Gene Expression of Survivin in Colorectal Cancer

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Abstract- Colorectal cancer (CRC) is the third most common cancer type in humans, the fourth most common cause of death annually. Survivin (known as SVV and BIRC5) is the smallest member of the inhibitor of apoptosis protein (IAP) family and it correlates with not only inhibition of apoptosis and a decreased rate of cell death, but also resistance to chemotherapy and tumor aggressiveness.The main goal of this study was to evaluate gene expression of survivin by RT-PCR as well as estimate the level of Oncostatin-M and antioxidants in the peripheral blood of patients with colorectal carcinoma and its relation to metastasis. **Results:** Our study shows high expression of survivin (p <0.001) and Oncostatin-M (p <0.001) in patients with colorectal cancer more than healthy persons. The present study revealed that CEA concentration was increased in colorectal carcinoma patients group compared to control group, p value was 0.010. Additionally, this study shows high elevation of antioxidants as catalase (p <0.001) , malondialdehyde (p <0.001) in patients group than control group. This proves that early detection of survivin and Oncostatin-M has important role in early screening of colorectal cancer.

Index Terms- survivin, colorectal cancer, antioxidants, oncostatin-m .

I. INTRODUCTION

Cancer is an abnormal growth of cells resulting from multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and eventually develops into a population of cells. These cells can invade tissues and metastasize to distant sites, causing significant morbidity and death, if untreated[1]**.** However, colorectal cancer incidence has remained relatively stable in the UK over the last decade[2]. In the past 2 decades, the overall morbidity and mortality of colorectal cancer are higher in city urban and in male as compared with rural district and female[3].

There are common mechanisms for colorectal cancer (CRC) formation. Several are connected with oxidative stress-induced cell apoptosis and others are related to imbalanced homeostasis or intake of drugs/toxins[4]. Survivin (known as SVV and BIRC5) is the smallest member of the Inhibitor of Apoptosis Protein (IAP) family, and it is about 16.5 kDa.

The protein comprises 142 amino acids organized in two domains: an N-terminal baculovirus-IAP repeat (BIR) domain, linked to a C-terminal α-helix. SVV presents during fetal development, but undetectable in terminally differentiated adult tissues[5]. The survivin gene locus encodes multiple genetic splice variants with unique properties and functions [6][7].

Survivin accumulates in the centromeres in G2 phase, and then it starts to diffuse to the chromosome arms and is abundant at the inner centromeres in prophase and metaphase of mitosis[8]. In anaphase, survivin re-localizes to the central spindle because it fails to associate with the centromeres[9]. Survivin secreted as a molecule with much expanded role in cellular homeostasis. It is involved in inhibition of apoptosis and regulation of cell proliferation and cell death. Survivin with aurora B and ICP forms a chromosomal passenger complex that bind to their target sites including centromere, midplate and cleavage furrow, where it regulates proper chromosome segregation and cytogenesis[10]**.** In addition,it correlates with not only inhibition of apoptosis and a decreased rate of cell death, but also resistance to chemotherapy and tumor aggressiveness [11].

Oncostatin M (OSM), an inflammatory cytokine belonging to the interleukin-6 (IL-6) superfamily, plays a vital role in multitude of physiological and pathological processes[12]**.** OSM is unique in this family as it can signal using heterodimers of gp130 and have a unique helical loop between its B and C helices that is not found on other IL-6 family cytokines [13]**.** It plays an important role in various biologic actions, including cell growth, neuronal development, and inflammatory responses. OSM decreased fatty acid content in the hepatocytes through expression regulation of several key enzymes of hepatic lipid metabolism which make OSM a novel therapeutic target for metabolic syndrome[14].OSM can directly or indirectly participate in tumorigenesis and insulin resistance development[15]. Recently, OSM has been emerged as a potent driver of tumorigenesis, metastasis, and therapy failure[16].

Under normal conditions, a balance between both the activities and the intracellular levels of these antioxidants is essential for the survival of organisms and their health. Antioxidant capacity is derived both exogenously (from food, beverage and sunlight) and endogenously (from enzymatic and non-enzymatic pathways)[17].SOD is known to catalyse the dismutation of superoxide to hydrogen peroxide and oxygen and has a central role in the defense against oxidative stress[18].

