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RESEARCH ARTICLE

Prognostic implications of glutathione peroxidase2 (GPx2) and the stemness-associated marker SOX2 in colonic carcinoma

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ABSTRACT

Background: Glutathione peroxidases are major antioxidant molecules, involved in redox homeostasis in cancer cells and cancer stem cells (CSCs). Cancer stem cells are implicated in tumor progression, recurrence, and therapy resistance. **Aim:** This study aims to evaluate the prognostic value of glutathione peroxidase2 (GPx2) and the stem cell-related marker (SOX2) immunohistochemical expression in relation to survival in colonic carcinoma patients. **Patients and Methods:** GPx2 and SOX2 immunohistochemical expressions were assessed in 85 cases of stage II and III colonic carcinomas and their relation to the clinicopathologic parameters and patients' survival was evaluated. **Results:** High GPx2 expression was detected in 51.8% of cases, while only 25.9% of cases showed high SOX2 expression. GPx2 expression was significantly related to tumor grade, lymph node status, pathological tumor stage, lymphovascular invasion, as well as perineural infiltration. High SOX2 expression was significantly associated with tumor grade, lymph node invasion, pathological stage, and lymphovascular invasion. A significant relation was also detected between GPx2 and SOX2 expression. Both high GPx2 and high SOX2 were significantly related to poor overall survival (OS) and disease-free survival (DFS). **Conclusions:** High expression of GPx2 and SOX2 in colonic carcinoma is associated with features of aggressive tumor behavior, including poor tumor differentiation, lymph nodal status, advanced stage, lymphovascular invasion, and poor patients' survival. Additionally, the expression of GPx2 in colonic carcinoma was significantly related to SOX2 expression. These markers can be considered prognostic markers for colonic carcinoma.

Keywords: Cancer stem cells, Colon carcinoma, Oxidative stress

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INTRODUCTION

Colorectal carcinoma (CRC) ranks as the third most common cause of cancer-related death worldwide, after breast and lung carcinomas (Siegel *et al.,* 2021). Despite progress in diagnosis and therapy, recurrence and distant metastasis remain major problems in CRC patients, influencing long-term disease-free survival. About 15-30% of stage II colon carcinoma patients treated by colectomy and adjuvant chemotherapy suffer a recurrence within 5 years (Ribeirinho-Soar *et al.,* 2019). Hence, it is mandatory to highlight novel biomarkers implicated in the progression and recurrence of colonic carcinoma to improve therapeutic strategy and clinical outcome.

Reactive oxygen species (ROS) produced during cellular aerobic metabolism, play a complex role in the regulation of various cellular processes including cellular proliferation, differentiation, and survival. However, excessive accumulation of ROS is harmful due to its damaging effect on DNA, peroxidation, and alteration of cellular lipids and proteins. Cellular homeostasis depends mainly on a balance between oxidant and antioxidant mechanisms, which protects cells against the harmful effect of ROS (Zhao *et al.,* 2022).

Imbalance of the regulatory mechanisms triggers oxidative stress and results in pathological conditions including cancer, through prolonged DNA damage with further genomic instability and alteration of signal transduction pathways (Emmink *et al.,* 2014). Moreover, cancer cell survival and proliferation depend mainly on the signaling activity of modest levels of ROS. However, excessive accumulation of ROS

suppresses tumor growth and induces tumor cell apoptosis (Huang *et al.,* 2012). Therefore, malignant cells commonly upregulate defensive antioxidant scavenging mechanisms. Yet, redox homeostasis in cancer cells remains unclear.

Glutathione peroxidases represent a major part of the antioxidant cellular defense mechanisms. They catalyze hydrogen peroxide using glutathione as a substrate, thereby reducing intracellular oxidative stress. They are composed of 8 isozymes (GPx1-8), of which five members (GPx1-4 and 6) are selenocysteine (Sec)-containing proteins and three are cysteine-containing proteins. They are implicated in various cellular processes including proliferation, survival, apoptosis, chemoresistance, and immune response (Zhao *et al.,* 2022). Glutathione peroxidase 2 (GPx2) is a gastrointestinal tract–enriched antioxidant enzyme (Liu *et al.,* 2017).

Cancer stem cells (CSCs) constitute a small population of cancer cells with a slow proliferative rate and resistance to anticancer therapeutics including chemotherapy, radiotherapy, and targeted therapy. Cancer stem cells are implicated in cancer initiation, and they can be considered the main cause behind relapse and metastasis following cancer therapy, therefore investigating markers associated with CSCs is necessary (Takeda *et al.,* 2018). The role of oxidative stress and antioxidant enzymes in the maintenance of CSCs has been emphasized in previous research with inconsistent results (Shi *et al.,* 2012).

SOX2 is a member of the sex-determining region Y (SRY) related HMG-box (SOX) family of genes. It is a transcription factor that is implicated in the self-renewal and maintenance of stemness of embryonic and neuronal stem cells (SCs), reprogramming somatic cells into induced pluripotent stem cells (Mamun *et al.,* 2020 and Novak *et al.,* 2020). Moreover, SOX2 overexpression has been reported in several tumors conferring cancer stem cell characteristics. Furthermore, SOX2 plays a critical role in cancer cell proliferation, differentiation, and invasion (Lundberg *et al.,* 2014).

This study aimed to evaluate immunohistochemical expression of GPx2 and the stem cell-related marker (SOX2) in colonic carcinoma patients and their relation to clinicopathologic parameters and patients' survival. Moreover, we assessed the relation between both markers' expressions in the studied cases.

PATIENTS AND METHODS

The current prospective study included 85 cases of colonic carcinoma presented to the Oncology Department, Faculty of Medicine, Tanta University Hospitals during the period from July 2020 to February 2023. The study protocol was approved by the research ethics committee (approval code number: 36132) and the included patient signed an informed consent.

Inclusion and exclusion criteria

Patients fulfilling the following criteria were included in the study, age: > 18 years and < 75 years, Performance status: 0-2 according to Eastern cooperative oncology group (ECOG), histopathological proof of colonic carcinoma with American joint committee of Cancer (AJCC) Stage II or III, adequate hepatic function, renal function and proper blood picture count, no history of severe neuropathy, ventricular arrhythmia, congestive heart failure, myocardial infarction, or any co-morbidity. Patients with poor organ functions or a history of other malignancies were excluded from the study.

