JBUON 2021; 26(5): 1893-1897
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Impact of peroxiredoxin-6 expression on colon adenocarcinoma

Evangelos Falidas[1], Eirini Kitsiouli[2], Dimitrios Tsounis[3], Asimina Kalogirou[4], Evangelos Tsiambas[5], George Tsouvelas[6], Stylianos Papadopoulos[2], Michail Mitsis[7], Marilena Lekka[2], Konstantinos Vlachos[7]

[1]Department of Surgery, Halkida General Hospital, Halkida, Greece; [2]Laboratory of Biochemistry, Chemistry Department, University of Ioannina, Ioannina, Greece; [3]Department of Gastroenterology, 251 AF Hospital, Athens, Greece; [4]Department of Pathology, Halkida General Hospital, Halkida, Greece; [5]Department of Cytology, 417 VA (NIMTS) Hospital, Athens, Greece; [6]Department of Nursing, University of West Attica, Athens, Greece; [7]Department of Surgery, University Hospital of Ioannina, University of Ioannina, Ioannina, Greece

Summary

Purpose: *Peroxiredoxins (Prdxs) represent a family of proteins that act as antioxidant enzymes and are involved in a variety of metabolic functions including mainly the intracellular hydrogen peroxide (H_2O_2) levels reduction. Especially, Prdx-6 protein encoded by the PRDX6 gene (1q25.1) regulates also phospholipid modifications and induces response to oxidative stress and injuries. Our aim was to investigate the expression of Prdx-6 in colon adenocarcinoma (CA).*

Methods: *A series of 30 formalin-fixed, paraffin-embedded primary CAs tissue sections were used and analyzed. Immunohistochemistry was performed using an anti-Prdx-6 antibody. Digital image analysis was also implemented for evaluating objectively the protein expression levels on the corresponding stained cells.*

Results: *Prdx-6 protein overexpression (increased immunostaining levels) was observed in 12/30 (40%) cases, whereas 18/30 (60%) CA tissues demonstrated low to moderate protein levels, respectively. Prdx-6 overall expression was strongly associated with the stage of the examined tumors (p=0.011), whereas other statistical significances were not assessed (inflammatory infiltration: p=0.364; carcinoma location: p=0.93; differentiation grade: p=0.517; tumor diameter: p=0.983; ulceration: p=0.622).*

Conclusions: *Prdx-6 overexpression is observed in a significant subset of CAs correlating with aggressive biological behavior (advanced stage). Prdx-6 is a crucial enzyme for oxidative stress/injury endogenous cell response and should be an interesting agent as a biomarker and potential therapeutic target.*

Key words: *carcinoma, colon, peroxiredoxin, immunohistochemistry, oxidative stress*

Introduction

Among biochemical and metabolic deregulations that occur in the intra-cellular micro-environment during carcinogenetic process, elevated oxidative stress is a crucial event [1]. Interestingly, the role of aberrant H_2O_2 intra-cellular concentration is critical leading to enhanced oxidative stress motivating also redox signaling pathways [2,3]. Concerning colon adenocarcinoma (CA), it represents a well analyzed model of genomic instability. Extensive molecular analyses have shown that two main mechanisms of genetic deregulation are involved in the malignant transformation of normal colon glandular epithelia [4]. Gross chromosomal instability, as the result of chromosomal polysomy/aneuploidy, is detected in the majority (~85%) of CAs, whereas the rest demonstrate chromosomal

Corresponding author: Evangelos Tsiambas, MD, MSc, PhD. 17 Patriarchou Grigoriou E΄ Street, Ag. Paraskevi, 153 41 Athens, Greece.
Fax: +30 210 6526259; Email: tsiambasecyto@yahoo.gr
Received: 16/05/2021; Accepted: 07/06/2021

stability (diploid predominantly patterns). A subset of the chromosomally unstable cases are characterized by microsatellite instability (MSI) due to MLH1 gene promoter CpG island hypermethylation and also to specific mutations in other DNA mismatch repair (MMR) genes, including MSH2 and MSH6 that are involved in the cases of hereditary non-polyposis colorectal cancers (HNPCC) [5,6]. Finally, a small fraction of patients with CA is characterized by a complex genetic signature and molecular heterogeneity due to a combination of these mechanisms [7,8].

In the current study we explored the role of peroxiredoxin-6 (prdx-6) - a member of peroxiredoxin antioxidant enzymes family- protein expression patterns in a series of CAs.

