Original Article

Expressions of Poly (ADP-ribose) Glycohydrolase (PARG) and Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) in Colorectal Carcinoma

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ABSTRACT

Objective: To investigate the significance of Poly (ADP-ribose) glycolhydrolase (PARG) and membrane type 1 matrix metalloproteinase (MT1-MMP) expressions in human colorectal carcinoma.

Methods: Immunohistochemical staining for PARG and MT1-MMP was carried out on colorectal adenoma-carcinoma tissue microarrays containing normal colorectal mucosae, adenoma, adenoma with malignant transformation and adenocarcinoma (total 130 specimens). The expressions of PARG and MT1-MMP in the GLTN [Gallotannin]-treated and GLTN-untreated lovo cells were detected by Western Blot.

Results: PARG expression in adenocarcinoma (83.1%) and adenoma with malignant transformation (66.7%) was significantly higher than that in normal colorectal mucosa (10%) and adenoma (10.5%). Expression of MT1-MMp in normal colorectal mucosa and adenoma was negative, while the expression in adenocarcinoma (80.3%) and adenoma with malignant transformation (72.2%) was high. The expressions of PARG and MT1-MMP in adenocarcinoma with metastasis and in late tumor stages were significantly higher than those in adenocarcinoma with no metastasis and in early tumor stages. Thus, PARG expression shows a positive correlation with the expression of MT1-MMP. The expressions of PARG and MT1-MMP in GLTN-treated lovo cells were weaker than that in GLTN-untreated lovo cells.

Conclusion: The expression of PARG was probably related to the development of colorectal carcinoma. PARG may play an important role for the regulation of MT1-MMP expression in colorectal carcinoma.

Key words: PARG; MT1-MMP; Colorectal carcinoma; Tissue microarray; Immunohistochemistry

INTRODUCTION

Poly (ADP-ribose) glycohydrolase (PARG) and poly (ADP-ribose) polymerase (PARP) play important roles in regulating cell functions by catalyzing the metabolism of poly (ADP-ribose), which is a post-translational modification in eukaryotic cells. PARP uses ADP-ribose units from its substrate NAD+ to build a polyanionic poly (ADP-ribose) polymer onto Glu residues on its target proteins when activated by DNA single strand breaks[1]. PARP has been shown to be involved in many disease processes, such as in inflammatory reactions, leukemias, prostatic carcinoma, etc.[2, 3]. PARG plays an important role in regulating PARP, it can reactivate PARP that binds to PAR which is deactivated by hydrolysis, and is removed through the cycling of PAR[1]. PARG also plays an important role in the

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development of inflammation. Studies have shown that by knocking out PARG gene or by inhibiting PARG, there can be the down-regulation of cellular adhesion molecules (CAM) expressions, such as ICAM-1, P-selectin, VCAM-1, which play a role in inflammatory processes[4]. The inhibition of PARP or PARG can lead to the damage of bowels caused by ischemia and reperfusion at a certain coordinated degree, suggesting that inhibiting PARG may inhibit the activation of PARP[5]. Our previous studies[6] have shown that levels of PARG and PARP were significantly higher in colorectal carcinoma than those in control group, however we did not make any comparative study for normal colorectal mucosa, adenoma, adenoma with malignant transformation and adeno-carcinoma.

Matrix metalloproteinases (MMPs) are responsible for the turnover of many proteins in the extracellular matrix, and can promote tumor angiogenesis[7], which are important factors in promoting tumor growth, invasion, and metastasis. Membrane type 1 matrix metalloproteinase (MT1-MMP) is a significant member of MMPs. It is located at cytolemma by a short transmembrane domain and a cytoplasmic tail. MT1-MMP acts as an important mediator of proteolytic events on the cell surface in cancer cells, and it is engaged in the pericellular proteolysis of the extracellular matrix components. It also can activate other members of MMPs on cell surface such as MMP-2, MMP-13, as well as up-regulating the expression of VEGF-A, which is a promoter of tumor angiogenesis. So it is closely associated with tumor invasion, and metastasis[8].