Patient evaluation, treatment plan, and follow-up

All patients underwent colectomy with en-bloc dissection of mesenteric lymph nodes followed by adjuvant chemotherapy. Patients received adjuvant chemotherapy for 6 months starting 4–6 weeks post-surgery with either FOLFOX (oxaliplatin 85 mg/m^2 on day1, leucovorin 400 mg/m^2 on the day1, both by intravenous infusion, fluorouracil 400 mg/m^2 on day1 by direct intravenous injection, and fluorouracil 2,400 mg/m^2 by continuous intravenous infusion for 46 hours, repeated every 2 weeks for a total of 12 cycles) or XELOX (oxaliplatin 130 mg/m2 day1 by intravenous infusion and oral capecitabine 1,000 mg/m2 day1–day14, repeated every 3 weeks for a total of 8 cycles).

Patients were evaluated by careful history taking and clinical examination aiming to assess performance status and detect signs suggesting distant metastasis. Complete blood count, liver

function tests, kidney function tests, fasting, and post-prandial blood sugar were done for all patients at initial presentation and before the start of every cycle of chemotherapy. Serum tumor markers including Carcinoembryonic Antigen (CEA) and Cancer Antigen 19.9 (CA19.9) were assessed following colectomy. Moreover, patients were subjected to chest, abdomen, and pelvis Computed Tomography (CT) with contrast post-surgery.

Follow up: After finishing adjuvant chemotherapy, patients were checked up regularly every 3 months with a detailed medical history, physical examination, CEA, CA19.9; radiological investigation, including*, chest, abdomen, and pelvic CT with contrast every 6-12 months*, and colonoscopy every one year after surgery. Progression was confirmed based on clinical presentation, laboratory and radiological investigations, and pathological examination if feasible.

Histopathological evaluation

Biopsies from included cases were subjected to routine Hematoxylin and Eosin (H&E) staining for histopathologic diagnosis and assessment of pathologic features including histopathological tumor subtype, grade of differentiation, depth of invasion, lymph nodal status, presence of lymphovascular invasion, and perineural infiltration. Cases were classified and graded according to 2019 5th edition World Health Organization (WHO) classification of colorectal tumors (Ahadi *et al.,* 2021). Tumors were staged according to the recommendation of the 8th edition of the American joint committee of Cancer (AJCC) staging system for colorectal tumors (Weiser *et al.,* 2018).

Immunohistochemical staining

The following primary antibodies were used for immunohistochemical staining of the studied cases: anti-GPx2 antibody, a mouse monoclonal IgG1 antibody (1:200; sc-133160; Santa Cruz Biotechnology Inc., Dallas, USA). Anti-SOX2 antibody, a mouse monoclonal IgG1 antibody (1:500; clone E-4, Santa Cruz Biotechnology, Dallas, USA).

Immunostaining was performed according to the protocol of DAKO automated immunostainer (Link-48). Briefly, deparaffinization of sections and antigen retrieval were performed in a Dako PT Link unit using high and low pH EnVision TM FLEX Target Retrieval Solutions at 97°C for 20 minutes. Then, the slides were incubated with the primary antibodies for 20–30 minutes, following treatment with a peroxidase-blocking reagent for 5 minutes with the subsequent addition of horseradish peroxidase reagent for 20 minutes and diaminobenzidine chromogen solution for 10 minutes. Hematoxylin was applied for counterstaining.

Interpretation of immunohistochemical staining

Assessment of GPx2 immunohistochemistry: GPx2 immunoreactivity was detected as positive cytoplasmic staining in the neoplastic cells. A semi-quantitative score was used to evaluate the staining by multiplying the percentage of stained neoplastic cells by the intensity of staining. The intensity was classified into mild, moderate, strong on a scale from (1 to 3). The percentage of staining was classified into; 0 (less than 10% staining), 1 (10-25% staining), 2 (25-50% staining), 3 (50-75 % staining) and 4 (more than 75% staining) (Murawaki *et al.,* 2008).

Assessment of SOX2 immunohistochemistry: SOX2 immunoreactivity was detected as nuclear staining in the neoplastic cells; cytoplasmic staining if present was ignored. The immunoreactivity was semi quantitatively classified, according to the percentage and intensity of stained tumor cells. Intensity scores were categorized into no (0), weak (1), moderate (2), and strong staining (3). Percentage of positive epithelial cells [0%–10% (1), 11%–30% (2), 31%–50% (3), and >50% (4)]. The final score was determined by multiplying the score for the percentage of positive cells (0-3) by the staining intensity (1-4) to attain a final maximum total score of 12 (Zamzam *et al.,* 2021). For statistical purposes, cases with scores less than 5 were classified as low expression, whilst scores equal to or more than 5 denoted high immunohistochemical expression (for both GPx2 and SOX2).

Statistical analysis

Data were analyzed using SPSS software package version 23.0. **(**Armonk, NY: IBM Corp**)**. Categorical

data were represented as numbers and percentages. The chi-square test was applied to investigate the association between the categorical variables. Alternatively, Monte Carlo correction was applied when more than 20% of the cells have an expected count less than 5. Continuous data were tested for normality by Shapiro-Wilk test. Quantitative data were expressed as a range (minimum and maximum) and median or mean, standard deviation. Student t-test was used to compare two groups for normally distributed quantitative variables. Kaplan Meier method was used to estimate overall and disease-free survival in correlation to GPx2 and SOX2 expression. Log-rank test compared survival curves. Cox regression model analysis was used to correlate survival data with clinicopathologic features. The significance of the obtained results was judged at the 5% level. Disease-free survival (DFS) is measured from the day of surgery until the date of documented disease progression or to the last follow-up. Overall survival (OS) is calculated from the day of surgery until death from any cause or to the date of the last follow-up.

