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Unraveling the Interplay between Sterol Regulatory Element-Binding Protein 1 (SREBP-1) and the Lipophagy Receptor Oxysterol-Binding Protein-Related Protein 8 (ORP8) in Colorectal Carcinoma

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ABSTRACT

Background: Autophagy is a cellular self-degradation process. Lipophagy is a selective autophagy of lipid droplets. Colorectal carcinoma has high incidence worldwide. Both sterol regulatory element-binding protein 1 (SREBP-1) and lipophagy participate in maintaining lipid homeostasis during tumor growth. Oxysterol-Binding Protein-Related Protein 8 acts as a lipophagy receptor, which interacts with microtubule-associated protein 1 light chain 3, and gamma-aminobutyric acid type A receptor-associated protein (LC3/GABARAPs). Autophagy may affect chronic inflammation and oxidative stress. **Objective:** This study aimed to elucidate the interplay between SREBP-1 and the lipophagy receptor oxysterol-binding protein-related protein 8 (ORP8) in colorectal carcinoma and their potential contribution in disease pathogenesis.

Patients and methods: The study comprised 12 colorectal carcinoma patients, enrolled into two groups: Group 1 (having the tumor specimens), and group 2 (having their corresponding safety margin specimens). The levels of interleukin 4 (IL-4), interleukin 6 (IL-6), malondialdehyde (MDA), LC3, catalase activity, gene expression levels of SREBP-1, ORP8, and GABARAP together with immunohistochemical expression of beclin-1 were determined.

Results: The current research displayed elevated gene expression levels of SREBP-1, immunohistochemical expression level of beclin 1, and protein levels of IL-4, IL-6, LC3, and MDA. Gene expression levels of ORP8, GABARAP, and catalase activity showed downregulation. There was negative relationship between SREBP-1 and ORP8 in colorectal carcinoma. **Conclusion:** SREBP-1 and ORP8 might contribute in disease pathogenesis.

Keywords: Lipophagy, Sterol Regulatory Element-Binding Protein 1, Autophagy, Colorectal carcinoma, Oxysterol-Binding Protein-Related Protein 8.

INTRODUCTION

Adenocarcinoma accounts for more than 90% of CRCs [1]. SREBP-1 is a transcription factor that controls the expression of β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) reductase. Therefore, it affects cholesterol synthesis [2]. SREBP enhances lipid droplet synthesis in tumor tissue [3]. SREBP-1, not only regulates lipid homeostasis, but also acts as a regulator of endoplasmic reticulum stress, cellular growth, apoptosis, and autophagy [4].

Lipid droplets (LDs) are intracellular organelles of phospholipid monolayers surrounding neutral lipids. They are separated from hydrophilic cytosol by proteins known as perilipins [5]. Lipophagy is a selective autophagy process for degradation of intracellular triacylglycerols and cholesterol stored in LDs via lysosomal acid lipases, to maintain cellular lipid homeostasis [6]. Lipophagy includes protein-mediated sequestration of LD within double-membrane vesicles forming a phagosome followed by its fusion with lysosome forming autophagolysosome where LD are degraded by lysosomal lipases [7].

The autophagy related gene 8 (Atg 8) family proteins are subdivided into: The gamma-aminobutyric acid receptor-associated protein (GABARAP) and microtubule-associated protein 1 light chain 3 (LC3) subfamilies. They play important role in autophagy induction and autophagosome-lysosome fusion [8]. Beclin 1 is an autophagy specific protein that contributes in autophagosome formation by forming a complex with class III phosphoinositide 3-kinase [9]. Oxysterol-Binding Protein-Related Protein 8 (ORP8) acts as a receptor for lipophagy that mediates LD turnover, [10] through direct interaction between autophagosomal membranes and phagophore-anchored LC3/GABARAPs. ORP8 acts also as lipid transporters for phosphoserine from endoplasmic reticulum to plasma membrane [11].