Our previous studies[9] showed that the expressions of MMP-2 and MMP-9 were weakened by inhibiting PARP, and the expressions of PARG and PARP were correlated positively in colorectal carcinoma. PARG inhibition suppresses the expressions of both PARG and PARP[6]. But we did not know if the expressions of PARG was related to MT1-MMP in colorectal adenocarcinoma. So we made tissue microarrays (including normal colorectal mucosa, adenoma, adenoma with malignant transformation, well-differentiated adenocarcinoma, moderately-differentiated adeno-carcinoma, poorly-differentiation adenocarcinoma), and detected the protein expressions of PARG and MT1-MMP by immunohistochemical staining. Then, we detected the expressions of PARG and MT1-MMP in GLTN treated and GLTN untreated lovo cells by Western Blot. The main aim was to investigate the significance of PARG and MT1-MMP expressions in colorectal carcinoma.

MATERIALS AND METHODS

Materials

In all, 130 specimens from patients who underwent curative surgical resection or endoscopic biopsy from January 2001 to December 2003 at the Southwest Hospital of Third Military Medical University were examined. None of them had received chemotherapy or radiotherapy before surgery. The specimens included normal colorectal mucosa (n=10), adenoma (n=20), adenoma with malignant transformation (n=20), well-differentiated adenocarcinoma (n=20), moderately-differentiated adenocarcinoma (n=40), and poorly-differentiated adenocarcinoma (n=20).

Lovo cell line was kindly supplied by Professor Tang WX (Chongqing Medical University). Rabbit anti-PARG polyclone antibody was purchased from ABcam Biotechnology, whereas rabbit anti-MMP14 (MT1-MMP) polyclone antibody was purchased from NeoMarkers Biotechnology. GLTN was purchased from SIGMA Biotechnology. M-PER@ Mammalian Protein Extraction Reagent was bought from Pierce Biotechnology.

Preparation of the Tissue Microarray

A tissue microarray was prepared as described by Kononen J, et al.[10]. Briefly, using the H&E sections as a template, representative areas of each tumor or normal tissue were identified and marked on a section of the donor block. Approximately three-millimeter thick tissue cylinders (0.6-mm diameter) were punched from each donor paraffin block using a tissue microarray instrument (Beecher Instruments, Silver Spring, MD, USA). The donor cores were placed into the corresponding recipient block holes that were punched ahead of time by the same tissue microarray instrument. After construction, serial 4-μm thick sections were cut and mounted onto polylysine-coated sections for H&E and immunohistochemical staining.

Immunohistochemical Analysis

Immunohistochemical staining of PARG and MT1- MMP was performed on the tissue microarrays by the SP method following the manufacturer's instructions. Appropriate positive and negative controls were employed throughout. The positive control was a section of known positive tissue. PBS was used to replace the first antibody in negative control slides. The individual

diluting strength of PARG and MT1-MMP antibodies were 1:200 and 1:400.

Scores of expression of PARG were referred to Shimizu's[11] method with slight modification: none (not stained) =0, focal (less than one third of cells stained)=1, multifocal (less than two third of cells stained)=2, and diffuse (more than two third of cells stained)=3. The intensity of staining was graded as follows: none (not stained)=0, strong (clearly identified)=2, and mild (between 0 to 2)=1. The scores for distribution and intensity were added and graded as follows: 0=(-), 2=(+), 3=(++), 4=(+++). The positive should be ≥(+).

Scores of expression of MT1-MMP were calculated on the basis of the percentage of positive cells and the intensity of staining[12]. When the percentage of positive cells was less than 5%, the score was set as 0, when the percentage was 5%–25%, the score was 1, when the percentage was 26%–50%, the score was 2, and when the percentage was more than 50%, the score was 3. The intensity of staining was graded as follows: none (not stained)=0, mild staining=1, and strong staining=2. The product of the multiplication of the percentage and intensity was regarded as the final scores and graded as follows: ≤1=(-), 2=(+), 3=(++), 4=(+++).