Methods

Study group

For the purposes of our study, 30 archival, formalin-fixed and paraffin-embedded tissue specimens of histologically confirmed primary CAs were used. The hospital ethics committee consented to the use of these tissues in the Department of Pathology, Halkida General Hospital, Greece for research purposes, according to World Medical Association Declaration of Helsinki guidelines. The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for establishing the corresponding histopathological diagnoses. All lesions were classified according to the histological typing and staging criteria of World Health Organization (WHO) digestive system tumors classification [9]. Clinicopathological data of the examined cases are demonstrated in Table 1.

Antibodies and immunohistochemistry assay (IHC)

We selected and applied the rabbit monoclonal anti-prdx-6 (clone EPR3754, Abcam, USA; dilution 1:250) antibody. Immunohistochemistry (IHC) protocol for the antigen detection was carried out on a 3 μm-thick paraffin sections of the current blocks. Tissue sections initially deparaffinized in xylene and rehydrated via graded ethanol - were immunostained according to the EN Vision+ (DAKO, Denmark) assay using an automated staining system (I 6000 - Biogenex, CA, USA) and according to the corresponding antibodies manufacturer's

Table 1. Clinicopathological parameters and total Prdx-6 expression results

Clinicopathological parameters		*Prdx-6*		*p value*
CAs (n=30)	*n (%)*	*OE 12/30 (40%)*	*LE 18/30 (60%)*	
Gender				1.000
Male	15 (50)	6 (20)	9 (30)	
Female	15 (50)	6 (20)	9 (30)	
Grade				0.517
I	1 (3.3)	0 (0)	1 (3.3)	
II	22 (73.3)	10 (33)	12 (40)	
III	7 (23.3)	2 (6.7)	5 (16.7)	
Stage				0.011
I	3 (10)	0 (0)	3 (10)	
II	13 (43.3)	2 (6.7)	11 (36.7)	
III	10 (33.3)	7 (23.3)	3 (10)	
IV	4 (13.3)	3 (10)	1 (3.3)	
Anatomical location				0.930
Sigmoid	13 (43.3)	6 (20.3)	7 (6.7)	
Rectum	8 (13.3)	2 (13.3)	6 (20)	
Ascending-Caecum	9 (23.3)	4 (13.3)	5 (16.7)	
Inflammatory infiltration				0.364
Yes	25 (83.5)	9 (23.3)	16 (50)	
No	5 (16.7)	3 (10)	2 (6.7)	
Ulceration				0.622
Yes	5 (16.5)	1 (3.3)	4 (13.3)	
No	25 (83.5)	14 (46.7)	11 (36.7)	

CAs: colon adenocarcinomas, Prdx-6: peroxiredoxin 6, OE: overexpression (high/moderate expression) staining intensity values ≤ 130 at stained cells, LE: low expression staining intensity values >130 at ≤ 160 at stained cells

instructions. This specific assay is based on a soluble, dextran-polymer system preventing endogenous biotin reaction and increasing the quality of the stained slides. Briefly, the sections, after peroxidase blocking, were incubated with primary antibody for 30 min at room temperature and then incubated with Horseradish peroxidase labeled polymer-HRP LP for 30 min. A wash with TBS was performed. The antigen-antibody reaction was visualized using 3-3, diaminobenzidine tetrahydrocloride (DAB) as a chromogen substrate (8 min at room temperature). Finally, the tissue sections were slightly counterstained with hematoxylin for 30 sec, dehydrated and mounted. For negative control slides, the primary antibodies were omitted. Focal or diffuse cytoplasmic/sub-membranous staining pattern was considered to be acceptable for the marker. Normal liver epithelia tissue sections demonstrating prdx-6 expression were used as positive markers for its immunostaining pattern, according to antibody manufacturer's instructions (Figure 1a-d).