Oxidative stress may increase mutation rate and initiate pro-carcinogenic signaling pathways [12]. Malondialdehyde (MDA) is an oxidative stress marker derived from lipid peroxidation [13]. The catalase enzyme has anti-oxidant cellular defense through the dismutation of hydrogen peroxide into water and molecular oxygen [14]. Additionally, chronic inflammation is a hallmark of colorectal carcinoma [15]. Interleukin-6 (IL-6) is one of the pro-inflammatory cytokines, which regulate tumor cell growth. Interleukin-4 (IL-4) promotes cell growth and resistance to apoptosis [16].

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Although various articles studied the role of lipophagy in tumor pathogenesis, the interaction between SREBP-1 and the lipophagy receptor ORP8 in colorectal carcinoma hasn't been fully elucidated. The current research aimed to elucidate the interplay between SREBP-1 and the lipophagy receptor ORP8 in CRC in order to clarify their potential contribution in disease pathogenesis.

PATIENTS AND METHODS

Study Design: The current research involved 12 patients suffering from colorectal carcinoma presented to Tanta University Hospital. The clinical and demographic data of the patients were reported. The patients were subjected to the required laboratory investigations. In addition to colonoscopy for diagnosis and tissue biopsy acquisition by the colonoscopes (EC-760ZP-V/L and FEZ1500DL). Patients were prepared for surgical excision with safety margin. Pathological examination was conducted to confirm diagnosis. The tissue specimens were stored at -80 °C for further assay. The tissue specimens were enrolled into two groups: **Group 1 (G1):** The corresponding normal tissue from safety margin specimens were collected from the 12 CRC patients and were enrolled into the control group (G1). **Group 2 (G2):** The primary CRC specimens of the 12 patients were enrolled into group 2 (G2).

Inclusion criteria: Patients suffering from primary colorectal carcinoma who received no preoperative radiotherapy or chemotherapy were included in the study.

Exclusion criteria: Other types of carcinoma, patients who received preoperative radiotherapy or chemotherapy, history of essential hypertension, diabetes mellitus, renal failure, hepatic failure, or any chronic disease.

Tissue homogenate preparation: Following tissue collection, each group's tissue specimens were divided into two sections: The first was used to prepare tissue homogenate and the second was kept in 10% formalin for histopathological analysis, which established the identity of every tissue.

Preparation of lysis buffer: Volume was adjusted for 1 L by adding distilled water after adding 100 ml of 0.5 Tris HCl (pH 8), 100 ml of glycerol, 10 ml of 100 Mm EDTA, 10 ml of 1M sodium pyrophosphate, 30 ml of 5 M NaCl, and 5 ml of Triton X-100 [17]. Tissue fragments were homogenized at a ratio of 1/5 w/v in lysis buffer (pH 8). After centrifugation for 15 min at 15,000 rpm and 4 °C, the supernatants were separated and stored at – 80 °C.

Biochemical assessment

Assessment of LC3, IL-4 and IL-6 using enzyme-linked immunosorbent assay (ELISA): Interleukin 4 (IL4), interleukin 6 (IL6), and LC3 levels were assayed using kits from (My Biosource Inc., California, USA; catalog number: MBS1603826, MBS702269, and MBS2707843 respectively).

Assessment of redox status parameters: The enzyme activity of catalase, and MDA level were assayed by kits from (Bio-diagnostic, Giza, Egypt) (CAT# CA 25 17), and (CAT# MD 25 29) respectively.

Real-time quantitative RT-PCR analysis of ORP8, SREBP-1 and GABARAP: Total RNA was isolated from tissue samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA, 15596026) according to the manufacturer's instructions. Reverse transcription of total RNA was carried out using RevertAid H Minus Reverse Transcriptase (Thermo Scientific, USA) to obtain cDNA to be used as a template to assess the relative expression of ORP8, SREBP-1, and GABARAP genes using the Step One Plus RT- PCR system (Applied Biosystem, USA). The primers were made with the Primer 5.0/ 7 program, and their sequences were listed in table (1). After calculating the cycle threshold (Ct) values of target and housekeeping genes, the relative gene expression was evaluated using the $2^{-\Delta\Delta Ct}$ method [18].