RESULTS

Clinicopathologic characteristics

Eighty-five (85) cases of colonic carcinoma were included in the current study. Included histopathological subtypes were conventional adenocarcinoma (68.2%), mucinous carcinoma (17.6%), signet ring cell carcinoma (9.4%), and medullary carcinoma (4.7%). Fifty-six cases (65.9%) exhibited T3 depth of invasion, and 58.8% of cases showed positive nodal metastasis (N1 or N2); which were classified also as stage III tumors. Fifty-five cases were grade II carcinomas; representing (64.7%) of cases, and the remainder (35.3%) were grade III carcinomas. Perineural and lymphovascular were detected in 44.7% and 38.8% of cases, respectively. The clinicopathologic features of the studied cases are represented in (Table 1).

Immunohistochemical results

Relation of GPx2 immunoreactivity with clinicopathologic parameters of the studied cases

High expression of GPx2 was detected in 51.8% of cases (Figure 1). GPx2 immunohistochemical expression was significantly related to the lymph node stage (p=0.017). Also, GPx2 expression was significantly associated with pathological tumor stage (p=0.007) as 64% of stage III tumors exhibited high GPx2 expression, whereas 65.7% of stage II tumors showed low GPx2 expression. Moreover, a statistically significant relation was detected between high GPx2 expression and grade of differentiation, as 83.3% of grade III tumors showed high GPx2 expression. Furthermore, high expression of GPx2 was significantly related to lymphovascular invasion as well as perineural infiltration (p<0.001).

Table 1. Distribution of the studied cases according to clinicopathologic parameters (n = 85)

	Number (%)
Sex	
Male	41 (48.2%)
Female	44 (51.8%)
Age (years)	
Mean ± SD.	50.8 ± 15.3
Median (Min. – Max.)	52 (22 – 80)
Location	
Right sided	33 (38.5%)
Left sided	52 (61.2%)
Histopathological subtype	
Mucinous	15 (17.6%)
Adenocarcinoma	58 (68.2%)
Signet ring carcinoma	8 (9.4%)
Medullary carcinoma	4 (4.7%)
T (Depth of invasion)	
pT2	4 (4.7%)
pT3	56 (65.9%)
pT4	25 (29.4%)
N (Lymph node status)	
N0	35 (41.2%)
N1	26 (30.6%)
N2	24 (28.2%)
Stage	
II	35 (41.2%)
III	50 (58.8%)
Grade	
II	55 (64.7%)
III	30 (35.3%)
Perineural invasion	
Negative	47 (55.3%)
Positive	38 (44.7%)
Lymphovascular invasion	
Negative	52 (61.2%)
Positive	33 (38.8%)
GPx2 expression	
Low	41 (48.2%)
High	44 (51.8%)
SOX2 expression	
Low	63 (74.1%)
High	22 (25.9%)

SD: Standard deviation

On the other hand, GPx2 immune expression was not significantly related to patients' age, sex, location of the tumor, histopathologic tumor subtype, pT tumor stage (depth of invasion). The immunohistochemical expression of GPx2 in relation to clinicopathologic features is illustrated in Table 2.

Relation of SOX2 immunoreactivity with clinicopathologic features of the studied cases

High SOX2 expression was detected in 25.9% of cases (Figure 2). High SOX2 expression showed a statistically significant positive association as regard lymph node stage (p=0.002) and pathological stage (p=0.002) as 91.4% of stage II tumors exhibited low SOX2 expression. Moreover, SOX2 expression was significantly associated with histological tumor grade (P<0.001), and the presence of lymphovascular invasion (p=0.006).

No significant relation was detected between SOX2 expression and patients' age, sex, location of the tumor, histopathologic tumor subtype, pT tumor stage (depth of invasion) or perineural invasion. The immunohistochemical expression of SOX2 in relation to clinicopathologic features was illustrated in Table 2.

Relation between GPx2 and SOX2 immunohistochemical expressions in the studied colonic carcinoma cases

A statistically significant relation was detected between GPx2 and SOX2 immunohistochemical expression (p<0.001), as 86.4% of cases with high SOX2 immunoreactivity showed high GPx2 expression (Table 2).

Prognostic value of GPx2 and SOX2 expression in colonic carcinoma

The median follow-up period was 25 months (ranging from 13 to 30 months). At two years, recurrence or metastasis occurred in twenty-two (25.9%) patients. Out of the forty-four cases with high GPx2 expression, twenty patients (45.5%) developed relapse, while 2 patients (4.9%) of the low GPx2 group developed relapse. DFS was higher in patients with low GPx2 expression than those with high expression (94% Versus 56%) with a statistically significant distribution (P=0.003). Only one patient with low GPx2 expression died in comparison to fifteen patients with high GPx2 expression which was translated as significantly higher OS (P=0.012) in patients with low GPx2 expression in comparison to patients with high GPx2 expression (96% Versus 72%) as shown in Figure 3.

Out of the twenty -two cases who had high SOX2 expression, seventeen patients (77.3%) had recurrence or metastasis while 5 patients (22.7%) of the low SOX2 group developed progression. DFS was higher in patients with low SOX2 expression than those with high expression (94% Versus 48%) with a statistically significant distribution. Also, death occurred in sixteen patients (18.8%), of which eleven patients (68.75%) were in the high SOX2 group and five patients (31.25%) were in the low SOX2 group.

A statistically significant distribution of mortality was detected in favor of the low SOX2 group in comparison to the high SOX2 group (93% Versus 57%). Both OS and DFS rates were significantly higher in low SOX2 patients in comparison to high SOX2 patients (P=0.004, P=0.001, respectively) as shown in Figure 3.

Univariate and multivariate Cox regression analyses of factors predicting shorter OS and DFS

On performing univariate analysis of the prognostic markers in relation to OS, high SOX2 expression, high GPx2, high nodal stage, pathological tumor stage, high tumor grade, presence of lymphovascular or perineural invasion were found to be significantly associated with unfavorable prognosis. High SOX2 retained significance with poor OS on multivariate analysis (Table 3).

As regard the analysis of the prognostic parameters in relation to disease-free survival, high SOX2, high GPx2 and presence of perineural invasion were significantly related to shorter disease- free survival on univariate analysis. On performing multivariate analysis of these significant parameters, only high SOX2 was found to be statistically significant as illustrated in Table 4.

