Peroxiredoxins in colorectal neoplasms

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Summary. Peroxiredoxins (Prxs) are novel group proteins with efficient antioxidant capacity, and some of them also have effects on cell proliferation, differentiation, apoptosis, and chemotherapy and radiotherapy resistance. Altogether six distinct Prxs expressions were investigated in histological samples of colorectal neoplasm and the distant normal tissues and investigated associatedly with parameters such as clinical stage and lymphnodes metastasis. Normal colorectal tissues were almost negative for Prxs, except Prx4 (15/32). In colorectal cancer tissues, the most prominent reactivity was observed with Prx2 in 23/32 cases, while the corresponding figures for others was 21/32 (Prx1), 18/32 (Prx3, Prx5, Prx6) and 8/32 (Prx4). Prx1 (P=0.023), Prx2 (P=0.012), and Prx5 (P=0.028) were the isoforms that showed significantly increased expression in colorectal cancer patients with stage III or lymphnodes metastasis-positive cases. There was a significant relationship between the expression of Prx1 and Prx2 (rs=0.425, P=0.015) and between Prx3 and Prx4 (rs=0.364, P=0.041). Additionally, 8 cases were studied by western analysis. Prx1, 2, 3, 5 and 6 were particularly elevated in tumors compared to nonmalignant tissue as assessed by immunohisto-chemistry. It appeared that some Prxs were upexpression in colorectal cancer tissues and may have some prognostic significance; the induction of Prxs could be explained by increased production of reactive oxygen species in carcinomatous tissue.

Key words: Peroxiredoxin, Colorectal neoplasms, Reactive oxygen species, Antioxidant

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Introduction

Organisms living under aerobic conditions need to be protected against the damage caused by reactive oxygen species (ROS) such as hydroxyl radicals (OH), superoxide anions (O_2), and hydrogen peroxide (H_2O_2) (Roessner et al., 2008), which arise from either in pathological or physiological conditions. The most important sources of ROS are the electron transport chains of mitochondria and endoplasmic reticulum, because electron transport chains have the tendency to leak electrons to oxygen, resulting in ROS formation. It can be estimated that in the aerobic metabolism, 1-2% of total O_2 consumption could result in the production of ROS. This is the reason that certain amounts of ROS are constantly generated by aerobic organisms in physiological conditions (Roessner et al., 2008). In pathological conditions, both preneoplastic stages and cancer cells are associated with a large number of ROS production (Pelicano et al., 2004). The long-term presence of even a small amount of ROS is a risk to cells because they participate in a number of pathophysiologic processes, including protein damage, DNA damage and lipid peroxidation, and are considered to be a key factor in tumor development (Memon et al., 2005; Rhee et al., 2005). In addition, exposure to external agents such as light, ionizing radiation, some redox drugs or UV radiation, can also lead to ROS generation (Fridovich and Freeman, 1986; Sies, 1993; Jin et al., 1997).To counter these deleterious processes, cells use several protective systems that either repair the various types of damage or destroy the ROS (Rabilloud et al., 2002). The most important cellular protective mechanisms against ROS are antioxidant enzymes, like catalase, glutathione peroxidase, and peroxiredoxins (Prxs) (Memon et al., 2005; Rhee et al., 2005). More than a century after the discovery of catalase, our understanding of mammalian cell based antioxidant defenses was rapidly developing. Prxs were initially identified in yeast and subsequently

showed that Prxs are present in organisms from all kingdoms (Rhee et al., 2005). They constitute a family of antioxidant enzymes with no homology with conventional antioxidant proteins (Chae et al., 1993, 1994; Yim et al., 1994). They were first named thioredoxin peroxidases because they reduced H_2O_2 to water using thioredoxin as an intermediate electron donor. As it became clear that not all these proteins used thioredoxin, they were renamed peroxiredoxins (Chae et al., 1994).