Human catalase is a heme-containing peroxisomal enzyme. It breaks down hydrogen peroxide to water and oxygen. Catalase is implicated in ethanol metabolism, inflammation, apoptosis, aging and cancer [19]. Catalase is a key enzyme which its expression and localization is markedly altered in cancer[20]. In colorectal carcinogenesis significant increase in the level of lipid

peroxidation products like malondialdehyde (MDA) was estimated[21] .

II.   PATIENTS AND METHODS

Patients

The study was carried out on 50 pre – treatment colorectal cancer patients of both sexes attending to Mansoura University Oncology Center (MUOC), Mansoura, Egypt, their mean of age was 53.8 years with SD ±13.9 and 50 apparently healthy age & sex matched controls, their mean of age was 52.8 years with SD ±5.8 . To confirm the presence of colorectal cancer (CRC), samples were screened morphologically and pathologically. Colorectal cancer patients who received medical treatments, radiotherapy or chemotherapy are excluded.

Blood collection

Morning blood samples were collected from the study participants to be estimated after applying all the precautions. By using a sterile plastic syringe for each patient, 5 ml of venous blood were withdrawn and divided into 1ml that was put in a tube containing EDTA for survivin and 4ml were put in a clean and dry test tube then allowed to centrifuge to separate serum for estimation of Oncostatin-M (OSM), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and glutathione(GSH).

III.   DETECTION OF SURVIVIN BY REAL TIME PCR

Total RNA was extracted from venous blood by using Thermo Scientific GeneJet RNA Purification utilizing the method of **Chomczynski and Sacchi** [22], **and Boom et al**.[23] . The extracted RNA was transcript to cDNA by SensiFAST™ cDNA Synthesis Kit (Bioline USA Inc., USA) based on the instructions of manufacturer. We used the housekeeping gene (GADPH) to normalize mRNA concentrations. Quantitative PCR was to compare expression levels of survivin transcripts. The relative expression levels of reference samples and survivin in the blood samples were determined by quantitative PCR using real time RT-PCR analyses (The Applied Biosystems™ StepOne . RT-PCR were performed using a sensi Fast™ SYBR NO-ROX kit (Bioline USA Inc., USA) in a final volume of 20 ml. An initial PCR activation step was 2 min at 95°C followed by 35 cycles of 5 s at 95°C , 20 s at 61°C, 10 s at 72°C and 5-10 min at 72°C. The sequence of primers used in real-time PCR was designed according to kit of Biosearch Technologies Custom Oligonucleotide Synthesis. The sequence of primers used in real-time PCR are shown in **Table (1).**

Full Name of the gene	Sequences (5′_3′)	Reference number
(GADPH)	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTC	SS368309-13 SS367846-54
Survivin	F: AGAACTGGCCCTTCTTGGAGG R:CTTTTTATGTTCCTCTATGGGGTC	SS367846-52 SS367846-51

Table (1). It was used to reverse transcribe and expand the RNA template for 35 cycles so finally CT was estimated.

Assessment of the biochemical parameters

Quantitative determination of Oncostatin M (OSM) was done by using Abcam's Oncostatin–M Human ELISA Kit based on **Croci DO et al** [24], Ltd. ,USA based on the manufacturer's instructions. Determination of serum catalase activity was done by the method of **Aebi [25]** and **Fossati et al [26]** by using a commercially available kit (Biodiagnostic, Dokki, Giza, Egypt), by adding 0.5 ml of reagent 1 (chromogen buffer) to 50 μl of serum sample then 100μl of reagent 2 (H_2O_2). Then adding 0.2 ml of reagent 3 (catalase-inhibitor) and 0.5 ml of reagent 4 (peroxidase enzyme). Measure the pink colour that appeared at 510 nm.

