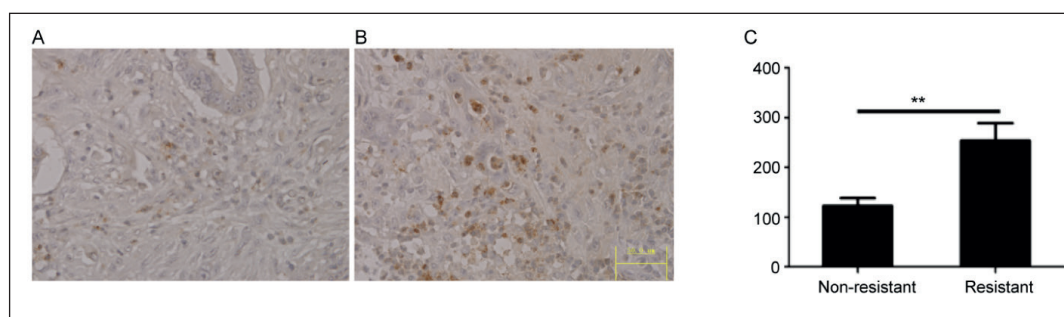

Figure 1. Expression of FPRL2 in specimen of resistant and non-resistant colon cancer. The IHC detection of FPRL2 had only sporadic expression (magnification: 40×). (**A**) in the tissues of non-resistant patients and significantly increased in tissues of drug-resistant patients (magnification: 40×). (**B**). Histological score (**C**).
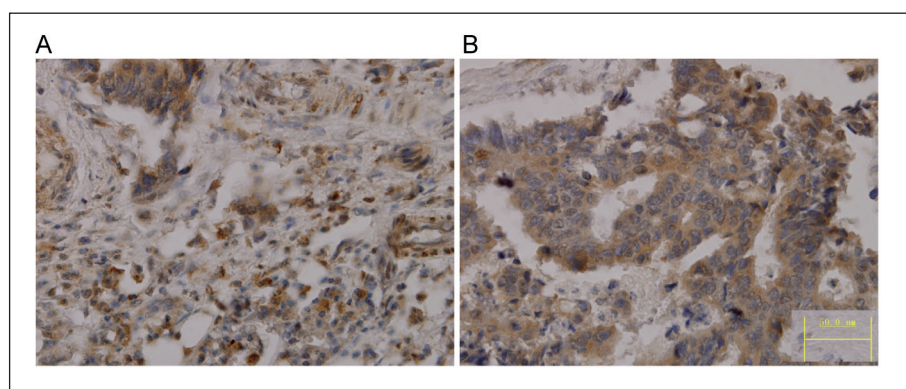
Figure 2. Expression of FPRL2 ligand F2L in specimen of resistant and non-resistant colon cancer. The IHC detection of F2L was significantly expressed both in drug-resistant and non-resistant tissues; there was no difference in staining between the two groups (magnification: 40×).

Expression of FPRL2 and F2L/HBP (1-21) in colon cancer cells

In qRT-PCR analysis, there are 60% (3/5) colon cancer cells expressed FPRL2, and 100% (5/5) colon cancer cells expressed F2L/HBP (1-21) (Figure 3A, B).

LC50

As shown in Table III, LC50 of both 5-Fu and MMC in HCT-116 cells (FPRL2-positive) were significantly higher than that in those of SW620 cells (FPRL2-negative).

p-AKT expression

The expression of p-AKT in HCT-116 cells was higher in SW620 cells treated with or without 5-Fu and MMC (Figure 4).

Discussion

Colon cancer is the third common malignant tumor in the world, a prevalence for 1.2-5% in total malignant tumors. In recent years, incidence and mortality of colorectal cancer increased significantly[1]. An in-depth study of the biological characteristics of colon cancer helps to find a solution to this common tumor.

The highly expressed formyl peptide receptor family in inflammatory cells has three subtypes: FPR1, FPR2 and FPRL2. The homology of FPR1 with FPR2, FPRL2 and FPR1 and FPR2 was 56% and 83%, respectively. This study confirmed the expression of FPRL2 in colon cancers. Meanwhile, pathogenic-derived synthetic peptides[9, 10] and endogenous substances derived from the host, such as hydrolyzate of the heme binding protein were ligands for FPRs[11,12]. The study also found that heme binding proteins were distributed in all detected colon cancer cell lines and patients' specimens, indicating that the FPRs may be involved in certain biological characteristics of colon tumors.