All Prxs proteins contain a conserved cysteine (Cys) residue in the NH2-terminal portion of the molecule, and most contain an additional conserved Cys in the COOH-terminal region (Seo et al., 2000). A small number of Prxs proteins lack the COOH-terminal Cys (Kang et al., 1998). So six Prxs isoforms in mammalian cells can thus be divided into three subgroups as follows: designated 2-Cys subgroup include Prx1 through Prx4, share two conserved motifs centered on Cys residues (Rhee et al., 2005); Prx5, atypical 2-Cys subgroup, because its C-terminal cysteine is not in the conserved position (Knoops et al., 1999; Yamashita et al., 1999); Prx6, 1-Cys subgroup, conserves only the Cys nearer the NH2-terminus, which is the catalytic site (Shen and Nathan, 2002).

Prxs play a key role in several cellular functions, such as protein and lipid protection against oxidative injury, cell proliferation, differentiation and apoptosis (Sánchez-Font et al., 2003). Previous studies indicated that the expression of Prxs were different in certain cells and tissues, but not in others. And the cell type- and tissue-specific expression of Prxs would also contribute to their respective activities. To better understand the role of these proteins in human colorectal carcinomas, all six Prxs expressions were investigated in tumor and distant normal tissues by immunohistochemistry and western blotting. The results were also correlated with patient clinical and pathological date, such as stage and lymphnodes metastasis.

Materials and methods

Tissue specimens

Tumor tissue samples and distant normal tissues were obtained from patients with a pathological diagnosis of colorectal adenocarcinoma determined by two pathologists. Patients were operated in Department of Gastrointestinal Surgery, the First Affiliated Hospital, Chongqing Medical University during the period from March 2009 to May 2009. There were 32 colorectal carcinoma patients participating in the study. The patients' characteristics: male 13, female 19; age 38–75, average 61 years old; all moderately differentiated cancer; TNM staging: 20 cases in stage I–II and 12 cases in stage III according to CRC staging standard by International Union Against Cancer (UICC). No patients had undergone preoperative radiotherapy or

chemotherapy. The principal committee of the First Affiliated Hospital of Chongqing Medical University authorized this research.

Immunohistochemistry

Specimens were fixed in 4% paraformaldehyde and were processed through a series of increasing ethanol concentrations for paraffin embedding. 4 μm sections were obtained, then deparaffinized in xylene and rehydrated through descending ethanol series. Then they were immersed in 10 mM citric acid monohydrate (pH 6.0) for 10 min, boiled in a microwave oven at 850 W for 2 min and at 350 W for 8 min (Karihtala et al., 2003). After that, Rabbit anti-human primary polyclonal antibodies (Prx1, 3 and 5, Abcam; Prx 2, 4 and 6, Proteintech) were incubated on the slides overnight at 4°C in wet room, then with secondary antibody (goat anti-rabbit, Santa Cruz). The expressions of Prxs were visualized by Chromogen 3,3-diaminobenzidine immunolabeling. Finally, the sections were counterstained with hematoxylin.

Western blotting analysis

Altogether 8 tumor specimens and a corresponding nonmalignant sample of colorectal from distant areas of the same patient were selected (Table 1) for western analysis to assess the protein intensities of Prx1-6 in neoplastic and nonneoplastic colorectal tissue. The protein extracts were separated via sodium dodecyl sulfate-polyacdene gel electrophoresis and transferred onto nitrocellulose membranes. The membrane was then blocked with the primary polyclonal antibodies against Prx1–6 (as described previously) and ß-actin (rabbit anti-human, Santa Cruz) at 4°C overnight. After being washed with TBS containing 0.05% Tween 20 the membranes were incubated with secondary antibody (goat anti-rabbit, Santa Cruz) for 2 hours. They were visualized by chemiluminescence system according to the manufacturer's instruction.

Statistical analysis

SPSS 10.1 for Windows was used for statistical

Table 1. Clinical information on cases analyzed for western blotting.

number	sex	age	stage	lymphnodes metastasis
1	female	56	III	yes
2	male	73	III	yes
3	male	64	III	yes
4	female	72	II	no
5	female	39	III	yes
6	female	68	II	no
7	female	42	I	no
8	male	69	II	no

analysis. The significance of the associations was determined using Fisher's exact probability test, chi-square text and correlation analysis. Statistical significance was assumed when p<0.05.