Determination of blood reduced glutathione content was done by the method of **Beutler et al [27]** using a commercially available kit (Biodiagnostic, Dokki, Giza, Egypt), by adding 0.5 ml of reagent 1 (trichloroacetic acid) to 0.1 ml of blood sample, then centrifuge. Then adding 1.0 ml of reagent 2 (buffer) to 0.5 ml of supernatant .After that, add 0.1 ml of reagent 3 (5,5′-dithiobis(2-nitrobenzoic acid)). Measure the colour at 405 nm.

Malondialdehyde was determined by the method of **Ohkawa et al [28]**by using Thiobarbituric acid (TBA) by adding 1 ml of it to 0.2 ml of serum then heat in boiling water bath for 30 minutes, cool and read the absorbance of the pink product at 543nm. Superoxide dismutase (SOD) was determined by using **Nishikimi et al [29]**method which relies on the ability of the enzyme to inhibit the phenazinemethosulfate (PMS)- mediated reduction of nitroblue tetrazolium dye.

IV.   STATISTICAL ANALYSIS

Data obtained from the present study were analysis using SPSS versions 20. Continuous data were expressed in the form of mean ± SD while categorical data were expressed in the form of number and percent. Student t test, ANOVA and regression analysis by utilizing univariate and multivariate were applied for continuous data. Whereas, categorical data were done using Chi-square test.

V.   RESULTS

Our study shows significant differences in patients group compared to control group according to carcinoembryonic

antigen (CEA), catalase, superoxide dismutase (SOD), malondialdehyde (MDA), and Oncostatin M (OSM) concentration. Whereas, glutathione concentration (GSH) shows no significant difference between patients group compared with control groups.

Table (2). Comparison of some laboratory data between CRC patients and control groups.

Variant	Control group (N=50)	Patients group N=50
CEA(ng/ml) Mean±SD *P*	2.5±0.8	3.3±1.1 0.010*
SOD (U/ml) Mean±SD *P*	332.4±90.8	54.9±15.3 <0.001**
Catalase (U/L) Mean±SD *P*	288.7±86.2	539.2±149.7 <0.001**
GSH(mg/dl) Mean±SD *P*	1.2±0.4	0.9±0.3 0.190
MDA(nmol/ml) Mean±SD *P*	2.4±0.6	9.4±3.1 <0.001**
OSM (pg/ml) Mean±SD *P*	4±1.1	11.5±3.3 <0.001**

P <0.001, highly significant. **CEA: Carcinoembryonic antigen & **SOD**: Superoxide dismutase **GSH**: Glutathione & **MDA**: Malondialdehyde & **OSM**: Oncostatin-M

Table (3) shows that mean survivin expression was 1.5. Cases showed significantly higher survivin concentration (upregulation) when compared to control group**.**

		Control N=50		Cases N=50	*P*
Survivin expression	Mean±SD	1	0	1.5±0.5	<0.001[T***]
	Median (min-max)	1	(1-1)	1.2 (0.9-3.3)	

SD: stander deviation, T:Student T test, Survivin expression is expressed as mean±SD.
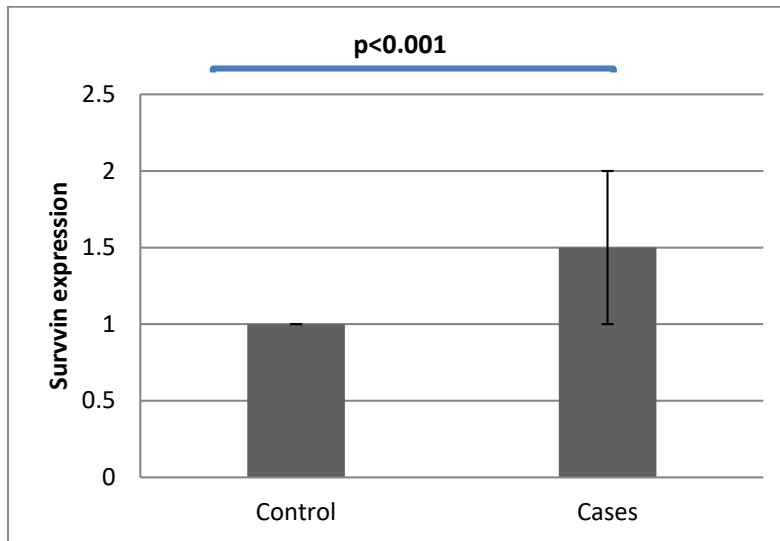**Table(3)** Comparison of survivin expression between CRC patients and control groups.

