

ORIGINAL ARTICLE

A study on the correlations of MRP-1 expression with the pathogenesis and prognosis of colorectal cancer

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Summary

Purpose: To investigate the expression level of multidrug resistance-associated protein 1 (MRP-1) and its correlation with prognosis in the pathogenetic process of colorectal cancer.

Methods: 116 patients with colorectal adenocarcinoma and 50 patients with colorectal adenomas were studied. Thirty cut-end normal tissue sections were subjected to immunohistochemical staining, real-time polymerase chain reaction (RT-PCR) and Western blotting, to detect the expression levels of MRP-1 gene and protein in tissues. Besides, the correlations of the expression of MRP-1 in colorectal adenocarcinoma tissues with clinicopathological features and prognosis were analyzed.

Results: MRP-1 was mainly expressed in the cell membrane and cytoplasm in colorectal adenocarcinoma. The positive expression rates of MRP-1 in colorectal adenocarcinoma tis-

ues, colorectal adenoma tissues and normal tissues were 73.28, 46.0 and 20.0%, respectively, showing statistically significant differences ($p < 0.05$). In adenocarcinoma tissues, MRP-1 expression level was associated with the differentiation grade, TNM staging and whether there was lymph node metastasis ($p < 0.05$ in all comparisons). The 5-year survival rates of patients with negatively expressed MRP-1 protein, no lymph node metastasis and high/moderate grade of differentiation as well as in stage I+II were remarkably higher ($p < 0.01$ in all comparisons).

Conclusion: In colorectal adenocarcinoma tissues, the expression of MRP-1 is elevated and patients with negatively expressed MRP-1 have a better prognosis. Therefore, MRP-1 can be a reference indicator for clinical diagnosis and prognosis.

Key words: colorectal cancer, MRP-1, prognosis

Introduction

Colorectal cancer is currently one of the most common malignant gastrointestinal tumors in China. At present, people's living standards are gradually improving, the proportion of the elderly is increased, and the number of patients with colorectal cancer is also on the rise with steady steps [1]. The treatment methods of colorectal cancer mainly include surgery as well as radiotherapy and chemotherapy. However, the 5-year survival rate of colorectal cancer patients is generally low with poor prognosis, so finding biomarkers related

to prognosis is particularly important. According to reports, over 80% of colorectal cancers originate from malignant transformation of adenomas [2]. Some authors pointed out that the malignant transformation process is usually accompanied by abnormal overexpression of ATP-binding cassette (ABC) transporters [3]. Multidrug resistance-associated protein 1 (MRP-1) is one of the main members of the ABC transporter family multidrug-resistance (MDR). At present, it has been found that MRP-1 is highly expressed in breast cancer, lung cancer

and other diseases, and the effect of chemotherapy in patients with high expression of MRP-1 is poor [4,5]. In this study, immunohistochemical staining, reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting were mainly applied to detect the expression of MRP-1 in three kinds of tissues (colorectal adenocarcinoma, colorectal adenoma and normal tissues). In the present study, the correlations of MRP-1 expression with clinicopathological features in adenocarcinoma and prognosis were investigated.

Methods

Data

A total of 116 patients definitely diagnosed with colorectal adenocarcinoma and treated in Chinese PLA General Hospital from April 2010 to April 2012 were studied, including 56 cases of colon cancer, 60 cases of rectal cancer and 50 cases of colorectal adenomas. Among them, there were 69 males and 47 females, aged 38-81 years. These patients were classified into lymph node metastasis (n=41) and no lymph node metastasis (n=75), high differentiation (n=33), moderate differentiation (n=49) and low differentiation (n=34), and stage I+II (n=77) and stage III+IV (n=39) in tumor-node-metastasis (TNM) staging. Besides, 30 cut-end normal tissues were collected. The included tissues in this study were all pathologically verified. The patients had complete clinical data and received no radiotherapy or chemotherapy before surgery. All patients and family members signed informed consent.

Reagent consumables

All consumables were purchased from the different companies.