Results

The expressions of Prxs were investigated in tumor tissues and distant normal colorectal tissues by using isoform-specific antibodies for better understand the role of them in human colorectal carcinomas. In our 32 tumor lesions, Immunohistochemistry indicated that Prx1 was present in 65.63% of cases (n=21), Prx3, Prx5 and Prx6 positive staining were seen in 56.25% of cases (n=18). The most intensive expression was shown by Prx2,

71.88% were positive (n=23). But the positive rate of Prx4 was only 25.00% (n=8) (Fig. 1) (Table 2).

Distant normal colorectal tissues showed weak or undetectable expression of Prxs compared with those of carcinomatous tissue, except Prx4. 46.88% were positive (n=15), which was a near-significant difference with tumor tissues (P=0.068) (Table 2).

Altogether, 8 tumor specimens and a corresponding nonmalignant sample of colorectal from distant areas of the same patient were selected for western analysis in order to assess the protein intensities of Prx1-6 in neoplastic and nonneoplastic colorectal tissue. In line with the immunohistochemistry, western blotting analysis revealed Prx1, 2, 3, 5 and 6 proteins to be significantly higher in carcinomas than in control colorectal tissues. Prx4 expression by western blotting analysis was slightly stronger in controls than in carcinomas (Fig. 2).

When analyzing Prxs with clinicopathological parameters, such as sex, age, clinical stage and lymphnodes metastasis, none had significant association with sex or age. Prx3, 4 and 6 were the Prxs isoforms that did not have significant association with clinical stage and lymphnodes metastasis. However, Prx1 (P=0.023), Prx2 (P=0.012), and Prx5 (P=0.028) showed significantly increased expression in colorectal cancer patients with stage III or lymphnodes metastasis-positive cases (Table 3). There was a significant relationship between the expression of Prx1 and Prx2 (rs=0.425,

Table 2. The expression of Prxs in colorectal neoplasms and distant normal tissues.

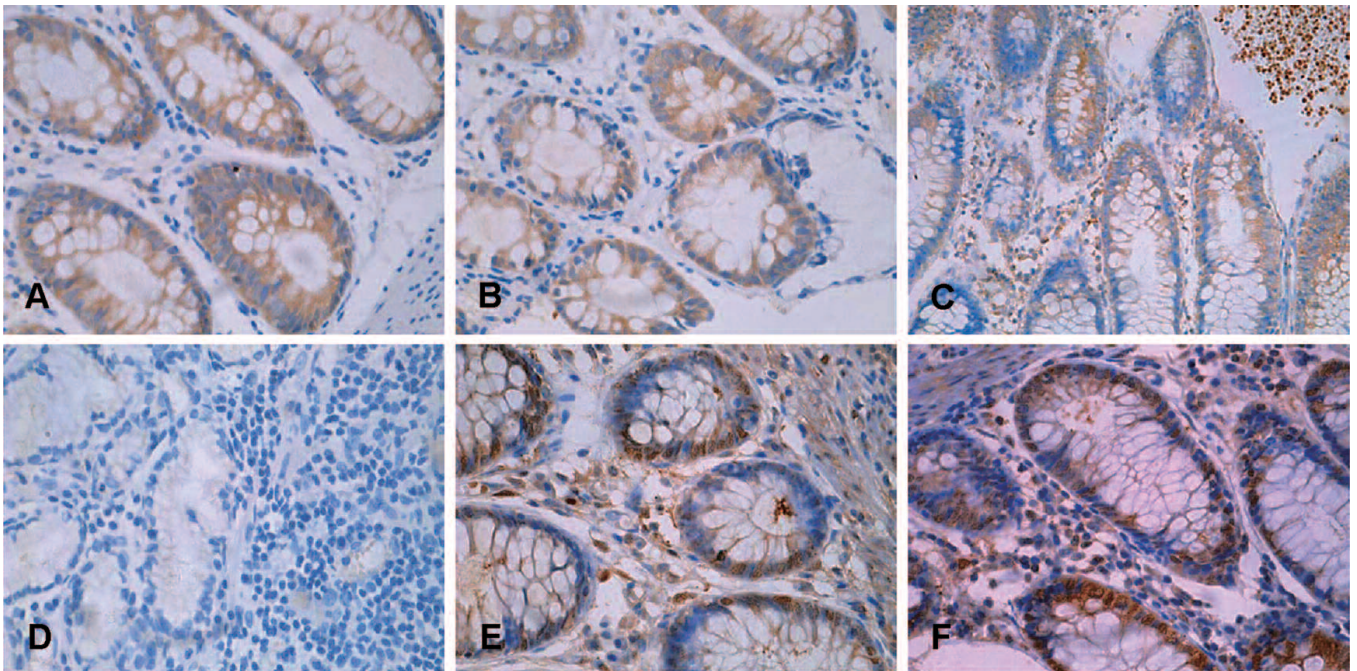|	normal tissues (N=32)		tumor tissues (N=32)		P
	positive	positive rate (%)	positive	positive rate (%)	
Prx1	5	15.63	21	65.63	<0.01
Prx2	7	21.88	23	71.88	<0.01
Prx3	3	9.38	18	56.25	<0.01
Prx4	15	46.88	8	25.00	=0.068
Prx5	3	9.38	18	56.25	<0.01
Prx6	4	12.50	18	56.25	<0.01