Cell Culture and Treatment

Lovo human colorectal carcinoma cells were cultured in RPMI 1640 (Invitrogen, Gibco, Calif., USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and 100 μg/ml streptomycin, and were maintained in a 37°C incubator with 5% CO_2 humidified air. For the experiment, the cells were divided into two groups, the treated group (treated with GLTN) and the control group (treated without GLTN).

Western Blot

Lovo cells at logarithmic growth phase were treated with GLTN (final concentration was l00 μmol/L), and in the control group same volume of physiological saline was added. After 12 h, the lovo cells of the two groups were collected respectively[6], then washed with ice-cold PBS and lysed with M-PER (Mammalian Protein Extraction Reagent, Pierce), following the manufacturer's instructions. The total proteins were extracted, following the manufacturer's instructions. The protein concentrations of the two groups were determined by the Bradford protein assay respectively. Equal amounts of protein (20–40 μg) were subjected to electrophoresis in SDS polyacrylamide gels (8%) and were transferred onto polyvinylidene fluoride membrane. The membranes were blocked with 5% fat-free milk for 1 h to block nonspecific binding sites, then were incubated with appropriate antibodies against PARG, MT1-MMP and β-actin and kept over night at 4°C. After being washed three times with TBST for 5 min, the membranes were incubated with horseradish peroxidase conjugated secondary antibodies for 1 h at 37°C, then immunoreactive bands were visualized by ECL reagent. The protein actin was served as an internal loading control. The results were analyzed. The above experiments were performed 6 times.

Statistical Analysis

All statistical analyses were performed with the SPSS 11.5 software package. The associations of PARG expression and MT1-MMP expression with clinicopathological parameters were evaluated by the chi-square test or Fisher's Exact test accordingly. The correlation between PARG expression and MT1-MMP expression was assessed by the Spearman correlation test. Comparisons between GLTN-treated group and control group were assessed by *t* test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Quality of Tissue Microarrays

A tissue microarray containing 118 specimens was well prepared including 10 cases of normal colorectal mucosae, 19 adenoma, 18 adenoma with malignant transformation, and 71 adenocarcinoma (containing 15 well differentiated, 38 moderate differentiated and 18 poorly differentiated).

Expressions of PARG and MT1-MMP in Normal Colorectal Mucosa, Adenoma, Adenoma with Malignant Transformation and Adenocarcinoma

PARG was localized in the cytoplasm and/or nucleus of the tumor cells. The positive rates of PARG were 83.1% in colorectal adenocarcinoma, 66.7% in adenoma with malignant transformation, 10.5% in adenoma, and 10% in normal colorectal mucosa. PARG expressions were much higher in adenocarcinoma and adenoma with malignant transformation than in adenoma and normal

colorectal mucosa (both $P<0.05$) (Table 1; Figure 1, A, B).

Positive staining of MT1-MMP was located in membrane and/or cytoplasm of tumor cells. No positive staining of MT1-MMP was observed in normal colorectal mucosa and adenoma. The positive rates of MT1-MMP were 80.3% in colorectal adenocarcinoma, and 72.2% in adenoma with malignant transformation. The MT1-MMP expressions were significantly higher than those in adenoma and normal colorectal mucosa (both $P<0.05$) (Table 1; Figure 1, C, D).

Table 1. *Expressions of PARG and MT1-MMP in normal colorectal mucosa, adenoma, adenocarcinoma and adenoma with malignant transformation*

Item	Case	PARG		MT1-MMP	
		Positive	Negative	Positive	Negative
Normal colorectal mucosa	10	1	9	0	10
Adenoma	19	2	17	0	19
Adenoma with malignant transformation	18	12*	6	13*	5
Adenocarcinoma	71	59*	12	57*	14

*$P<0.05$, compared with the expressions in normal colorectal mucosa and adenoma.