Fig.1 clarifies survivin concentration. It was significantly higher in patients group compared with control group, P <0.001.

The patients group was divided according to the location of cancer into 3 groups: colon cancer (25 patients), colorectal cancer (14 patients), and rectum cancer (11 patients). **Fig.(2)** reveals that there were no significant differences in survivin expression between colon cancer subgroup, *p* value was 0.6
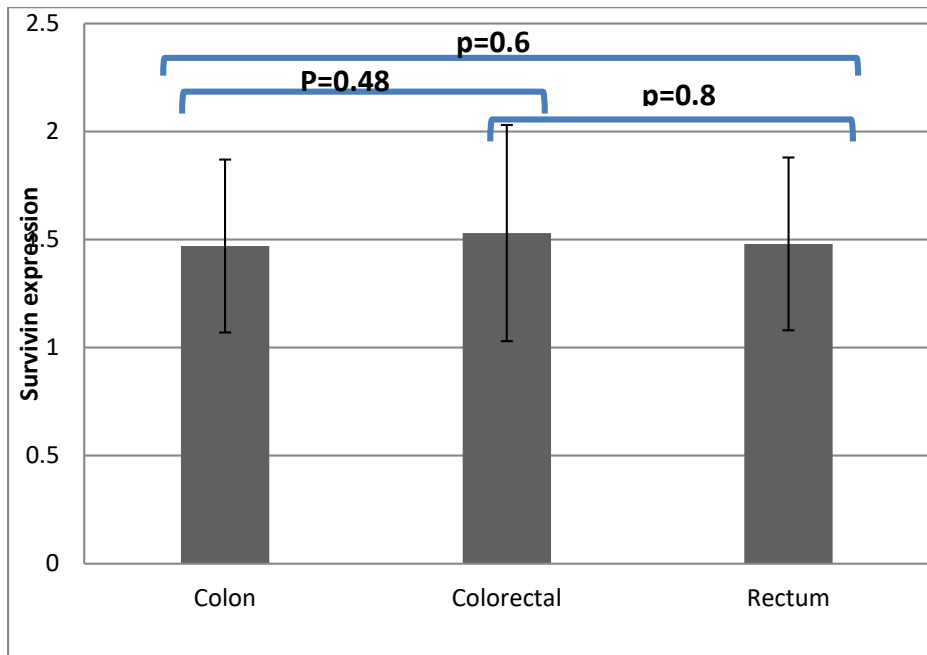

Fig.(2) Survivin espression in colon cancer, colorectal cancer and rectal cancer patients.