Fig. 1. Immunohistochemical staining of Prxs in human colorectal cancer tissues. Prx1 (**A**), Prx2 (**B**) and Prx3 (**C**) show strong cytoplasmic immunoreactivity. Immunostaining of Prx4 is weak (**D**). Nuclear and cytoplasmic staining of Prx5 and Prx6 are seen in **E** and **F**. x 200

Table 3. The correlation of Prxs expression with clinicalpathology in colorectal neoplasms.

		clinicopathological parameter					
		sex		age		stage (lymphnodes metastasis)	
		male	female	≤60	>60	I-II(no)	III(yes)
	N	13	19	14	18	20	12
Prx1	positive	8	13	10	11	10	11
	positive rate (%)	61.54	68.42	71.43	66.67	50.00	91.67
	P		0.721		0.712		0.023
Prx2	positive	10	13	12	11	11	12
	positive rate (%)	76.92	68.42	85.71	61.11	55.00	100.00
	P		0.704		0.235		0.012
Prx3	positive	8	10	7	11	12	6
	positive rate (%)	61.54	52.63	50.00	61.11	60.00	50.00
	P		0.725		0.721		0.718
Prx4	positive	4	4	4	4	5	3
	positive rate (%)	30.77	21.05	28.57	22.22	25.00	25.00
	P		0.684		0.703		1.000
Prx5	positive	7	11	9	9	8	10
	positive rate (%)	61.54	57.89	71.43	50.00	40.00	83.33
	P		1.000		0.490		0.028
Prx6	positive	8	10	7	11	10	8
	positive rate (%)	61.54	52.63	50.00	61.11	50.00	66.67
	P		0.725		0.721		0.471
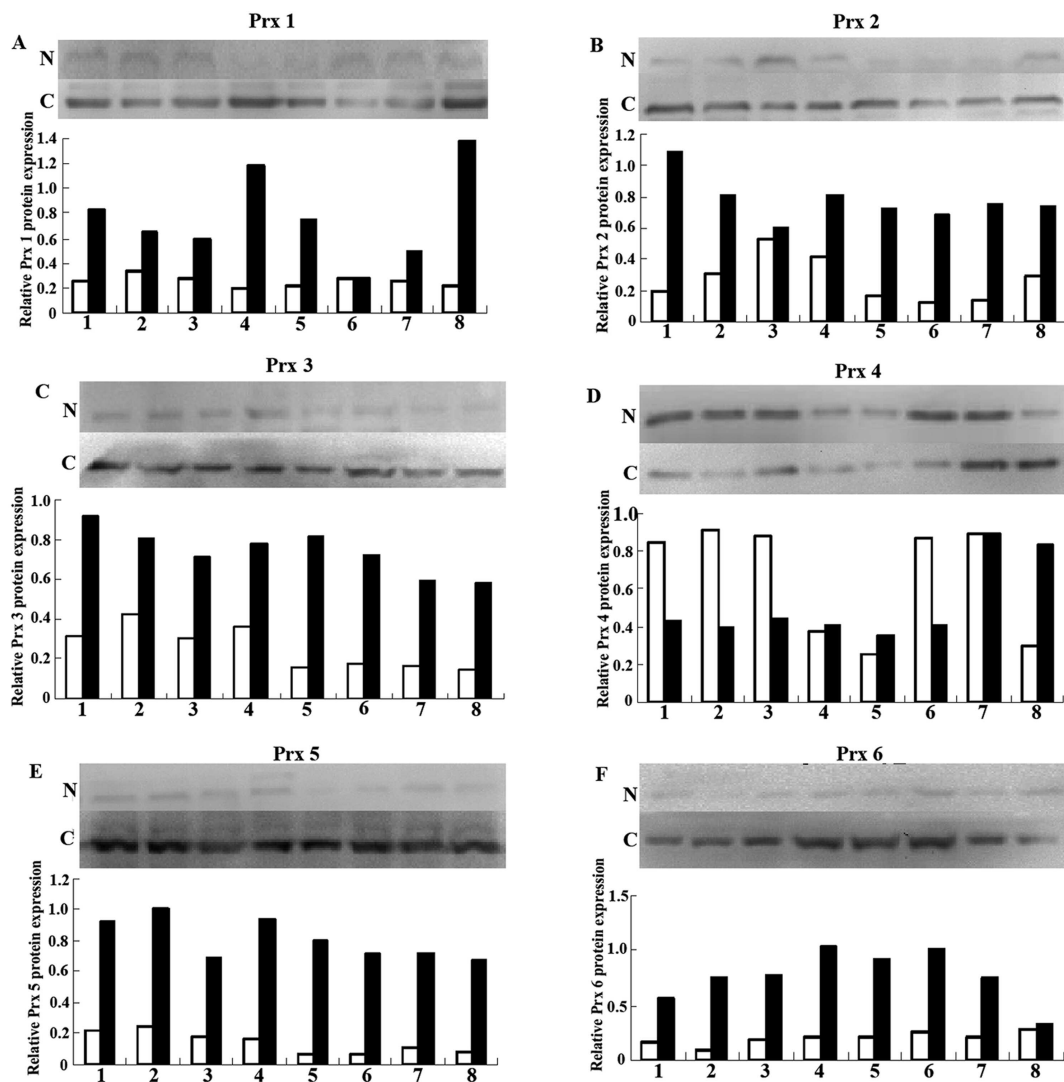
Fig. 2. Western blotting analysis of Prx1 (**A**), Prx2 (**B**), Prx3 (**C**), Prx4 (**D**), Prx5 (**E**) and Prx6 (**F**) expression in paired samples of adenocarcinoma (black columns) and distant nonmalignant colorectal tissues (white columns) of the same patient (n=8). (C- colorectal cancer tissues; N- distant normal colorectal tissues)