DISCUSSION

Oxidative stress is considered a major contributor to cellular as well as DNA damage, with subsequent development and progression of cancer. GPx2 is a double-edged weapon in carcinogenesis and cancer progression, and its role varies according to the stage of the disease. GPx2 can protect cells from the mutagenic effect of ROS through its antioxidant role, thus preventing carcinogenesis.

Table 2. Relation between GPx2 and SOX2 expressions with clinicopathologic parameters (n=85)

	GPx2 expression			SOX2 expression		
	Low (n = 41)	High (n = 44)	Test of Sig. (p)	Low (n = 63)	High (n = 22)	Test of Sig. (p)
Sex						
Male	22 (53.7%)	19 (46.3%)	χ^2=0.933	29 (70.7%)	12 (29.3%)	χ^2=0.473
Female	19 (43.2%)	25 (56.8%)	(p=0.344)	34 (77.3%)	10 (22.7%)	(p=0.491)
Age (years)						
Mean ± SD.	53.20 ± 13.33	48.59 ± 16.86	t=1.401	52.24 ± 14.09	46.73 ± 18.24	t=1.460
Median (Min. – Max.)	55 (28 – 79)	45 (22 – 80)	(p=0.165)	55 (25 – 80)	44.50 (22 – 77)	(p=0.148)
Location						
Right sided	15 (45.5%)	18 (54.5%)	χ^2=0.167	22 (66.7%)	11 (33.3%)	χ^2=1.561
Left sided	26 (50%)	26 (50%)	(p=0.683)	41 (78.8%)	11 (21.2%)	(p=0.212)
Histopathological subtype						
Mucinous carcinoma	7 (46.7%)	8 (53.3%)		11 (73.3%)	4 (26.7%)	
Adenocarcinoma	31 (53.4%)	27 (46.6%)	χ^2=4.485	46 (79.3%)	12 (20.7%)	χ^2=4.669
Signet ring carcinoma	3 (37.5%)	5 (62.5%)	(^{MC}p=0.216)	4 (50%)	4 (50%)	(^{MC}p=0.164)
Medullary carcinoma	0 (0%)	4 (100%)		2 (50%)	2 (50%)	
T (Depth of invasion)						
pT2	2 (50%)	2 (50%)	χ^2=2.233	4 (100%)	0 (0%)	χ^2=1.121
pT3	30 (53.6%)	26 (46.4%)	(^{MC}p=0.316)	40 (71.4%)	16 (28.6%)	(^{MC}p=0.625)
pT4	9 (36%)	16 (64%)		19 (76%)	6 (24%)	
N (Lymph node status)						
N0	23 (65.7%)	12 (34.3%)	χ^2=8.143*	32 (91.4%)	3 (8.6%)	χ^2=12.759*
N1	11 (42.3%)	15 (57.7%)	(p=0.017*)	19 (73.1%)	7 (26.9%)	(p=0.002*)
N2	7 (29.2%)	17 (70.8%)		12 (50%)	12 (50%)	
Stage						
II	23 (65.7%)	12 (34.3%)	χ^2=7.280*	32 (91.4%)	3 (8.6%)	χ^2=9.295*
III	18 (36%)	32 (64%)	(p=0.007*)	31 (62%)	19 (38%)	(p=0.002*)
Grade						
II	36 (65.5%)	19 (34.5%)	χ^2=18.505*	48 (87.3%)	7 (12.7%)	χ^2=14.058*
III	5 (16.7%)	25 (83.3%)	(p<0.001*)	15 (50%)	15 (50%)	(p<0.001*)
Perineural invasion						
Negative	35 (74.5%)	12 (25.5%)	χ^2=28.975*	38 (80.9%)	9 (19.1%)	χ^2=2.485
Positive	6 (15.8%)	32 (84.2%)	(p<0.001*)	25 (65.8%)	13 (34.2%)	(p=0.115)
Lymphovascular invasion						
Negative	34 (65.4%)	18 (34.6%)	χ^2=15.776*	44 (84.6%)	8 (15.4%)	χ^2=7.694*
Positive	7 (21.2%)	26 (78.8%)	(p<0.001*)	19 (57.6%)	14 (42.4%)	(p=0.006*)
SOX2 expression						
Low	38 (60.3%)	25 (39.7%)	χ^2=14.231*			
High	3 (13.6%)	19 (86.4%)	(p<0.001*)			

SD: Standard deviation, t: Student t-test, χ^2: Chi square test, MC: Monte Carlo, *: Statistically significant at p<0.05

Table 3. Univariate and multivariate COX regression analysis for the parameters affecting overall survival (OS)

OS	Univariate		Multivariate	
	HR (95% C.I)	P value	HR (95% C.I)	P value
Sex	2.008 (0.656 – 6.832)	0.203		
Age	1.875 (0.854-4.632)	0241		
Histopathological subtype	1.865 (0.741 – 5.127)	0.109		
Location	0.675 (0.219 – 2.079)	0.382		
T (Depth of invasion)	2.745 (0.439 – 7.452)	0.379		
N (Lymph node status)	3.512 (1.746 – 9.542)	0.017*	2.963 (0.952 – 4.965)	0.217
Stage	5.797 (1.217 – 7.614)	0.026*	1.759 (0.872 – 6.325)	0.137
Grade	3.501 (1.105 – 11.082)	0.032*	2.175 (0.365 – 4.754)	0.228
Perineural invasion	0.037 (0.005 – 0.301)	0.019*	0.421 (0.208 – 7.432)	0.389
Lymphovascular invasion	0.167 (0.048 – 0.582)	0.009*	0.224 (0.174 – 0.451)	0.198
GPx2 expression	8.667 (2.324 – 14.903)	0.010*	3.652 (0.721 – 8.749)	0.102
SOX2 expression	4.750 (3.968 – 5.829)	0.001*	2.965 (1.098 – 4.534)	0.017*

HR: Hazard ratio, C.I: Confidence interval, #: All variables with p<0.05 was included in the multivariate, *: Statistically significant at p<0.05

Table 4. Univariate and multivariate COX regression analysis for the parameters affecting disease-free survival (DFS)