MDA concentration showed significant positive correlation with OSM concentration and negative correlation with survivin expression as shown in **Fig(3)** and **Fig(4).**
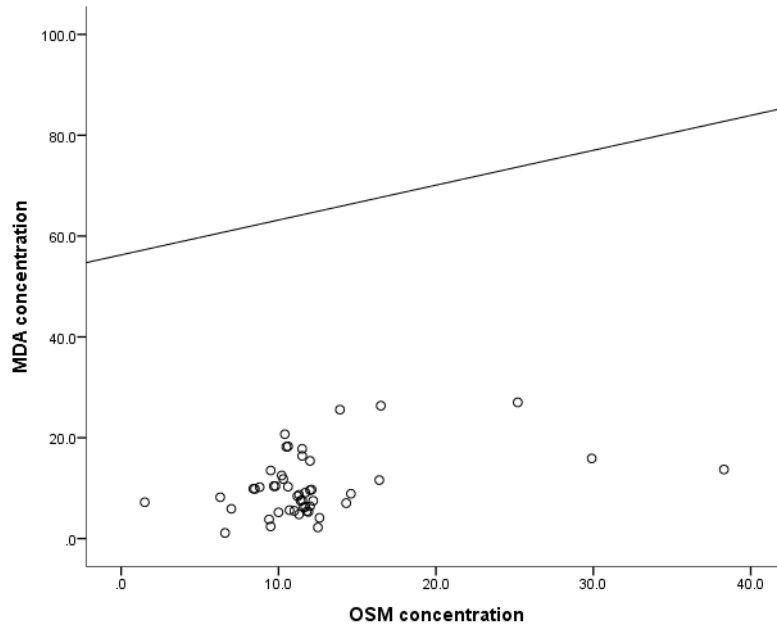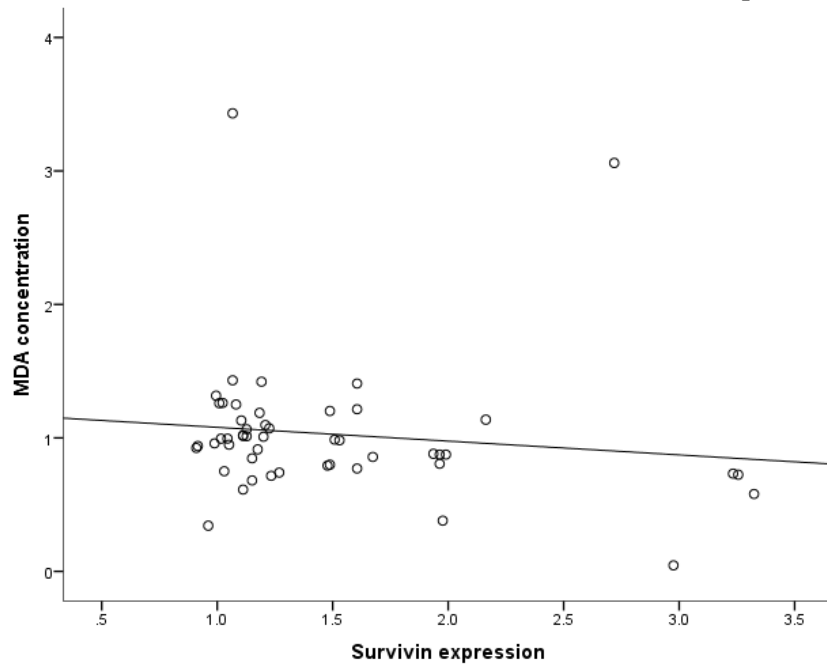
Fig (3). Linear Pearson correlation of MDA with OSM concentration in patients group.

Fig(4) Linear Pearson correlation of MDA with survivin expression in patients group.

ROC curve for some laboratory markers were conducted for discrimination between cases and controls. GSH AUC failed to discriminate, while CEA showed poor discrimination between both groups. On the other hand, SOD, catalase, MDA, OSM showed excellent AUCs. AUCs of SOD, catalase, MDA, OSM showed significant increase when compared to CEA AUC. GSH AUC did not differ significantly than CEA AUC for discrimination between cases and controls. Performance characteristics and best cut off values are shown in **Table (5).**

	CEA	SOD	Catalase	GSH	MDA	OSM
AUC	0.626	1	0.929	0.576	0.963	0.942
Cut off	4.3	<164.7	350.3	<1.2	4.7	6.25
Sensitivity (%)	44	100	96	76	90	98
Specificity (%)	90	100	90	52	100	92
PPV (%)	81.5	100	90.6	61.3	100	92.5
NPV (%)	61.6	100	95.7	68.4	90.9	97.9
Accuracy (%)	67	100	93	64	95	95
P	-	**<0.001**	**<0.001**	0.211	**<0.001**	**<0.001**

AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; p1, probability for discrimination between colorectal carcinoma cases and control group; p2, comparison between AUC of CEA and AUC of each marker