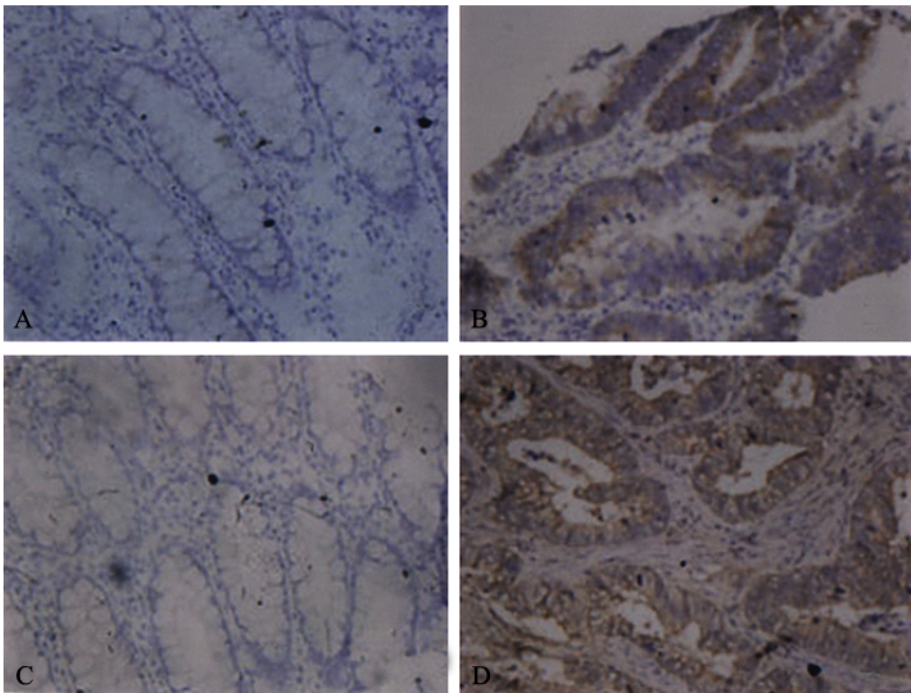

Figure 1. Expressions of PARG and MT1-MMP in normal colorectal mucosa and adenocarcinoma. A: Expression of PARG in normal colorectal mucosa was negative; B: Expression of PARG in adenocarcinoma was positive; C: Expression of MT1-MMP in normal colorectal mucosa was negative; D: Expression of MT1-MMP in adenocarcinoma was positive.

Correlation of PARG and MT1-MMP Expressions with Clinicopathological Characteristics of Colorectal Carcinoma

The expression of PARG was much higher in carcinoma involving the deep muscularis and serosal layers than in carcinoma within the superficial muscularis layer ($P<0.05$). PARG expression was also significantly higher in cases with lymph node metastasis compared to those cases without lymph node metastasis ($P<0.05$). Moreover, in cases with distant metastasis we noticed a higher PARG expression than those cases without any distant metastasis ($P<0.05$).

Furthermore, there was a high expression of PARG in cases with Duke's classification stage C and D compared to that in Duke's stage A and B. (P<0.05). The expression of PARG had no correlation with sex, age, site, size or differentiation (all P>0.05) (Table 2).

The expression of MT1-MMP was also much higher in carcinoma with deep muscularis and serosal layer invasion than carcinoma confined within superficial muscularis layer (P<0.05). It was also significantly higher in cases with lymph node metastasis than that in cases without lymph node metastasis (P<0.05). Cases with distant metastasis showed higher expression of MT1-MMP than those without distant metastasis (P<0.05). Expression of MT1-MMP in Duke's stage C and D was obviously higher than in Duke's stage A and B. (P<0.05). The expression of MT1-MMP had no correlation with sex, age, site, size or differentiation (all P>0.05) (Table 2).