Rabbit anti-human MRP-1 monoclonal antibody (Beijing Dingguo Changsheng Biotechnology Co., Ltd.), 3,3'-diaminobenzidine (DAB) color developing reagent and citrate buffer powder (Shanghai X-Y Biotechnology Co., Ltd.), fluorescence real-time quantitative PCR kit (Guangzhou Vipotion Biotechnology Co., Ltd.), TRIGene reagent (Shanghai Kang Lang Biological Technology Co., Ltd.), RTkit (Shanghai Boyi Biotechnology Co., Ltd.), and tissue total protein extraction kit (Jiangsu KeyGen Biotech Co., Ltd).

Immunohistochemical staining

All tissues were fixed in formalin solution, embedded in paraffin, sectioned, dewaxed, hydrated and washed with phosphate-buffered saline (PBS). Then, the sections were blocked for 15 min and sealed with 10% serum under non-specific background for 15 min at room temperature. After that, primary antibody (MRP-1 monoclonal antibody) was added, and sections were stored in a refrigerator at 4°C overnight, followed by washing with PBS after removal. Subsequently, biotin-labeled secondary antibody was added for incubation at room temperature for 30 min, followed by washing.

Streptomyces antibiotic protein-peroxidase solution was applied for incubation at room temperature for another 30 min. After DAB staining, the sections were subjected to washing, counterstaining, mounting and observation.

Real-time PCR

The total ribonucleic acid (RNA) was extracted from colorectal adenocarcinoma, adenoma and cut-end normal tissues according to the instructions of the TRIGene kit, and its concentration and purity were measured at the wavelength of 260 nm (A260)/A280 value of 1.8-2.0. Based on the RT kit [Reverted First Strand complementary deoxyribonucleic acid (cDNA) Synthesis Kit, Thermo, K1622] instructions, primer sequences were synthesized by Shanghai Jiran Biotechnology Co., Ltd. MRP-1. Forward primer: 5'-AGTTCTGCGGTGCTGTTGTG-3' and reverse primer: 5'-TTCGCTGAGTTCCTGCGTAC-3'. β -actin forward primer: 5'-CAGGAAGGAAGGCTGGAAG-3' and reverse primer: 5'-CGGAAATCGTGCCTGAC-3'. A total volume of 20 μ L reaction systems were reversely transcribed into cDNA on a RT-PCR instrument.

According to the real-time fluorescence instructions for quantitative PCR kit (2 \times RealStar Green Power Mix, GenStar, A311), the reaction conditions of 25 μ L reaction system were as follows: a total of 40 cycles of reaction at 95°C for 10min, at 95°C for 30s, at 59.4°C for 30s, and at 95°C for 15s, followed by cooling to 65°C. Then, the fluorescence value was read, and the relative expression of MRP-1 messenger RNA (mRNA) was calculated by RT-PCR with β -actin as an internal reference.

Western blotting

Total protein was extracted from colorectal adenocarcinoma, adenoma and cut-end normal tissues after surgery according to the total protein extraction kit and stored at -70°C for standby application, and the concentrations were assessed. Gels at different concentrations were prepared for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. According to the marker band, the site where the protein was located was selected and cut, and then the semi-dry protein was transferred onto the membrane for 30 min, followed by sealing for 90 min. After that, rabbit anti-human MRP-1 primary antibody solution (diluted at 1:100) was added for incubation at 4°C overnight, followed by addition of tris-buffered saline with Tween 20 (TBST) solution for washing. Subsequently, secondary antibody (diluted at 1:100) was added for incubation at room temperature for 1 hr, followed by addition of TBST for washing. Then, electrochemiluminescence (ECL) color developing solution was added dropwise in the dark for color development, followed by photographic fixing. The transfer membrane containing the target protein was scanned via the ChemiDocTM MP imaging system, image analysis was conducted using ImageJ professional image analysis software, and absorbance values were recorded.