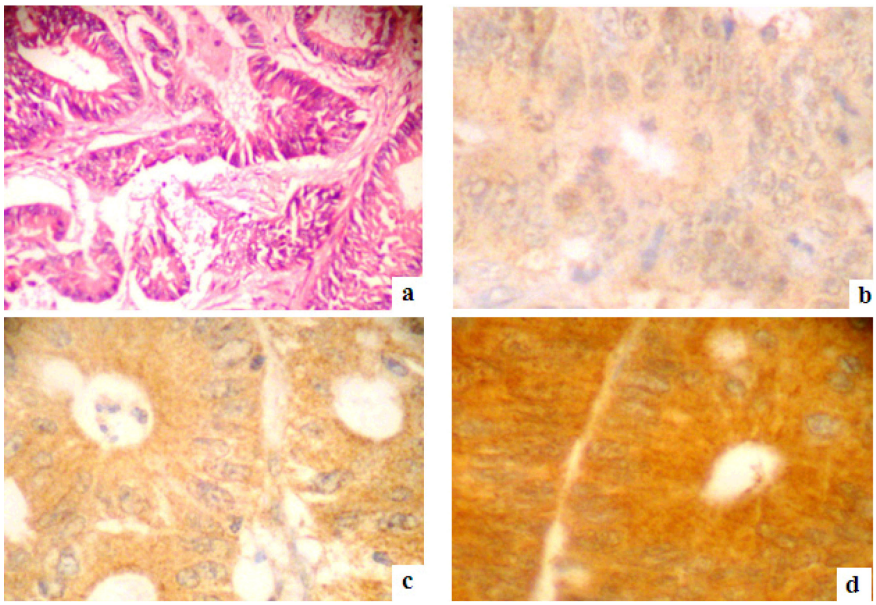

Figure 1. Peroxiredoxin-6 (prdx-6) protein expression in colon adenocarcinoma tissue sections. **a:** Colon adenocarcinoma histology (hematoxylin and eosin (H&E) stain, original magnification: 100x) **b-d:** Low expression level, Moderate expression level, High expression level, respectively (diffuse cytoplasmic staining pattern; anti- prdx-6, DAB stain, original magnification: 400×).

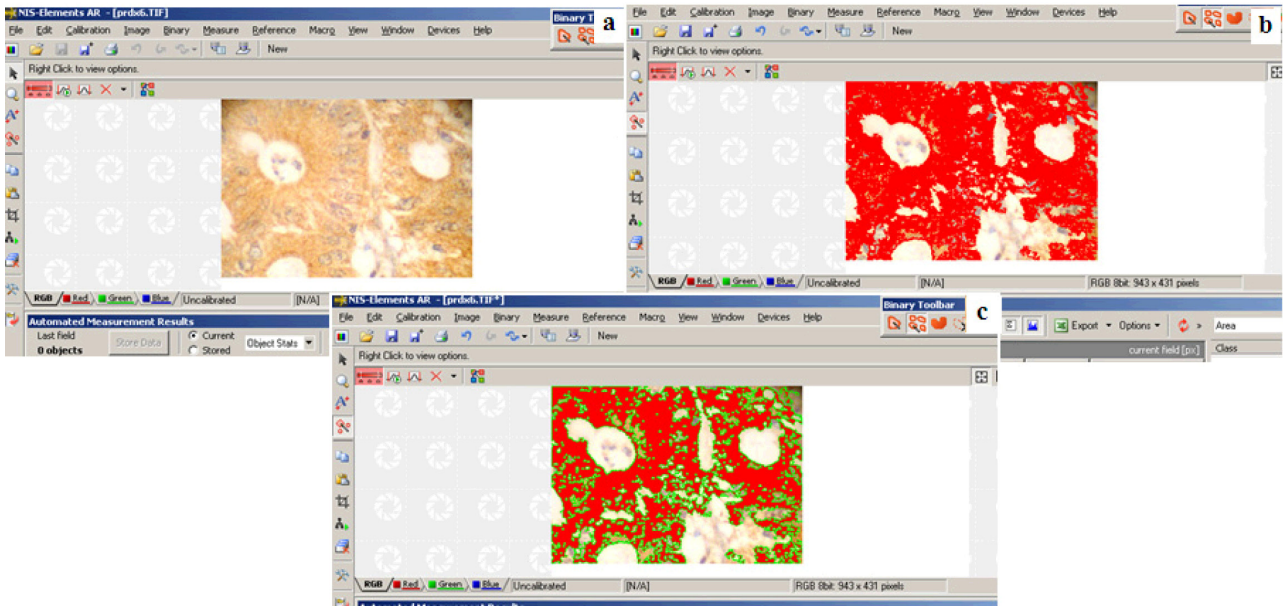

Figure 2. Peroxiredoxin-6 (prdx-6) protein digital image expression analysis in colon adenocarcinoma. **a-c:** Progressive measurements in three stages: red/green areas represent different levels of protein expression as staining intensity values (diffuse cytoplasmic staining pattern; anti- prdx-6, DAB stain, original magnification: 400×).