Table (1): Real-time PCR (qPCR) primer pairs

Gene	Nucleotide accession Number	Primers	Amplicon size
ORP8 (ID: 114882)	NM_001003712	Forward: 5′- ATGACAACATTAGGCCGCGA-3′ Reverse: 5′ - AGATCAGTAGCACCCCAGGT-3′	910
SREBP-1 (ID: 6720)	NM_004176.5	Forward: 5′- TTCCGAGGAACTTTTCGCCG-3′ Reverse: 5′- GGGAGGGCTTCCTGTAGAGA-3′	700
GABARAP (ID: 2568)	NM_014211.3	Forward: 5′- GCCCTAACAGAGCCTCAACA -3′ Reverse: 5′- TTGTCACTTCTGCCGACCTC -3′	174
B-actin (ID: 60)	NM_001101.5	Forward: 5′- CTTCGCGGGCGACGAT-3′ Reverse: 5′- CCACATAGGAATCCTTCTGACC-3’	104

Histopathological evaluation: Hematoxylin and eosin stained tissue sections were reevaluated according to WHO classification for digestive system tumors (2019). The histopathological assessment included histopathologic type (conventional adenocarcinoma, mucinous adenocarcinoma with mucinous component representing > 50% and adenocarcinoma with mucinous differentiation ≤ 50%), TNM staging, stage grouping, tumor grade, lymphovascular ,and perineural invasion, necrosis and mitotic count [19].

Immunohistochemical study: Tumor sections, of about 5 μm thickness, were left for 30 min, on positively charged slides, to dry at 37 °C. This is followed by deparaffinization and antigen retrieval, in Dako PT link unit, using high and low PH EnVision FLEX antigen retrieval solutions (reaching 97°C for 20 min). Dako Autostainer Link 48 was used for immunostaining. The application of peroxidase blocking reagent, was followed by incubation with primary antibodies for 30 min. Then horseradish peroxidase polymer was applied for 20 min and diaminobenzidine (DAB) was applied as chromogen. Hematoxylin was used for counterstaining. The primary antibody utilized was Beclin1 (Abcam, ab55878, Cambridge, UK).

The immunoreactivity of Beclin1 was evaluated in either the cytoplasm or nucleo-cytoplasmic in the tumor cells according to the intensity and percentage of positively stained cells. Immunostaining intensity was recorded as: 0 negative, 1 weak, and 2 strong. The grading of positively stained cells percentage was: Grade 0: 0-5 %, grade 1: 6 -25%, grade 2: 26-50%, grade 3: 51-75%, and grade 4: 76-100%. For calculating the immunoreactive score, the score of staining intensity, was added to the percentage score of positively stained cells (0-6). The immunoreactive score of tumors is designated negative if it ranges within 0-3, and is classified positive if it ranges within 4-6. Normal epithelial cells display no or weak cytoplasmic immunostaining.

Ethical approval: The current study was carried out according to the Declaration of Helsinki. The current study protocol got acceptance from The Research Ethics Committee, Faculty of Medicine, Tanta University, with approval code: 36264PR709/5/24. A written informed consents were obtained from all the patients who participated in the research.

Analysis of statistical data

Statistical Package for the Social Sciences; SPSS, Chicago, USA (version 21 for Microsoft Windows, USA) was used for data analysis. Categorical data were displayed as percentages. Quantitative data were expressed as mean and standard deviation. An unpaired t-test was used for comparison between two groups. Pearson coefficient was used for correlation. $P \leq 0.05$ is considered significant.

RESULTS

Demographic and clinical data: No significant variations among the studied groups regarding gender, age, and BMI. However, a significant difference has been estimated between studied groups in tumor marker carcinoembryonic antigen (CEA) level. The current study displayed activation of inflammatory, autophagic and oxidative stress pathways, as indicated by significant elevation in the levels of IL-4, IL-6, LC3, and MDA in group 2 as compared to group 1. Catalase activity displayed significant decline ($p < 0.001$*) as illustrated in table (2).