DFS	Univariate		Multivariate	
	HR (95% C.I)	P value	HR (95% C.I)	P value
Sex	1.609 (0.595 – 4.351)	0.327		
Age	2.174 (0.689-5.421)	0.239		
Histopathological subtype	1.745 (0.31 – 3.854)	0.295		
Location	2.100 (0.773 – 5.707)	0.112		
T (depth of invasion)	1.425 (0.785 – 4.125)	0.239		
N (lymph node status)	0.745 (0.298 – 2.458)	0.452		
Stage	0.044 (0.006 – 1.523)	0.126		
Grade	0.167 (0.057 – 1.486)	0.218		
Perineural invasion	6.109 (1.979 – 8.891)	0.028*	2.754 (0.529 – 4.531)	0.203
Lymphovascular invasion	4.737 (0.651 – 9.589)	0.106		
GPx2 expression	0.067 (0.014 – 0.315)	0.017*	0.486 (0.095 – 1.743)	0.231
SOX2 expression	0.131 (0.056 – 0.276)	0.002*	0.298 (0.109 – 0.754)	0.043*

HR: Hazard ratio, C.I: Confidence interval, #: All variables with p<0.05 was included in the multivariate, *: Statistically significant at p<0.05

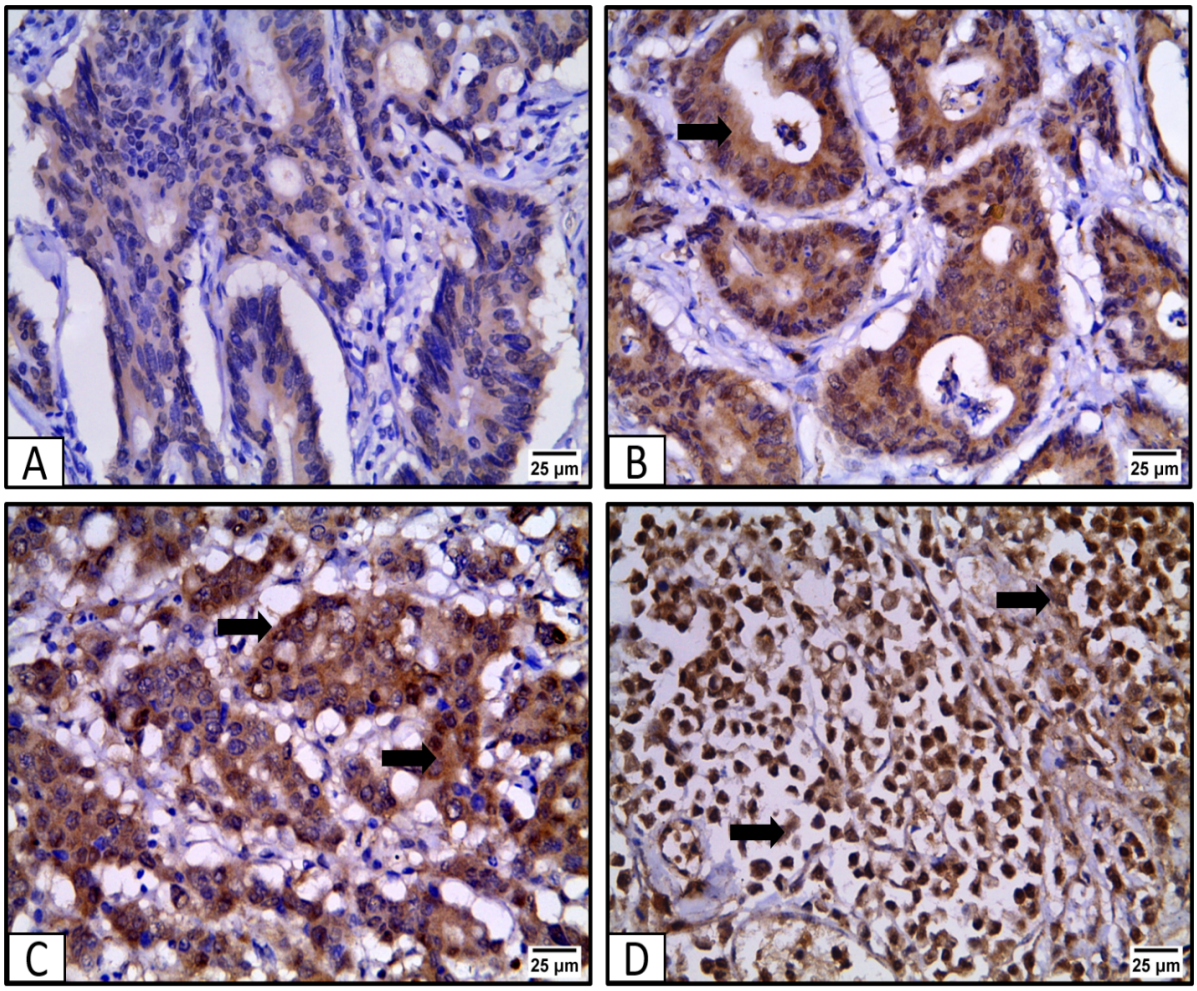

Figure 1. Immunohistochemical expression of GPx2 in colonic carcinoma cases: (A) a case of moderately differentiated adenocarcinoma (grade II) showing low score cytoplasmic expression for GPx2, (B) another case of moderately differentiated adenocarcinoma (grade II) showing high score cytoplasmic expression for GPx2, (C) a case of poorly differentiated adenocarcinoma (grade III) showing high score cytoplasmic expression for GPx2, (D) a case of signet ring cell carcinoma showing high score cytoplasmic expression for GPx2 (Original magnification x400, scale bar 25 um, cytoplasmic localization marked by black arrow).