Table 2. *Correlation of PARG and MT1-MMPexpressions with clinicopathological characteristics of colorectal carcinoma*

Item	N	PARG		P	MT1-MMP		P
		+	%		+	%	
Sex							
Male	49	39	79.6	>0.05	38	77.6	>0.05
Female	40	32	80.0		32	80.0	
Age(years)							
≥54	52	42	80.8	>0.05	40	76.9	>0.05
<54	37	29	78.4		30	81.1	
Site of colorectal carcinoma							
Colon	43	35	81.4	>0.05	37	86.0	>0.05
Rectum	46	36	78.3		33	71.7	
Size of colorectal carcinoma							
≥4.0	47	38	80.1	>0.05	38	80.1	>0.05
<4.0	42	33	78.6		32	76.2	
Histological differentiation							
Adenoma with malignant transformation	18	12	66.7		13	72.2	
Well-differentiated	15	10	66.7	>0.05	10	66.7	>0.05
Moderately-differentiated	38	32	84.2		32	84.2	
Poorly-differentiated	18	17	94.4		15	83.3	
Depth of invasion							
Superficial muscularis	20	9	45.0		6	30.0	
Deep muscularis	21	18	85.7	<0.05	18	85.6	<0.05
Serosal	48	44	91.6		46	95.6	
Lymph node metastasis							
Negative	57	40	70.0	<0.05	41	71.9	<0.05
Positive	32	31	96.9		29	90.6	
Distant metastasis							
Negative	79	61	77.2	<0.05	60	75.9	<0.05
Positive	10	10	100		10	100	
Duke's stage							
A–B	55	38	69.0	<0.05	39	71.0	<0.05
C–D	34	33	97.0		31	91.2	

Correlation Analysis for the Expressions of PARG and MT1-MMP in Colorectal Carcinoma

Correlation analysis showed that the expression of PARG was correlated to the expression of MT1-MMP (r=0.216, P<0.05) in colorectal carcinoma (Table 3).

Effects of GLTN on the Expressions of PARG and MT1-MMP in Lovo Cells

Expressions of PARG and MT1-MMP were

determined in lovo cells by Western blot. It was showed that both PARG and MT1-MMP expressions were significantly decreased in GLTN-treated cells when compared with the GLTN-untreated cells (P<0.05) (Table 4, Figure 2).

Table 3. *The correlation between the expressions of PARG and MT1-MMP in colorectal adenocarcinoma*

		PARG	
		+	–
MT1-MMP	+	59	11
	–	12	7

Correlation analysis r=0.216, P<0.05.

Table 4. *Effects of GLTN on the expressions of PARG and MT1-MMP in lovo cells*

Group	PARG	MT1-MMP
Control	0.823±0.154	0.912±0.168
GLTN treated cells	0.325±0.086*	0.563±0.107*

*P<0.05, compared with control group.

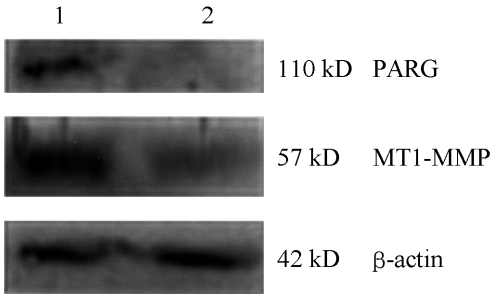

Figure 2. Expressions of PARG and MT1-MMP in GLTN-untreated and GLTN-treated lovo cells determined by western blot. The B-actin was used as an internal control. Lane 1: GLTN untreated group; Lane 2: GLTN treated group.