Evaluation of experimental results

Fields of view to be observed were randomly selected to count 100 cells, and the average number of cells in the fields of view was calculated as the positive cell

number of the expressed proteins in tissues. Color depth scores: 0-3 points represented no coloring, light yellow, pale brown and medium brown, respectively. Stained cell positive rate scores: 0-4 points showed that the percentages of positive cells were [0,5%], [5%,25%], [25%,50%], [50%,75%] and [75%,100%], respectively. Products of these two components: ≤ 1 point for negative, 2-4 points for weakly positive (+), 5-8 points for moderately positive (++) and ≥ 9 points for strongly positive (+++).

Statistics

In this study SPSS 17.0 professional software (Beijing Xinmei Jiahong Technology Co., Ltd.) was adopted for data processing and analysis. Analysis of variance (ANOVA) was applied for the comparisons among groups. The comparison of percentages was conducted using the χ^2 test, and $\alpha=0.05$ was used as the test statistical standard.

Results

Images of immunohistochemical staining

In normal tissues, MRP-1 was mainly expressed in the epithelial cells near the apical part of the intestinal mucosa. In adenocarcinoma tissues, MRP-1 was primarily expressed in the cytoplasm and membranes, presenting pale brown or medium brown color. In adenomas, MRP-1 was chiefly expressed in the cytoplasm (Figure 1).

MRP-1 expression in different tissues

The positive expression rate of MRP-1 was 20.0% (6/30) in 30 cases of normal tissues, 73.28% (85/116) in 116 cases of colorectal adenocarcinoma tissues and 46.0% (23/50) in 50 cases of colorectal adenoma tissues. The differences between normal tissues and colorectal adenocarcinoma tissues, between normal tissues and colorectal adenoma tissues and between colorectal adenocarcinoma tissues and adenoma tissues were statistically significant ($p<0.05$ in all comparisons) (Table 1).

Correlations of MRP-1 expression in colorectal adenocarcinoma with clinicopathological features

The positive expression of MRP1 protein in colorectal adenocarcinoma tissues was correlated with the grade of differentiation of adenocarcinoma tissues, TNM staging and whether there was lymph node metastasis ($p<0.05$ in all comparisons), but not related to the patient's age, gender and tumor site ($p>0.05$ in all comparisons) (Table 2).

MRP-1 mRNA expression in three types of tissues

The relative expression of MRP-1 mRNA was 0.14 ± 0.02 in 30 cases of normal tissues, 0.62 ± 0.08 in 116 cases of colorectal adenocarcinoma tissues and 0.45 ± 0.06 in 50 cases of colorectal adenoma tissues, displaying statistically significant differences in comparisons between normal tissues and colorectal adenocarcinoma tissues, between normal tissues and colorectal adenoma tissues and between colorectal adenocarcinoma tissues and colorectal adenoma tissues ($p<0.05$ in all comparisons) (Table 3).

MRP-1 protein expression in three types of tissues

The relative expression of MRP-1 protein was 1.53 ± 0.18 in 30 cases of normal tissues, 7.44 ± 1.07 in 116 cases of colorectal adenocarcinoma tissues and 5.38 ± 0.96 in 50 cases of colorectal adenoma

Table 1. MRP-1 expression in different tissues

MRP-1	n	Positive cases (n)	Positive rate (%)
Normal tissue ^a	30	6	20.0
Colorectal adenocarcinoma tissue ^b	116	85	73.28
Colorectal adenoma tissue ^c	50	23	46.0

a versus b, $\chi^2=29.491$ and $p<0.05$; a versus c, $\chi^2=4.247$ and $p<0.05$; and b versus c, $\chi^2=14.026$ and $p<0.05$