Table (5) Performance criteria, AUC and cut off values of some laboratory markers for discrimination between colorectal carcinoma patients group and control group.
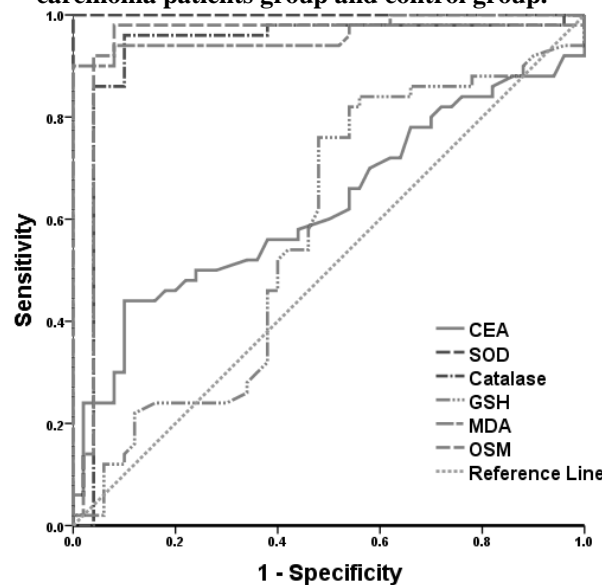

Fig (5). AUC and cut off values of some laboratory markers for discrimination between colorectal carcinoma cases and control group.

Regression analysis was conducted for prediction of colon, rectal and colorectal cancer within healthy control subjects, using age, gender, and laboratory data as covariates. Higher catalase, MDA, OSM ,survivin expression and lower hemoglobin concentration, SOD expression were associated with risk of colon, rectal and colorectal cancer development in univariate analysis. While in multivariate analysis, only higher MDA, OSM and survivin expression were considered as independent risk factors for colon , rectal and colorectal cancer development, these data were explained by **Table (6).**

	Univariable				Multivariable			
	p	**OR**	**95% CI**		**p**	**OR**	**95% CI**	
Age	0.376	1.207	0.120	1.699				
Gender	0.198	0.987	0.198	2.198				
Total leucocytic count	0.123	2.024	0.459	2.806				
Hemoglobin	**0.001**	0.206	0.109	0.389	0.072	0.601	0.435	1.832
Platelet count	0.889	1.000	0.995	1.004				
ALT (U/L)	0.898	0.998	0.964	1.033				
AST (U/L)	0.194	1.028	0.986	1.072				
Bilirubin	0.202	1.663	0.761	3.633				
Albumin	0.071	1.445	0.101	4.817				
Creatinine	0.087	1.456	0.287	2.198				
CEA	0.157	1.005	0.998	1.012				
SOD	**0.005**	0.998	0.997	0.999	0.130	2.824	0.180	8.731
Catalase	**0.002**	1.004	1.001	1.006	0.224	1.001	1.000	1.001

	p	OR	CI		p	OR	CI	
GSH	0.525	1.031	0.939	1.132				
MDA	**<0.001**	3.499	1.988	6.156	**0.009**	5.235	1.517	18.060
OSM	**<0.001**	1.555	1.325	1.826	**0.002**	1.085	1.030	1.143
Survivin expression	**0.014**	1.407	1.199	1.832	**0.006**	1.365	1.198	1.873

OR, odds ratio; CI, confidence interval.

Table (6). Regression analysis for prediction of colon, rectal and colorectal cancer .

VI. DISCUSSION

In the current study, the mean age of patients group was 53.8 ±13.9 years, while in control group was 52.8±5.8 years. There was no statistically significant difference between mean ages in our study groups. This is in line with **Chernyavskiy et al** [30] who found colon cancer was increased at younger ages (20-54 years). The relative different in age between our series and other studies may be attributed to different nature of daily behaviors, habitats, and time of correct diagnosis.

Regarding the sex in the current study, (34%) of patients were females and (66%) were males in patients group, while control group (40%) of patients were females and (60%) were males. There were no statistically significant differences between sex distributions in our study. This near-equal sex distribution was also reported by the studies of **Gies et al** [31] who determined 60% of patients were male and the study of **Ng et al** [32] the authors were noted not statistically significant in terms of age, gender.

Our results clarify hemoglobin concentration was significantly lower in patients group compared with control group (p <0.001). This is in agreement with **Al-Saeed et al** [33] who found that right-sided CRC had significantly low hemoglobin levels (P = 0.001) and it can be utilized as a prognostic markers in CRC patients. This is may be due to the exits of blood with human wastes (stool).