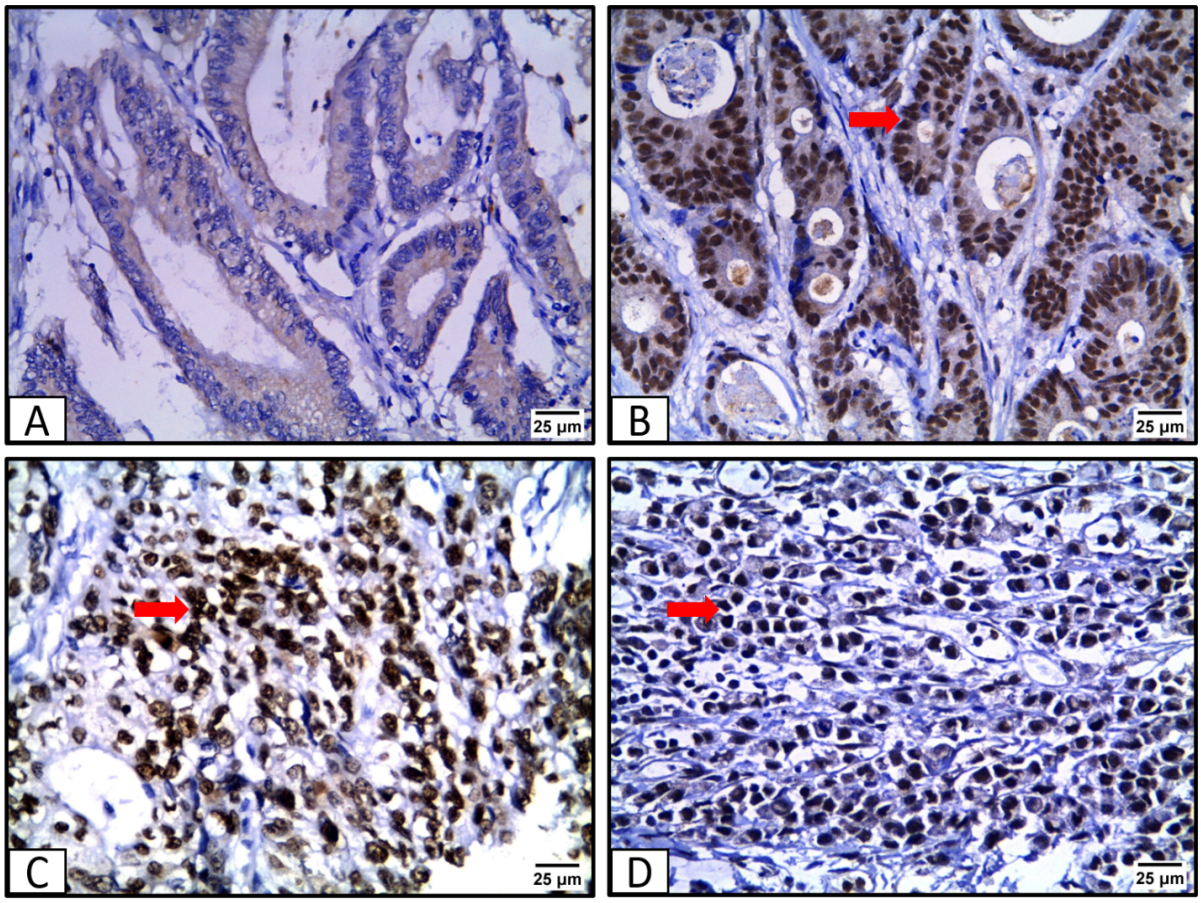

Figure 2. Immunohistochemical expression of SOX2 in colonic carcinoma cases: (A) a case of moderately differentiated (grade II) adenocarcinoma showing low score nuclear expression for SOX2, (B) another case of moderately differentiated adenocarcinoma (grade II) showing high score nuclear expression for SOX2, (C) a case of poorly differentiated adenocarcinoma (grade III) showing high score nuclear expression for SOX2, (D) a case of signet ring cell carcinoma showing high score nuclear expression for SOX2 (Original magnification x400, scale bar 25 um, nuclear localization marked by red arrow).

However, the same effect can preclude apoptotic elimination and add survival advantage to malignant cells, through the prevention of excessive accumulation of ROS (Yan and chen, 2006).

In the current work, high expression of GPx2 was detected in 51.8% of colonic carcinoma specimens. Murawaki *et al.* (2007) also demonstrated increased GPx2 expression in colorectal tumorous tissue; yet, with concomitant declining levels of other antioxidant molecules including GPx1 and GPx3. The mechanistic explanation of GPx2 in carcinogenesis is controversial. Muller *et al.* (2013) declared that the antiapoptotic function of GPx2 can be responsible for the maintenance of premalignant dysplastic foci and the development of colon cancer.

The loss of antiapoptotic effect in GPx2 knockout mice can be implicated in apoptotic elimination of premalignant epithelial lesions. On the other hand, GPx2 expression is claimed to minimize the number of tumors in the inflammation-induced colon cancer model suggesting anti-inflammatory function of GPx2.

In the present study, GPx2 expression was significantly related to poor tumor differentiation, advanced stage, the presence of nodal metastasis, perineural and lymphovascular invasion. Moreover, high GPx2 expression was associated with shorter OS and DFS. These results matched those of Liu *et al.* (2017), who studied GPx2 expression in gastric carcinoma and detected a correlation between GPx2 expression with aggressive diffuse and