Digital image analysis assay (DIA)

Prdx-6 protein expression levels were evaluated quantitatively by estimating the corresponding staining intensity levels (densitometry evaluation) in the stained malignant cells. We performed DIA using a semi-automated system (hardware: Microscope CX-31, Olympus, Melville, NY, USA, Digital camera, Sony, Tokyo, Jp; Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Areas of interest per tissue section were identified (5 optical fields at ×400 magnification) and filed in a digital database as snapshots. Measurements were performed by implementing a specific macro (cytoplasmic and sub-membrane expression for tumor cells, according to manufacturer's datasheet). Based on an algorithm, normal tissue sections (control) were measured independently and compared to the corresponding values in malignant tissue sections. A broad spectrum of continuous grey scale values (0-255) at the RedGreenBlue (RGB) analysis was available for discriminating different protein expression levels (Figure 2). Immunostaining intensity values decreasing to 0 represent a progressive overexpression of the marker, whereas values increasing to 255 show a progressive loss of its staining intensity. Total results and DIA values are demonstrated in Table 1.

Statistics

For statistical analyses, descriptive and inferential techniques were applied. Quantitative variables were presented as mean ± standard deviation, while the qualitative variables were presented in frequency tables. To evaluate the relationship between qualitative and quantitative variables, because of the small number of subjects in each group, the non-parametric Mann-Whitney and Kruskall-Wallis tests were applied. To evaluate the relationship between independent qualitative variables, where appropriate, the control x^2 test for linear trend and the control of Fisher test were applied. Statistical significance (p) was evaluated in pairs and differences <0.05 were considered statistically significant. Total IHC results and differences (p values) are described in Table 1.

Results

According to digital expression analysis, the examined immunostained CA tissue sections demonstrated different expression levels of prdx-6. Prdx-6 protein overexpression (increased immunostaining levels) was observed in 12/30 (40%) cases, whereas the rest 18/30 (60%) cases demonstrated low to moderate protein levels, respectively. Prdx-6 overall expression was strongly associated to the stage of the examined tumors (p=0.011). Interestingly, a progressive overexpression was observed from stage III to stage IV cases. Concerning the influence of prdx-6 expression in the other clinicopathological parameters, statistical significances were not assessed (inflammatory infiltration: p=0.364; carcinoma location: p=0.93; differentiation grade: p=0.517; tumor diameter: p=0.983; ulceration: p=0.622; gender: p=1.000)

Discussion

Prdxs represent a family of proteins -acting as antioxidant enzymes- involved in a variety of metabolic functions and pathways, including mainly the intracellular hydrogen peroxide (H_2O_2) levels reduction [10,11]. They also demonstrate a phospholipase activity and generally they participate in regulating intracellular homeostatic signaling pathways involved also in gene expression [12,13]. Because Prdxs enhance the survival ability of cells under oxidative stress conditions by reducing H_2O_2, their deregulation affect indirectly cellular mechanisms, such as apoptosis [14]. Concerning interactions with other molecules, the tumor suppressor Mst1 is responsible for prdx-1 phosphorylation and inactivation [15]. Prdx-6 protein encoded by the *PRDX6* gene (1q25.1) regulates phospholipid modifications – especially phospholipase A2 activity- and induces response to oxidative stress and injuries and also cell migration [16].

In the current study we explored the role of prdx-6 expression in CA. We observed a significant impact of the molecule in the stage of the examined tumors. Similar IHC studies have shown an upregulation of the molecule correlated to increased metastatic potential (lymph node positivity) [17]. Additionally, prdxs' elevated mRNA levels demonstrate strong relation with advanced pathological features (grade) in other malignancies, such as breast adenocarcinoma [18]. For this reason, they should be considered important endogenous regulators of cancer cell metabolic activity. Interestingly, the implication of prdx-6 in regulating acute and chronic colitis has been also investigated. An experimental study measured the mRNA levels of the molecule in transgenic mice-based colitis models [19]. The authors concluded that prdx-6 low expression or loss of it led to decreased antioxidant enzyme activity and low expression of pro-inflammatory cytokines, whereas mRNA expression of other antioxidant enzymes in colon samples was significantly increased. Concerning the relation between dietary agents and colon cancerous and non-cancerous epithelia, two studies have reported the critical role of catecholic colonic metabolites and roasted coffee beans that enhance the endogenous antioxidant defense system by upregulating enzymes -including the prdx-6 - which strongly respond to oxidative damages [20,21].