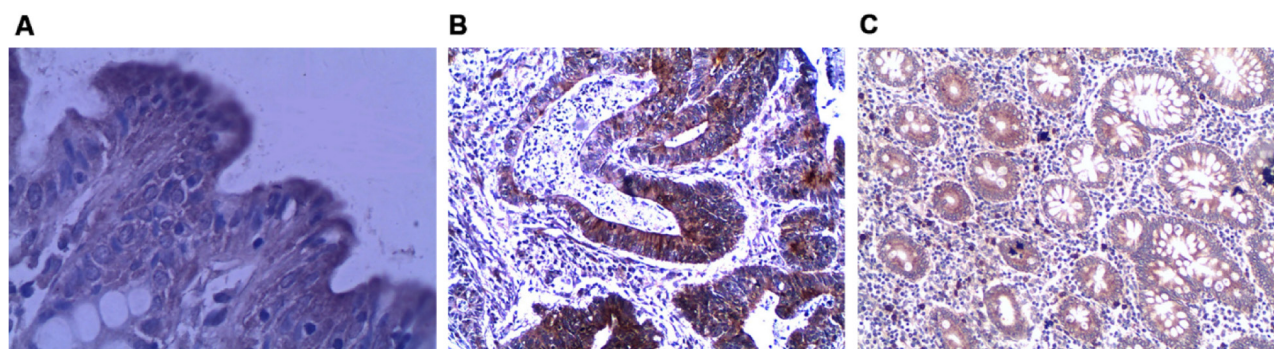


Figure 1. Immunohistochemical staining images of MRP-1 in different tissues. **A:** In normal tissues MRP-1 was mainly expressed in the epithelial cells. **B:** In adenocarcinoma tissues MRP-1 was primarily expressed in the cytoplasm and membranes. **C:** in adenomas MRP-1 was chiefly expressed in the cytoplasm.

Table 2. Correlations of MRP-1 expression in colorectal adenocarcinoma with clinicopathological features

Features	N	MRP-1 protein		
		Positive cases, n	χ^2	p
Age (years)				
<60	34	25	0.385	0.632
≥60	82	60		
Gender				
Male	69	58	3.591	0.077
Female	47	27		
Lymph node metastasis				
Yes	41	39	9.954	0.003
No	75	46		
Grade of differentiation				
High/moderate	82	54	12.028	0.002
Low	34	31		
Tumor site				
Colon	56	42	3.185	0.093
Rectum	60	43		
TNM stage				
I+II	77	47	9.477	0.002
III+IV	39	38		

Table 3. MRP-1 mRNA expression in three types of tissues

Tissues	n	MRP-1 mRNA expression Mean±SD
Normal tissue ^a	30	0.14±0.02
Colorectal adenocarcinoma tissue ^b	116	0.62±0.08
Colorectal adenoma tissue ^c	50	0.45±0.06

a versus b, $\chi^2=29.491$ and $p<0.05$; a versus c, $\chi^2=4.247$ and $p<0.05$; and b versus c, $\chi^2=14.026$ and $p<0.05$

Table 4. MRP-1 protein expression in three types of tissues

Tissues	n	Relative expression of MRP-1 protein mean±SD
Normal tissue ^a	30	1.53±0.18
Colorectal adenocarcinoma tissue ^b	116	7.44±1.07
Colorectal adenoma tissue ^c	50	5.38±0.96

a versus b, $\chi^2=29.491$ and $p<0.05$; a versus c, $\chi^2=4.247$ and $p<0.05$; and b versus c, $\chi^2=14.026$ and $p<0.05$

tissues, with statistically significant differences in comparisons between normal tissues and colorectal adenocarcinoma tissues, between normal tissues and colorectal adenoma tissues and between colorectal adenocarcinoma tissues and colorectal adenoma tissues ($p<0.05$ in all comparisons) (Table 4).

Correlation with prognosis

Analysis revealed that the survival rates of patients with negative expression of MRP-1 protein, no lymph node metastasis and high/moderate differentiation as well as in stage I+II were significantly higher than those of patients with MRP-1 protein positive expression, lymph node metastasis and low differentiation as well as in stage III+IV, showing statistically significant differences among groups ($p=0.0013$, $p=0.0018$, $p=0.0008$ and $p<0.0001$) (Figure 2).