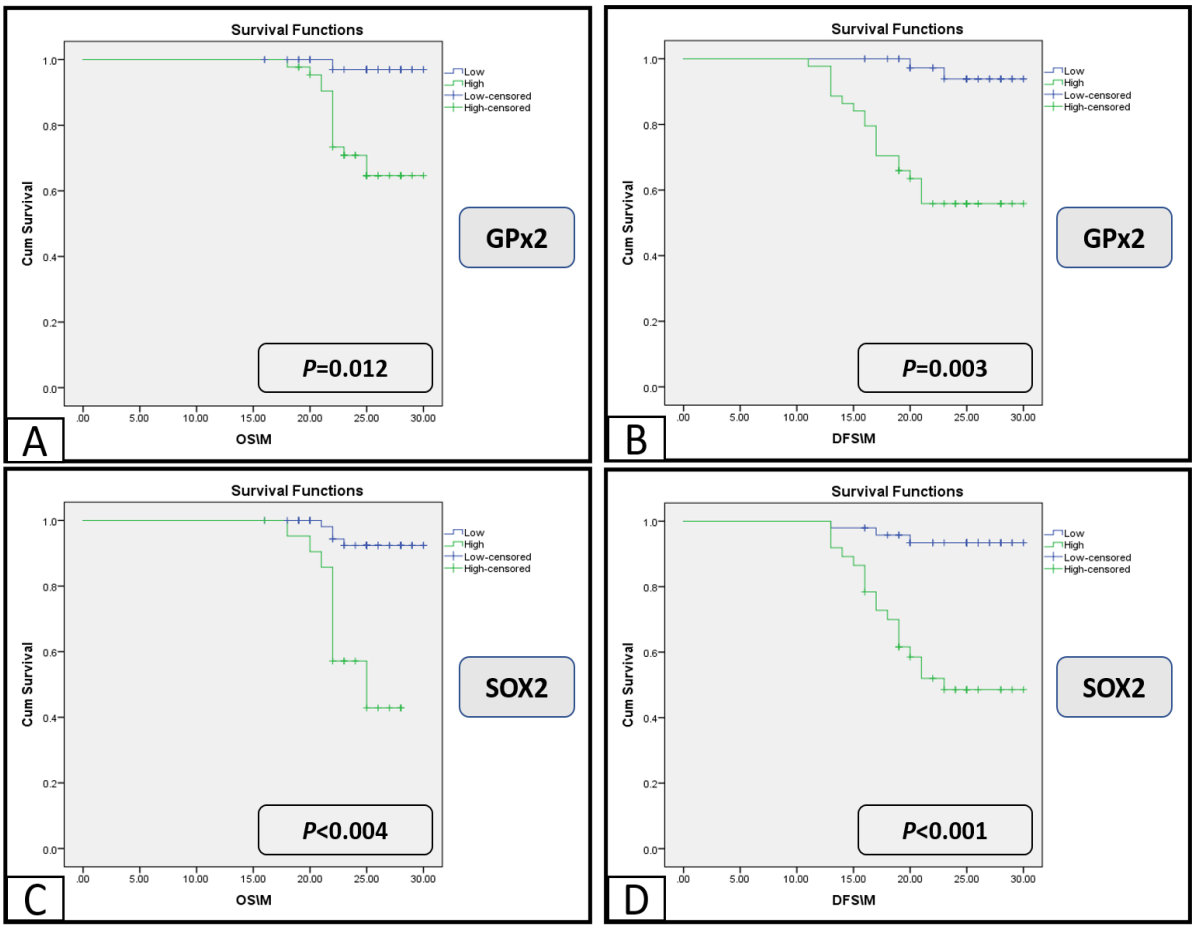

Figure 3. Kaplan–Meier survival curves for overall survival (OS) and disease-free survival (DFS) in relation to GPx2 and SOX2 immunohistochemical expression in colonic carcinoma patients: (A) Kaplan–Meier curves for OS in relation to GPx2 expression, (B) Kaplan–Meier curves for DFS in relation to GPx2 expression, (C) Kaplan–Meier curves for OS in relation to SOX2 expression, (D) Kaplan–Meier curves for DFS in relation to SOX2 expression.

signet ring histopathological patterns, Ki-67 labeling index, poor tumor differentiation, advanced nodal stage, and the presence of lymphovascular invasion. They also reported that patients with gastric cancer who had low GPx2 expression in their biopsies had significantly better OS than patients with high GPx2 expression levels, denoting that variation of GPx2 levels has a prognostic significance with respect to survival of gastric cancer patients. Additionally, Guo *et al.* (2021) documented that high GPx2 expression in glioblastoma was associated with poor patients' prognosis and shorter OS. Moreover, Emmink *et al.* (2014) found that high GPx2 expression in colorectal cancer was associated with early tumor recurrence and GPx2 silenced cell lines were more sensitized to chemotherapeutics, especially those associated with induction of high intracellular ROS levels, including Cisplatin.

On the other hand, low expression of GPx2 was associated with poor prognostic parameters and patients' survival in urothelial carcinoma of the upper and lower urinary tract (Chang *et al.,* 2015). Additionally, GPx2 expression significantly decreased in poorly differentiated esophageal squamous cell carcinoma, but wasn't related to clinical tumor stage or gross morphologic features, and loss of GPx2 expression was a predictive factor for shorter OS (Lei *et al.,* 2016).

Such controversial data highlights the hypothesis that GPx2 is differentially expressed in various tumors, with variable roles in cancer progression depending on interaction with several regulatory mechanisms. GPx2 is implicated in the regulation of several processes influencing cancer progression including proliferation, differentiation and invasion.

GPx2 gene has been reported to be up-regulated by β-catenin and ΔN isoform of p63, both inducing cellular proliferation (Muller *et al.,* 2013). It has been demonstrated that GPx2 silencing was associated with growth inhibition and decreased proliferative rate with a marked reduction of cyclin B1 and cell cycle arrest in castration-resistant prostate cancer cell lines (Naiki *et al.,* 2014).

Reports concerning the involvement of GPx2 in cancer metastasis are limited. Suzuki *et al.* (2013) demonstrated overexpression of GPx2 in aggressive highly metastatic hepatocellular carcinoma (HCC) cell lines compared with less metastatic lines. GPx2 silencing was associated with suppression of invasion and migration of HCC cell lines in vitro, and fewer metastatic tumor nodules in vivo with reduction of matrix metalloproteinase 9 (MMP9) including essential molecules in cancer metastasis.

Cancer stem cells (CSCs) have an important role in conferring aggressive tumor behaviors, such as chemoresistance, recurrence, and relapse, due to self-renewal and high metastatic capacity (Takada *et al.,* 2018). SOX2 is implicated in tumor initiation and progression through regulation of several pathways involved in cellular survival, proliferation, and differentiation with subsequent acquiring stem cell characteristics (Saigusa *et al.,* 2009). Moreover, SOX2 is a part of the transcriptional network including SOX2, OCT4 and NANOG which induce and maintain pluripotency in stem cells (Novak *et al.,* 2020). Lundberg *et al*. (2016) studied the role of SOX2 in the regulation of CSC characters in CRC cell lines. They demonstrated that SOX2-expressing cells exhibited a slow proliferative rate; with clongenic spheroid growth pattern and expression of stem cell markers (CD44 and CD24).