Table (2): Demographic, clinical, and biochemical data

Variable	Group 1 (control) [a] N= 12	Group 2 (CRC) [b] N= 12	P *value*
Age (years)	49.60 ± 5.84	50.93 ± 6.67	P > 0.05
BMI (kg/m2)	25.61 ± 1.55	26.61 ± 1.55	P > 0.05
CEA (µg/L)	1.2 ±0 .12 [a]	18.4 ± 2.1 [b]	P < 0.05
LC3 (ng/ml)	5.7 ± 1.3 [a]	20.34 ± 5.9 [b]	P < 0.05
IL4 (pg/ml)	147.60 ± 5.84 [a]	350.1.93 ± 5.67 [b]	P < 0.05
IL6 (pg/ml)	46.61 ± 4.55 [a]	126.61 ± 7.55 [b]	P < 0.05
Catalase (U/mL)	11.361 ± 1.003 [a]	3.5 ± 0.23 [b]	P < 0.05
MDA (nmol/mL)	3.880 ± 0.113 [a]	11.3 ± 1.6 [b]	P < 0.05

Data were presented as mean ± SD. P < 0.05 is significant. a: significant difference vs. CRC group. b: significant difference vs. control group.

Relative mRNA gene expression of ORP8, SREBP1, GABARAP

According to our results, SREBP1 mRNA gene expression showed significant upregulation in CRC cases ($p < 0.001$*). On the other hand, ORP8, and GABARAP gene expression levels displayed significant decline in CRC cases compared to group 1 ($p < 0.001$*) as in figure (1).

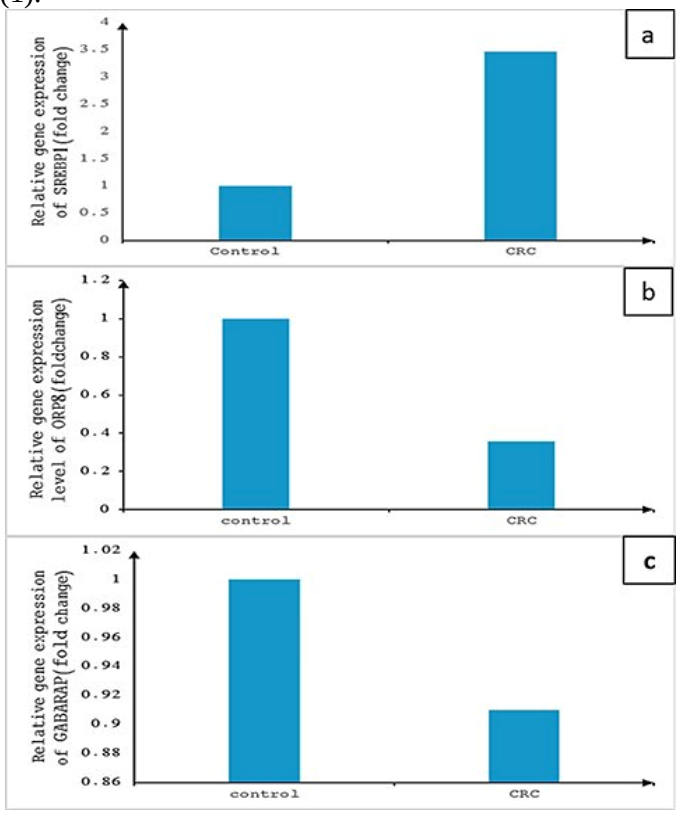

Figure (1): Relative mRNA gene expression (fold changes): (a) Relative gene expression of SERBP1 gene. (b) Relative gene expression of GABARAP gene. (c) Relative gene expression of ORP8 gene. $P < 0.05$ is significant.

Correlation among studied parameters: Correlations among all studied parameters were conducted using Pearson's correlation. All studied parameters displayed significant positive correlation, except catalase activity, ORP8, and GABARAP m RNA gene expression levels, as illustrated in table (3).