Discussion

MRP-1 is a drug-resistance protein that belongs to ABC transporter superfamily. Under normal condition, MRP-1 is often lowly expressed in most tissues in the body. MRP-1 protein exhibits various indispensable physiological functions in the body, such as involvement in the oxidative stress response caused by stimulation to external or internal factors [6], detoxification [7] and defense for the invasion of exogenous toxicants in the body [8]. It has been currently confirmed that chemotherapeutics can be pumped out of cells by MRP-1, thus leading to resistance of tumors to multiple drugs. This mechanism may be that with the constantly increased chemotherapy times or the prolongation of the treatment course, MRP-1 gene is abnormally overexpressed in tumor cells

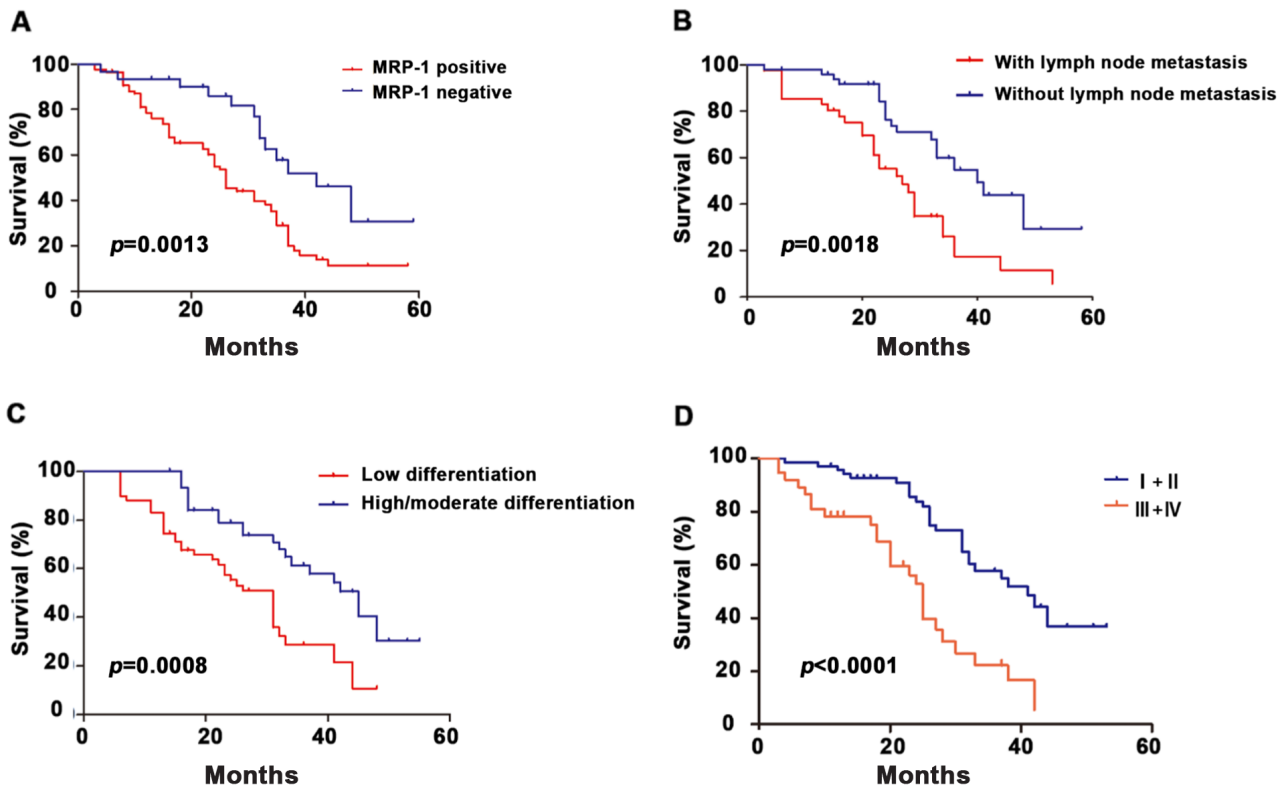


Figure 2. Correlation of MRP-1 protein with prognosis, lymph node metastasis, differentiation and stage.