In the current work, high SOX2 expression was detected in 25.9% of cases. High SOX2 expression was significantly related to the presence of lymph node metastasis, advanced pathological stage, poor tumor differentiation and presence of lymphovascular invasion. Moreover, SOX2 expression was an independent prognostic factor for both OS and DFS. Our results are consistent with those of Lundberg *et al.* (2014), Miller *et al.* (2017) and Chen *et al.* (2020). Lundberg *et al.* (2014) reported SOX2 overexpression in liver metastasis from CRC, suggesting that SOX2 overexpressing cells are the initiating and metastasizing cells in metastatic colon cancer with poor prognosis in those patients. Nevertheless, the prognostic significance of SOX2 in their studied group was stage dependent.

On the other hand, in a study by Ribeirinho-Soar *et al.* (2019) on a cohort of stage II CRC, SOX2 expression was not related to patients' prognosis. However, the absence of SOX2, when combined with high MUC2 and CDX2 expressions, was associated with longer DFS. Cancer progression and prognosis depend not only on a single molecule but on several interconnecting factors. Moreover, Miller *et al.* (2020) showed that SOX2 expression was not related to patients' prognosis and all patients improved on 5 fluorouracil chemotherapy irrespective of SOX2 expression. This controversy can be explained by different study cohorts and different methods of evaluation for SOX2 expression.

SOX2 is implicated in several pathways involved in tumor progression, including epithelial mesenchymal transition (EMT), which may contribute to migration, invasion, and proliferation of cancer cells (Song *et al.,* 2020). Han *et al.* (2012) demonstrated that SOX2 knockdown in CRC cell lines induced mesenchymal-epithelial transition, presented by upregulation of E- cadherin and downregulation of vimentin, which reduced cell migration and matrix metalloproteinase (MMP2) levels. Moreover, SOX2 expression was associated with lymph nodes and liver metastasis in CRC patients.

By contrast, Lundberg *et al.* (2016) demonstrated that SOX2 overexpression in CRC cell lines was not associated with upregulation of the EMT transcription factors, nor with upregulation of the mesenchymal marker vimentin although it was associated with E-cadherin downregulation. The implication of SOX2 in the regulation of WNT/β-catenin signaling pathway can be another mechanism for tumor progression and spread. Han *et al.* (2012) demonstrated that SOX2 knockdown

reduced WNT pathway activity in CRC cells. Neumann *et al.* (2011) found that CRC cases with co-expression of SOX2 and nuclear β-catenin exhibited higher rates of lymph node and distant metastasis than cases expressing either marker alone.

Database analysis revealed that SOX2 target gene set was enriched in poorly differentiated high-grade tumors, and was associated with chemotherapy resistance and recurrence of CRC. In xenograft, SOX2 overexpressing tumors exhibited higher microvascular density with lymphovascular emboli (Zheng *et al.,* 2017). Chen *et al.* (2020) illustrated that SOX2 expression in CRC promotes angiogenesis and vasculogenic mimicry and SOX2 knockdown inhibited tumorigenesis and angiogenesis.

Lundberg *et al.* (2016) hypothesized that poor prognosis associated with SOX2 expression in colonic carcinoma can be partially related to CDX2 downregulation. Moreover, Takada *et al.* (2018) demonstrated that high SOX2 mRNA levels in CRC patients was associated with shorter relapse-free survival rates. Additionally, Tang *et al.* (2013) demonstrated that high expression of SOX2 was significantly associated with overall survival and SOX2 level was an independent prognostic factor in laryngeal cancer patients. However, low expression of SOX2 has been reported to be associated with poor prognosis in some tumors, including gastric carcinoma and pulmonary squamous cell carcinoma (Wuebben and Rizzino, 2017).

Cellular metabolism is implicated in the regulation of cancer stem cell characters. In the current work, GPx2 expression in colonic carcinoma patients was significantly related to the stemness-associated marker; SOX2. GPx2 is predominantly expressed in the base of intestinal crypts; an area critical for the self-renewal of the intestinal epithelium. GPx2 knockout mice exhibited shortened villi with altered undifferentiated cells throughout the gut; suggesting GPx2 role in the maintenance of undifferentiated stem cells in gastrointestinal tissue (Jiao *et al.,* 2017).

On the other hand, Emmink *et al.* (2014) showed that GPx2 silencing in colorectal cancer cell lines decreased clone forming capacity, however, this was associated with increased expression of stem cell markers, including Nanog and SOX2; and speculated that GPx2 suppressed cells form slow-growing tumors with a stem-like phenotype but lack metastatic capacity.

Cancer stem cells are programmed to maintain tight redox homeostasis, which can be achieved by the upregulation of ROS scavengers. Redox homeostasis seems to be important for the preservation of the tumor-seeding cells (Shi *et al.,* 2012). ROS is considered the most important mutagen in CSC and modest levels of ROS are essential for the enhancement of several pathways involved in the maintenance of self-renewal and stem cell characters.

However, prolonged exposure to high ROS levels can have a determined effect on CSCs with induction of apoptosis and blockage of stem cell characters. Redox balance is also involved in the development of resistance against cancer therapeutics. Given that some chemotherapeutics exert their anti-cancer effect through the elevation of intracellular ROS levels, one can speculate that CSCs upregulate ROS scavengers to promote survival and tolerance to anti-cancer therapeutics (Abdal Dayem, 2010).

Given the importance of redox homeostasis in cancer cells and cancer stem cells, exploration and targeting molecular mechanisms involved in redox regulation in cancer can be an effective strategy to control progression and chemotherapy resistance in aggressive colonic cancers. Additionally, targeting factors associated with stemness, including SOX2, seems to be a promising strategy to improve the prognosis of colonic carcinoma.

CONCLUSION

High expression of GPx2 and SOX2 in colonic carcinoma is associated with poor prognostic parameters including; poor tumor differentiation, lymph nodal status, advanced stage, lymphovascular infiltration and poor patients' OS and DFS. Additionally, the expression of GPx2 in colonic carcinoma was significantly related to SOX2. Such markers can be considered prognostic markers for colonic carcinoma.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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DATA AVAILABILITY

Data are available with corresponding author upon request.

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