In conclusion, prdx-6 overexpression is observed in a significant subset of CAs correlating with aggressive biological behavior (advanced

stage). Prdx-6 is a crucial enzyme for oxidative stress/injury endogenous cell response and should be an interesting agent as a biomarker and potential therapeutic target. Further protein and molecular studies based on the molecule's deregulation mechanisms in cancerous and non-cancerous colon epithelia could reveal unexplored intracellular pathways regarding cell responses to oxidative damages in a variety of carcinomas, providing also targeted treatment solutions (ie autoantibodies) [22].

Conflict of interests

The authors declare no conflict of interests.

References

1. Sies H. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: oxidative eustress. Redox Biol 2017;11:613-9.
2. Sies H. Role of metabolic H2O2 generation: redox signaling and oxidative stress. J Biol Chem 2014;289:8735-41.
3. Sies H, Jones DP. Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. Free Radic Biol Med 2012;53:1522-30.
4. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759-67.
5. Koketsu S, Watanable T, Tada T, Kanazawa T, Ueda E, Nagawa H. Sporadic colorectal cancer in elderly people. Hepatogastroenterology 2003;50:1749-52.
6. Tang R, Changchien CR, Wu MC et al. Colorectal cancer without high microsatellite instability and chromosomal instability-an alternative genetic pathway to human colorectal cancer. Carcinogenesis 2004;25:841-6.
7. Salk JJ, Fox EJ, Loeb LA. Mutational heterogeneity in human cancers: origin and consequences. Ann Rev Pathol 2010;5:51-75.
8. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell 2011;144:646-74.
9. Nagtegaal ID, Odze RD, Klimstra D et al. The 2019 WHO classification of tumours of the digestive system. Histopathology 2020;76:182-8.
10. Wood ZA, Poole LB, Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science 2003;300:650-3.
11. Wood ZA, Schröder E, Robin Harris J, Poole LB. Structure, mechanism and regulation of peroxiredoxins. Trends Biochem Sci 2003;28:32-40.
12. Hall A, Nelson K, Poole LB, Karplus PA. Structure-based insights into the catalytic power and conformational dexterity of peroxiredoxins. Antioxidants Redox Signal 2011;15:795-815.
13. Fomenko DE, Koc A, Agisheva N et al. Thiol peroxidases mediate specific genome-wide regulation of gene expression in response to hydrogen peroxide. Proc Natl Acad Sci USA 2011;108:2729-34.
14. Kim SY, Kim TJ, Lee KY. A novel function of peroxiredoxin 1 (Prx-1) in apoptosis signal-regulating kinase 1 (ASK1)-mediated signaling pathway. FEBS Lett 2008;582:1913-8.
15. Rawat SJ, Creasy CL, Peterson JR, Chernoff J. The tumor suppressor Mst1 promotes changes in the cellular redox state by phosphorylation and inactivation of peroxiredoxin-1 protein. J Biol Chem 2013;288:8762-71.
16. Kwon J, Wang A, Burke DJ et al. Peroxiredoxin 6 (Prdx6) supports NADPH oxidase1 (Nox1)-based superoxide generation and cell migration. Free Radic Biol Med 2016;96:99-115.
17. Huang WS, Huang CY, Hsieh MC et al. Expression of PRDX6 Correlates with Migration and Invasiveness of Colorectal Cancer Cells. Cell Physiol Biochem 2018;51:2616-30.
18. Cha MK, Suh KH, Kim IH. Overexpression of peroxiredoxin I and thioredoxin1 in human breast carcinoma. J Exp Clin Cancer Res 2009;28:93-7.
19. Melhem H, Spalinger MR, Cosin-Roger J et al. Prdx6 Deficiency Ameliorates DSS Colitis: Relevance of Compensatory Antioxidant Mechanisms. J Crohns Colitis 2017;11:871-84.
20. Morales AM, Mukai R, Murota K, Terao J. Inhibitory effect of catecholic colonic metabolites of rutin on fatty acid hydroperoxide and hemoglobin dependent lipid peroxidation in Caco-2 cells. J Clin Biochem Nutr 2018;63:175-80.
21. Yazheng L, Kitts DD. Activation of antioxidant response element (ARE)-dependent genes by roasted coffee extracts. Food Funct 2012;3:950-4.
22. Fujita Y, Nakanishi T, Hiramatsu M et al. Proteomics-based approach identifying autoantibody against peroxiredoxinVI as a novel serum marker in esophageal squamous cell carcinoma. Clin Cancer Res 2006;12:6415-20.