P=0.015) and between Prx3 and Prx4 (rs=0.364, P=0.041). No associations among other Prxs expression could be found.

Discussion

Over the past 30 years, some studies on Prxs in different types of cancer have been carried out. However, as far as we know, this is the first study in which the expression of all of the six Prxs isoforms has been compared with clinicopathological parameters in colorectal cancer. According to previous studies, Prx1 was elevated in many cancers, including esophageal (Qi et al., 2005), pancreatic (Shen et al., 2004), thyroid (Yanagawa et al., 1999), oral (Yanagawa et al., 2000) and lung cancers (Chang et al., 2001). Both Prx1 and Prx2 were elevated in head-and-neck cancers (Park et al., 2000). Prx3 was upexpressed in prostate cancer (Lin et al., 2007). In addition, Prx1, 2, 3, 5, and 6 levels were increased in malignant mesothelioma (Kinnula et al., 2002) and breast cancer (Karihtala et al., 2003). These studies were consistent with our findings that most Prxs appeared to be more expressed in carcinomas than in our control colorectal tissues. Prx4 was overexpressed in osteosarcoma (Liu et al., 2009), lung (Park et al., 2008), prostate (Lin et al., 2007) and breast cancer (Karihtala et al., 2003). But we found that the positive rate of Prx4 was 46.88% (15/32) in normal colorectal tissues while only 25.00% (8/32) in tumor tissues. It was consistent with the reports of Kinnula et al. in malignant mesothelioma (Kinnula et al., 2002) and Jang et al. in stomach cancer (Jang et al., 2004).