DISCUSSION

PARG is a 110-kDa protein expressed in mammalian cells in cytoplasm and nucleus. PARG cleaves the PAR polymers with high specificity at the glycosidic bonds, generating free ADP-ribose[13]. PARP is deactivated when binding to PAR, while PARG reactivates it and removes it to the cycling of PAR[1]. PARG has been suggested to be involved in DNA damage responses and DNA repair. It was reported that PARG-deficient mice were hypersensitive to alkylating agents and ionizing radiation, and these mice were susceptible to streptozotocin-induced diabetes and endotoxic shock[14]. Deletion of the PARG gene in mice revealed a severe phenotype which had increasing sensitivity to cytotoxicity and early embryonic lethality[15]. All of these are probably related to PARP. It has been shown that overexpression of PARP in many malignant tumors, e.g. lung carcinoma and hepatoma[16, 17], was responsible for hyperplasia[18]. However, it is still unknown whether PARG is responsible for the development and progression of the tumor. In this study, we detected the expression of PARG in normal colorectal mucosa, adenoma, adenoma with malignant transformation and adenocarcinoma. The results showed that the expressions of PARG were significantly higher in adenoma with malignant transformation and adenocarcinoma than those in normal colorectal mucosa and adenoma. The expression of PARG had correlations with depth of tumor invasion, lymphatic metastasis, distant metastasis and Duke's stage classification. It is suggested that PARG is probably related to the development, invasion and metastasis of colorectal carcinoma.

Membrane type 1-matrix metalloproteinase (MT1-MMP, also called as MMP14) is a well characterized transmembrane metalloprotease which has a central role in the MMP activation cascade. The up-regulation of MT1-MMP can result in the downstream activation of several MMPs, most notably the collagenases and gelatinases[19]. MT1-MMP can cleave collagens types I, II, and III, and can degrade a range of extracellular macromolecules including fibronectin, laminin 1 and 5, vitronectin, fibrin, and aggrecan. It also activates proMMP-2 and proMMP-13 on the cell surface and enhances pericellular matrix proteolysis[20]. MT1-MMP can also up-regulate the expression of vascular endothelial growth factor (VEGF). So MT1-MMP is a key enzyme in cancer cell invasion, metastasis and angiogenesis. It was discovered that MT1-MMP was expressed in most epithelial carcinomas, but not expressed in normal or impaired endothelial cells[21]. Our data showed that the expression of MT1-MMP was only detected in adenocarcinoma and adenoma with malignant transformation, but not in normal colorectal mucosa and adenoma. This corresponded with the literature[7]. The expression of MT1-MMP had positive correlations with the depth of invasion, lymphatic metastasis, distant metastases and Duke's staging. It is suggested that MT1-MMP also plays an important role in invasion and metastasis.
It has been reported that PARG can regulate PARP in inflammation, while PARP is associated with nuclear factor NF-κB at pro-inflammatory stage.

Automodification of PARP up-regulates the formation of the NF-κB·DNA complex and enhances the NF-κB dependent gene expression. PARP inhibitors affect NF-κB activation and gene expression[22]. Our previous studies showed that the expressions of PARG and PARP were significantly increased in colorectal carcinoma. PARG inhibitor (GLTN) suppressed the expressions of both PARG and PARP in colorectal carcinoma cells[6]. PARP inhibitor (5-AIQ) inhibited the formation of the NF-κB·DNA complex and decreased NF-k B activity, thus down-regulating the expressions of NF-κB dependent genes, including, MMP-2 and MMP-9 in colorectal carcinoma cells[9]. It has been reported that Methylseleninic acid (MSeA) could have an effect on MT1-MMP expression through the suppression of NF-κB activity. NF-κB is an important transcriptional factor of MT1-MMP expression. The promoter region of the MT1-MMP gene contains binding sites for NF-κB[23]. Our data showed that PARG expression had positive correlation with the expression of MT1-MMP in colorectal carcinoma tissues. We also treated lovo cells with an inhibitor of PARG (GLTN) and observed the expressions of PARG and MT1-MMP. The result suggested that the levels of both PARG and MT1-MMP in GLTN-treated lovo cells were lower than that in GLTN-untreated lovo cells. Therefore, we propose that PARG regulates PARP by hydrolysis, while PARP affects the expression of MT1-MMP by decreasing NF-κB activity. Nevertheless, further studies are needed.

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