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Original Article

Multidrug resistance-associated protein 3 confers resistance to chemoradiotherapy for rectal cancer by regulating reactive oxygen species and caspase-3-dependent apoptotic pathway

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ABSTRACT

This study aimed to clarify the role of multidrug resistance-associated protein 3 (MRP3) in resistance to neoadjuvant chemoradiotherapy and long-term prognosis of advanced rectal cancer. Immunohistochemistry was used to measure MRP3 expression in biopsy specimens of 144 stage II–III rectal cancer patients who received preoperative chemoradiotherapy. The effect of MRP3 expression on short-term pathological response and postoperative long-term prognosis were assessed using the Cox proportional hazards model. Short interfering RNAs targeting MRP3 were synthesized and used to transfect human colorectal carcinoma cell lines. The effect of MRP3 down-regulation on cell proliferation and apoptosis in response to 5-fluorouracil and/or irradiation were examined *in vitro* and in xenograft mouse models, respectively. The content of intracellular reactive oxygen species and the activity of caspase-3-dependent apoptotic pathway in response to irradiation were further evaluated. High expression (immunoreactive score > 6) of MRP3 significantly predicted poor pathological response to chemoradiotherapy (tumor regression grade ≤ 2 vs. ≥3, $p = 0.002$) in univariate analysis and unfavorable long-term prognosis (5-year overall survival: HR = 1.612, 95% CI, 1.094–2.375, $p = 0.016$; 5-year disease-free survival: HR = 1.513, 95% CI, 1.041–2.200, $p = 0.030$) in multivariate Cox analysis. MRP3 down-regulation significantly increased 5-fluorouracil or irradiation-induced cell apoptosis and attenuated tumor growth following irradiation in animal models. MRP3 inhibition significantly reduced intracellular reactive oxygen species exporting from cells following irradiation, and increased expression of cleaved poly ADP-ribose polymerase and caspase-3. Aberrant expression of MRP3 in rectal cancer confers chemoradioresistance. MRP3 might be a predictive factor and an attractive target in treating advanced rectal cancer.

Abbreviations: 5-FU, 5-fluorouracil; APR, abdominoperineal resection; CA19-9, carbohydrate antigen 19-9; CCK-8, cell counting kit-8; CEA, carcinoembryonic antigen; CI, confidence interval; DCF, 2′, 7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DFS, disease-free survival; GFP, green fluorescence protein; HR, hazard ratio; LAR, low anterior resection; MRP, multidrug resistance-associated proteins; nCRT, neoadjuvant chemoradiotherapy; OS, overall survival; PARP, poly ADP-ribose polymerase; PBS, phosphate buffered saline; PCR, pathological complete regression; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SER, sensitization enhancement ratio; SF, surviving fractions; siRNA, small interfering RNA; TBST, tris-buffered saline tween; TME, total mesorectal excision; TRG, tumor regression grade.

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Introduction

Colorectal cancer is the third most common malignancy presenting serious threat to human health worldwide, and one-third is represented by rectal cancer, which accounts for approximately 40% of all colorectal cancer deaths [1]. Currently, preoperative 5-fluorouracil (5-FU) based neoadjuvant chemoradiotherapy (nCRT) followed by total mesorectal excision (TME) surgery has been adopted as a standardized multidisciplinary treatment for stage II–III rectal cancer [2]. However, the response to nCRT varies greatly, ranging from pathological complete regression (PCR) to complete resistance [3]. Understanding key molecules predisposing the resistance shows great promise in accurate prediction of therapeutic response.

Multidrug resistance-associated proteins (MRP, also named as ATP-binding cassette transporters) are important in chemoresistance [4]. Of them, MRP3 is a novel intracellular signaling molecule, which is situated at the basolateral membrane of epithelial cells and functions as an efflux pump, and actively mediates a broad spectrum transportation of different signaling molecules and cytotoxic drugs including glutathione, glucuronide, sulfate conjugates of xenobiotics and endogenous molecules, etoposide, teniposide and methotrexate across cellular membrane, and plays an important role in several kinds of human carcinomas resistance to chemotherapeutics [5]. Tissue distribution of MRP3 is mainly in colon, small intestine, liver, and adrenal gland [6]. It has been proved that fractionated irradiation could increase the expression of functionally relevant members of MRP family such as MRP1 [7], thus inducing a multi-drug resistance phenotype in colorectal carcinoma cell lines [8]. Intriguingly, MRP3 shares the highest degree of structural resemblance both in terms of amino acid alignment and protein topology with MRP1 (58% identity) [9]. It should be emphasized that chemoresistance is often associated with radioresistance in the treatment of rectal cancer, and there seems to be a cross regulatory mechanism.

In this study, we firstly analyzed the association of MRP3 expression in pretreatment biopsy specimens of stage II–III rectal cancer patients with pathological response and long-term prognosis. We carried out a series of experiments to determine the role of MRP3 in conferring resistance to chemotherapeutic agents and irradiation both *in vivo* and *in vitro*. Furthermore, we investigated the effects of MRP3 on reactive oxygen species (ROS) transportation across cell membranes and activity of cleaved poly ADP-ribose polymerase (PARP) and cleaved caspase-3 protein in cellular apoptotic pathway to reveal the potential molecular mechanism.