The formyl peptide receptor is a transmembrane G-protein coupled receptor that mediates
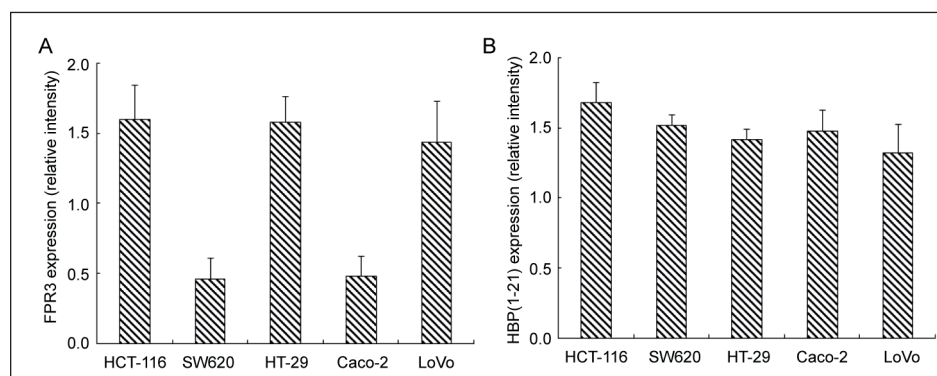

Figure 3. The mRNA expression of FPRL2 (**A**) and its ligand F2L (**B**) in colon cancer cell lines. The data are presented as the means±SD (n=3).

Table III. The IC50 for human colorectal tumor cells (μmol/L, n=3).

Cell line	5-FU	MMC
HCT-116	19.910±0.412*	0.713±0.010*
SW620	2.318±0.117	0.446±0.144

*$p<0.05$ vs. SW620 cell.

cellular responses. It is reported that MAPK, AKT and STAT signaling pathways are mainly activated after activation of FPRs, in which AKT pathway is closely related to apoptosis[8, 13]. We suggest that FPRs may be associated with tumor drug resistance. In this study, the expression of FPRL2 was significantly higher in resistant colon cancer tissues than those in non-resistant colon cancer tissues. LC50 of two major chemotherapeutic agents 5-Fu and MMC in FPRL2 positive cells were significantly higher than those in FPRL2 negative cells, consistent with previous reports[14]. The level of p-AKT in FPRL2 positive cells was higher than those in FPRL2 negative cells either treated with or without MMC and 5-Fu. These results indicate that the FPRL2 participates in at least part of the drug resistance through the AKT pathway.

Colorectal cancer resection is the main treatment. But for unresectable patients, high recurrence and poor prognosis are still the serious problem. Therefore, to improve the effect of adjuvant treatment of colon cancer is an important strategy to improve the prognosis of colon cancer. It is important to improve the sensitivity of colon cancer chemotherapy, since most colon cancer cells are resistant to chemotherapeutic

agents. Improve the prognosis of colon cancer is of great significance. However, the drug resistance research is poor. Multiple mechanisms involved in drug resistance, including the accumulation of drugs in the cells reducing the increased repair capacity[15], enhanced anti-apoptotic ability[16,17], and the survival of tumor environment conduced resistance to chemotherapy drugs[18-20].

Conclusions

This study presents another view of colon cancer cell resistance, which may increase the sensitivity of colon cancer to chemotherapeutic drugs.

Conflict of Interest

The authors declared no conflict of interest.

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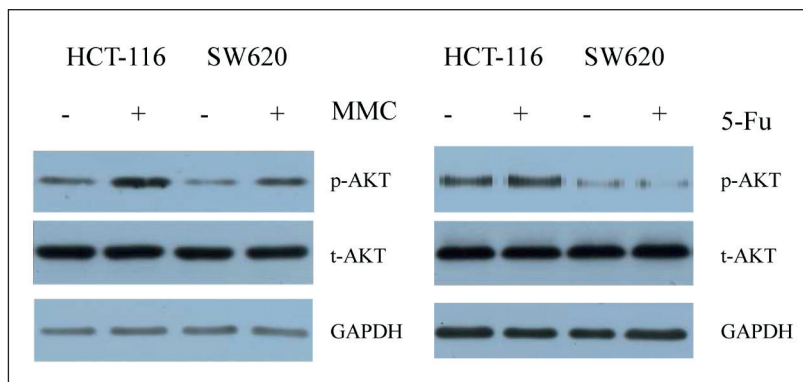
Figure 4. The expression of pAKT in cells treated with or without 5-FU and MMC. The level of pAKT in FPRL2-positive cells (HCT-116) was significantly higher than that in FPR3-negative cells (SW620). GAPDH was used as the loading control.

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