In addition to, mean values of white blood cells, and platelets were 8.35 +/-SD 2.44, and 311.6 x 10 ^{3} /mL +/-SD8.54, respectively which also was in line with previous studies. Mean values of white blood cells, and platelets were 11.9 +/- SD 2.3, 7.67 10 6 /mL+/- SD 2.44, and 343 x 10 3 /mL +/- SD 164.4, respectively **(Al-Saeed et al.,)** .The relative increased platelet count may be explained by it has an important role in metastasis of circulating tumor cells (CTCs) **(Liang et al** [34].

On the other hand, no significant differences were found regarding to clinical chemistry data that is comprised with **Park et al** [35] **and Tsai et al** [36] . This may be explained by none of our patients presented with colorectal liver metastasis. Furthermore, our results are in accordance with the study of **Launay-Vacher et al** [37] that found all colorectal patients have normal creatinine level.

The present study revealed that CEA concentration was increased in colorectal carcinoma patients group compared to control group, *p* value was 0.010. This is agreement with the study of **Yoo and Yeo** [38], that proved that CEA expression is evaluated to be higher in colon cancer cells , and is associated with response of rectal cancer to radiotherapy.

In respect to superoxide dismutase, lower SOD level was determined in patients group compared to control group, *p* value <0.001. Moreover, SOD concentration showed significant positive correlation with AST concentrations. This is in harmony with the aforementioned studies. Cancer cells have lower antioxidant enzyme activity (Superoxide dismutases, catalase and glutathione peroxidases) when compared with their normal counterparts (**Janicka et al** [39])**.**

In the current study, increased catalase concentration in patients group compared to control group, *p* value was <0.001. In agreement with, concentrations of catalase in the colon cancer group are significantly higher than controls (**Martin Mateo et al** [40] **)**

Bhagat et al [41]**,** revealed a significant increase in the level of serum lipid peroxide such as malondialdehyde (MDA) as an oxidant (P<0.001) which had an important role in progression and pathogenesis of colorectal cancer. Similar findings were noted by the current study which determined significant increase of MDA concentration in patients group as compared to control group (P<0.001).

Regarding the OSM assessment, there was significant increase of OSM concentration in patients group compared to control group, *p* value was <0.001. This is in accordance with the former study of **Gurluler et al** [42]**,** who noted significant higher serum OSM concentrations were detected in colon cancer patients than in controls (p < 0.001).

Our study clarifies survivin expression in colorectal cancer patients. It was determined significantly higher in patients group compared with control group, P <0.0001. Also, the study of **Gunaldi et al** [43]**,** found that serum survivin levels were significantly higher in cancer patients than healthy subjects (P = 0.019) and these patients had 4 times increased risk of cancer. The study of **Shojaei et al** [44]**,** showed that survivin is overexpressed in almost cancer types and has a crucial role in cancer progression, cancer cell resistance to anticancer drugs and ionizing radiation. Survivin has unique characteristics which make it a negative prognostic factor for patients with cancer (**Li et al** [45]).

Our results clarified that regression analysis was conducted for prediction of colon, rectal and colorectal cancer using age, gender, and laboratory data as covariates. Higher catalase, MDA, OSM, survivin expression and lower hemoglobin concentration, SOD were associated with risk of colon, rectal and colorectal cancer development in univariate analysis. While in multivariate analysis, only higher MDA, OSM and higher survivin expression were considered as independent risk factors for colon , rectal and colorectal cancer cancer development..

VII. CONCLUSIONS

Survivin expression is considered one of mechanisms utilized by cancer cells in order to progress. It has a key role in promotion of cell proliferation as well as inhibition of apoptosis in cancer cells. Moreover, counter act of survivin may be inhibit tumor growth and enhance cell apoptosis. Therefore, our study shows early detection of survivin, OSM and serum concentration of antioxidant mainly MDA have important role in early

screening of CRC. Additionally, their overexpression is conducted for prediction of colon, rectal and colorectal cancer within healthy control subjects.

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