In previous studies, Prx1 could be used as a tumor marker and attempted to connect with TNM classification and pathological changes (Yanagawa et al., 1999, 2000; Karihtala et al., 2003). In this study, Prx1 showed significantly increased expression in colorectal cancer patients with stage III or lymphnodes metastasis-positive cases (P=0.023), which was in line with the study of Yanagawa et al. in oral squamous cell carcinoma (Yanagawa et al., 2000). In addition, we also found a correlation of Prx2 and Prx5 expression in colorectal tumor with clinicopathological features. In our study, there was a significant relationship between the expression of Prx1 and Prx2 (rs=0.425, P=0.015) and between Prx3 and Prx4 (rs=0.364, P=0.041). In breast cancer there was a significant relationship between the expression of Prx3 and Prx4 and between Prx3 and Prx6 (Karihtala et al., 2003); between Prx1 and Prx6 and between Prx3 and Prx6 expression in malignant mesothelioma (Kinnula et al., 2002). Although our material is not considerably larger and all the samples are moderately differentiated colorectal cancer, in comparison with the results of mesothelioma and breast cancer, Prxs are suggested to show the difference of cancer type-specific in the expression of different Prx subtypes (Karihtala et al., 2003).

It has been estimated that nearly 20% of global human cancers are caused by infective and inflammatory diseases (Parkin, 2006). For example, in gastro-enterologic organs, chronic inflammatory bowel disease leads to colorectal cancer (Ekbom et al., 1990), chronic pancreatitis leads to pancreatic cancer (Nair et al., 2006), and alcoholic steatohepatitis leads to hepatocellular carcinoma (Loguerico and Federico, 2003). The activated inflammatory cells in these conditions are responsible for the production of high concentrations of different ROS (Roessner et al., 2008). In addition, cells in preneoplastic stages and cancer cells are metabolically active and need a high level of ATP supply to maintain their high proliferation rates. The highenergy production in the mitochondrial respiration chains is associated with increased ROS production (Pelicano et al., 2004). The mammalian Prxs isoforms are located in various subcellular locations (Rhee et al., 2005), including endoplasmic reticulum and mitochondria, areas where oxidative stress is most evident. So overexpression of Prxs in tumor tissues could be explained by cells responding to impending oxidative stress caused by ROS.

Cells have multiple pathways to transduce extracellular signals into the nuclear compartment. Oxidants and antioxidants represent different sets of signaling molecules. The delicate interplay inside cells between oxidants and antioxidants ultimately determines the activity profile for many transcription factors (Jin et al., 1997). Large bodies of data clearly demonstrated that besides its well-accepted antioxidant role, Prxs play a key role in several cellular functions (Sánchez-Font et al., 2003). In this respect, elevation of Prx1 correlates with resistance to chemotherapy in breast cancer (Iwao-Koizumi et al., 2005). In contrast, down-regulation of Prx1 has been shown to sensitize lung cancer cells to radiation and reduce metastasis of lung cancer xenografts (Chen et al., 2006). Similarly, cultured cells overexpressing Prx2 were much more resistant to apoptosis caused by hydrogen peroxide, serum deprivation, etoposide and ceramide (Zhang et al., 1997). And down-regulation of Prx2 sensitizes head-and-neck cancer cells to radiation (Park et al., 2000) and gastric carcinoma cells to cisplatin (Yo er al., 2002). Prx1 and Prx2 overexpression also protect thyroid cells from H_2O_2-induced apoptosis (Kim et al., 2000). Furthermore, Prxs also had an important effect on cell proliferation and differentiation (Lee et al., 2000; Hess et al., 2003).

Most human cancers markedly overexpress Prxs compared to normal cells in the same surgical specimens. Inhibiting antioxidant defenses in tumor cells may be an effective method to enhance radiotherapy, immunotherapy, or chemotherapy. Earlier experiments have established that individual Prxs may protect from different stresses (Shen and Nathan, 2002). Additional investigations are needed to verify the expression of Prxs and understand the biological significance of the multiplicity of these defenses in colorectal cancer. If Prxs are functionally redundant, it might be necessary to inhibit all of them to sensitize the tumor cells to improve

the treatment outcome; if not, inhibition of just one might sensitize the cells to achieve the therapeutic effect (Shen and Nathan, 2002).

In conclusion, several members of the Prxs protein family are highly expressed in human colorectal carcinoma, with differences among the various Prxs, the tumor clinicopathological parameter and their potential effects on tumor progression.

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