to pump drugs out of them, thus resulting in drug resistance [9]. Some authors have put forward the idea that the underlying mechanism may be that MRP-1 gene changes the power of hydrogen (pH) in the cytoplasm. When chemotherapeutics reach the site where they can exert effects, the drug concentration is obviously reduced, and MRP-1 can transport intracellular drugs to the extracellular space at a converse gradient concentration, thereby leading to drug resistance [10]. At present, it has been reported that MRP-1 is abnormally overexpressed in various malignant tumors, such as liver cancer [12], breast cancer [12], esophageal cancer [13] and lung cancer [14], and there are correlations of MRP-1 overexpression with tumor drug resistance, infiltration, metastasis and prognosis [15]. However, there are fewer reports about the relationship between MRP-1 and colorectal adenocarcinoma.

According to our study, MRP-1 could be expressed in colorectal adenocarcinoma tissues, colorectal adenoma tissues and cut-end normal adenocarcinoma tissues, but the expression of MRP-1 in colorectal adenocarcinoma tissues was notably higher than those in other groups, displaying statistically significant differences among groups ($p < 0.05$ in all comparisons). This suggests that MRP-1 overexpression may participate in the progressive deterioration of normal mucosal and colo-

rectal adenomas as well as the process of transition to colorectal adenocarcinoma, which is consistent with the results of the study on esophageal cancer of Wang et al. [16]. In addition, it was also found in this study that the expressions of MRP-1 protein in adenocarcinoma tissues of patients with stage III+IV and with lymph node metastasis were significantly higher than those in adenocarcinoma tissues of patients with stage I+II and with no lymph node metastasis, and the differences between the two groups were statistically significant ($p < 0.05$ in all comparisons). Therefore, it is speculated that MRP-1 overexpression may be involved in the infiltration and metastasis of colorectal adenocarcinoma. Moreover, the positive expression rate of MRP-1 protein in the high/moderate grade of differentiation was markedly lower than that in the lowly differentiated group ($p < 0.05$), which is consistent with the results of the study on liver cancer of Cole et al. [17]. Abdallah et al. [18] illustrated that when MRP-1 protein is overexpressed in liver cancer cells, these cells can exhibit features similar to hepatic progenitor cells. It is speculated that MRP-1 may be derived from the characterization of hepatic progenitor cell subtype liver cancer. There is a certain correlation between the overexpressed MRP-1 protein and microvascular infiltration of liver cancer. However, there were no significant correlations of the expressions of MRP-1 protein

in colorectal adenocarcinoma with age, gender and the site where adenocarcinoma occurs ($p > 0.05$ in all comparisons).

The follow-up revealed that the 5-year survival rates of patients with positively expressed MRP-1 protein, lymph node metastasis and histologically low differentiation, and in stage III+IV were relatively low, suggesting that when cancer metastasis occurs, the prognosis of patients was poor, which is consistent with the results of the study on lung cancer of Ko et al. [19]. Generally, the mechanisms of the occurrence and development of cancer is the result of combined actions by multiple factors, multiple stages and multiple steps. Cai et al. [20] pointed out in their search for clinical prognostic indicators of colorectal cancer that ABC C1 is overexpressed in cancer tissues, which is associated with Duke's stage and poor prognosis. It is speculated that MRP-1 may be involved in the occurrence and development of colorectal adenocarcinoma together with other ABC transporters. However, the specific mechanism of action has not been explained in this study, which needs to be further verified at a later period.

In summary, MRP-1 expression is elevated in colorectal adenocarcinoma tissues, and the prognosis of patients with negatively expressed MRP-1 is better, thus providing reference indicators for clinical diagnosis and prognosis.

Ethics approval

This study was approved by the ethics committee of Chinese PLA General Hospital and informed consents were signed by the patients and/or their guardians.

Authors' contributions

JY collected and analyzed the general data of patients. JY and PS were responsible for immunohistochemical staining. JY and GZ performed PCR and western blot. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflict of interests.

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