Materials and methods

Ethics approval

The study protocol conformed to the 1975 Declaration of Helsinki Principles and was approved by the human ethic committee of Changhai Hospital and Second Military Medical University (Shanghai, China). All animal treatments were strictly in accordance with international ethical guidelines concerning the Care and Use of Laboratory Animals, and the experiments were carried out with the approval of the Committee of Experimental Animal Administration of the Second Military Medical University. Signed informed consents were provided from all patients participating in this study.

Patients and specimens

A total of 144 stage II–III rectal cancer patients receiving nCRT and radical surgery in Department of Colorectal Surgery, Changhai Hospital from January 2006 to January 2009 were enrolled in this study. The mean age was 53.36 (±13.62) years (median, 52 years; range, 23–88 years). Pretreatment biopsy specimens were obtained before they received nCRT, and radical surgery was performed according to the criteria of TME (91 patients underwent low anterior resection LAR, and 53 patients underwent abdominoperineal resection APR) 6–8 weeks after the completion of nCRT. Circumferential resection margin status of each case was considered negative and final tumor staging were determined according to postoperative pathology reports. Adjuvant chemotherapy was individually tailored to each patient according to the National Comprehensive Cancer Network guidelines [10]. Clinicopathological and postoperative follow-up data were collected through patient records in our standard registration and follow-up system [11]. Five-year overall survival (OS) and disease-free survival (DFS) were applied to evaluate long-term prognosis.

Immunohistochemistry and tumor regression grade (TRG)

Pre-treatment biopsy rectal mucosa specimens harvested under colonoscopy were examined to evaluate the expression and subcellular localization of MRP3 immunohistochemically. Formalin fixed and paraffin embedded specimens were cut into 4 μm sections. The sections were deparaffinized in xylene, dehydrated in a graded ethanol series, and then immersed in methanol with 0.3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase activity. Thereafter, the sections were immunostained using mouse monoclonal antibody against human MRP3 (ab3375, Abcam, Cambridge, UK) at 1:50 dilution, rinsed 5 times with phosphate buffered saline (PBS), and then incubated with peroxidase-conjugated rabbit anti-mouse secondary antibody (Cell Signaling Technology, Beverly, MA). The sections were then developed with 3, 3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. Expression of MRP3 was estimated by a semi-quantitative score according to the percentage of positive tumor cells and immunostaining intensity, and pathological response was scored according to a 5-scale TRG criterion basing on the extent of fibrosis and residual tumor cells by postoperative histological examination independently by two pathologists (LT & JX) who were blinded to clinical information [12]. There was a close agreement on staining intensity (89%) and staining extent (90%) between the two pathologists. Disagreements were resolved by consensus.

Cell lines and irradiation

The human colorectal carcinoma cell lines HT-29 and SW-480, which have been proven to be resistant to irradiation [13,14], were purchased from the Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). All cells were routinely cultured in RPMI-1640 medium (Thermo, Fremont, CA) supplemented with 10% fetal bovine serum (Thermo), 100 μg/ml streptomycin, and 100 U/ml penicillin in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. In order to simulate the radiotherapy condition in clinical setting, irradiation to both cell samples and xenograft models was performed using a 6 MV X-ray linear accelerator (Clinac 21EX, Varian Medical Systems, Palo Alto, CA) at 100 cm source-skin distance in a dose rate of 400 cGy/min with a horizontal treatment field of 30×30 cm at room temperature.

Down-regulation of MRP3 by small interfering RNA (siRNA)

According to MRP3 gene coding sequence (NCBI Genbank accession number: NM 003786), three sequences were selected as the RNA interference targets and three pairs of oligo were specially designed and synthesized as annealed siRNA of standard purity according to the manufacturer's instructions (Genepharma, Shanghai, China) to exclude the off target effect. A non-silencing green fluorescence protein (GFP)-siRNA with no homology with mammalian genes was used as a negative control. All siRNAs were 21 nucleotides in length and contained symmetric 3′ overhangs of two deoxythymidines (Table 1). After screening, MRP3-homo-1019 was confirmed as the most validated target sequence to down-regulate MRP3 expression. For transfection, cells (2×10^4/mL) were seeded in 96-well plates for 24 h, resulting in a 70% to 90% confluence. Diluted MRP3-homo-1019 or negative control GFP-siRNA (100 nM, respectively) were mixed with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) in serum-free Opti-MEM (Invitrogen) for 20 min at 37 °C to form siRNA/lipofectamine complex, and then added to the cultured cells. After incubation for 6 h at 37 °C, the medium was replaced with the standard medium. GFP was optimized under an inverse fluorescence microscope (Nikon, Japan) to determine efficiency of transfection 24 h later.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen) and reverse transcription was carried out according to the manufacture's protocol (TaKaRa, Dalian, China). The house keeping gene GAPDH was used as an internal control. The

Table 1
Gene sequences used as the target of RNA interference.

	5′-3′ (sense)	5′-3′ (antisense)
MRP3-homo-1019	GUGCCUGCUUCAAGCUUAUTT	AUAAGCUUGAAGCAGGCACTT
MRP3-homo-1170	GCUGAUCUUACAACACUAUTT	AUAGUGUUGUAAGAUCAGCTT
MRP3-homo-1502	CCUUCCAGGUAAAGCAAAUTT	AUUUGCUUUACCUGGAAGGTT
Negative control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGA ATT