Table (3): Correlation among studied parameters

		CEA (µg/L)	LC3 (ng/ml)	IL4 (Pg/ml)	IL6 (Pg/ml)	Catalase (U/mL)	MDA (nmol/ml)	ORP8 expression	GABARAP expression
LC3 (ng/ml)	**r**	0.6302							
	P	< 0.001							
IL4 (Pg/ml)	**r**	0.6571	0.9898						
	P	< 0.001	< 0.001						
IL6 (Pg/ml)	**r**	0.7642	0.9279	0.9446					
	P	< 0.001	< 0.001	< 0.001					
Catalase (U/mL)	**r**	-0.901	-0.857	-0.8586	-0.95				
	P	< 0.001	< 0.001	< 0.001	< 0.001				
MDA (nmol/ml)	**r**	0.7876	0.8144	0.7615	0.75	-0.8672			
	P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001			
ORP8	**r**	-0.8768	-0.407	-0.4455	-0.6866	0.8076	-0.529		
	P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		
GABARAP	**r**	-0.1927	-0.2936	-0.307	-0.3998	0.1676	-0.1551	0.0873	
	P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	
SREBP1	**r**	0.4861	0.4403	0.382	0.224	-0.3888	0.758	-0.05366	-0.6835
	P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

$P < 0.001$ is significant.

Histopathological results: The size of tumor masses ranged between 3-6 cm, only 70% of patients showed lymph node metastasis, and 60% of patients showed distant metastasis. 66.6% of tissue specimens of tumors were well differentiated. 75% of tumor tissue specimens displayed beclin 1 immunohistochemical expression. Microscopic examination of studied cases revealed normal colon mucosa of control group as showed in figure (2), and adenocarcinoma with malignant infiltrating glands in G2 group. One case showed mucinous differentiation with mucin lakes formation. Another case displayed signet ring adenocarcinoma as showed in figure (3).

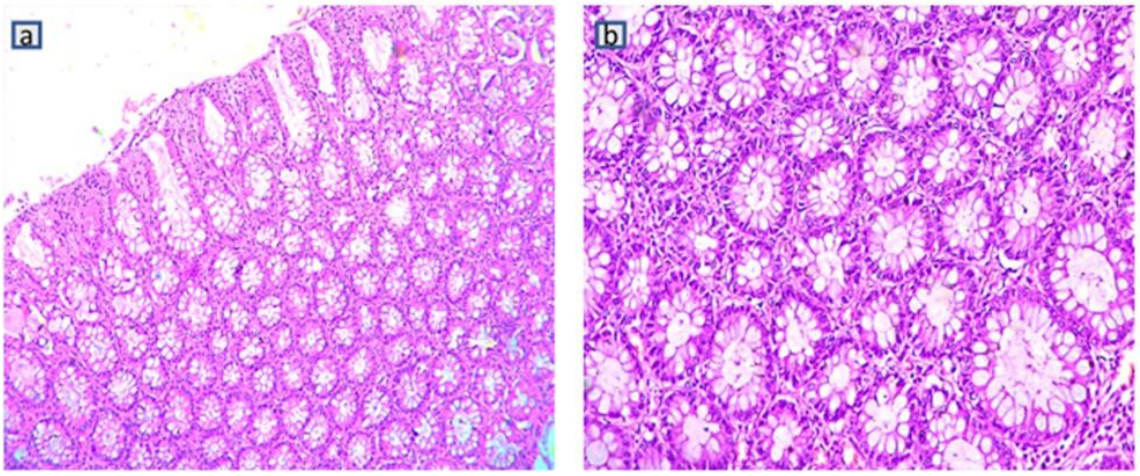

Figure (2): Normal colon mucosa of control group showing colonic mucosal glands lined by simple columnar mucin secreting epithelial cells with intervening stroma showing some immune cells and vascular spaces (by H&E,aX100 and bX200).

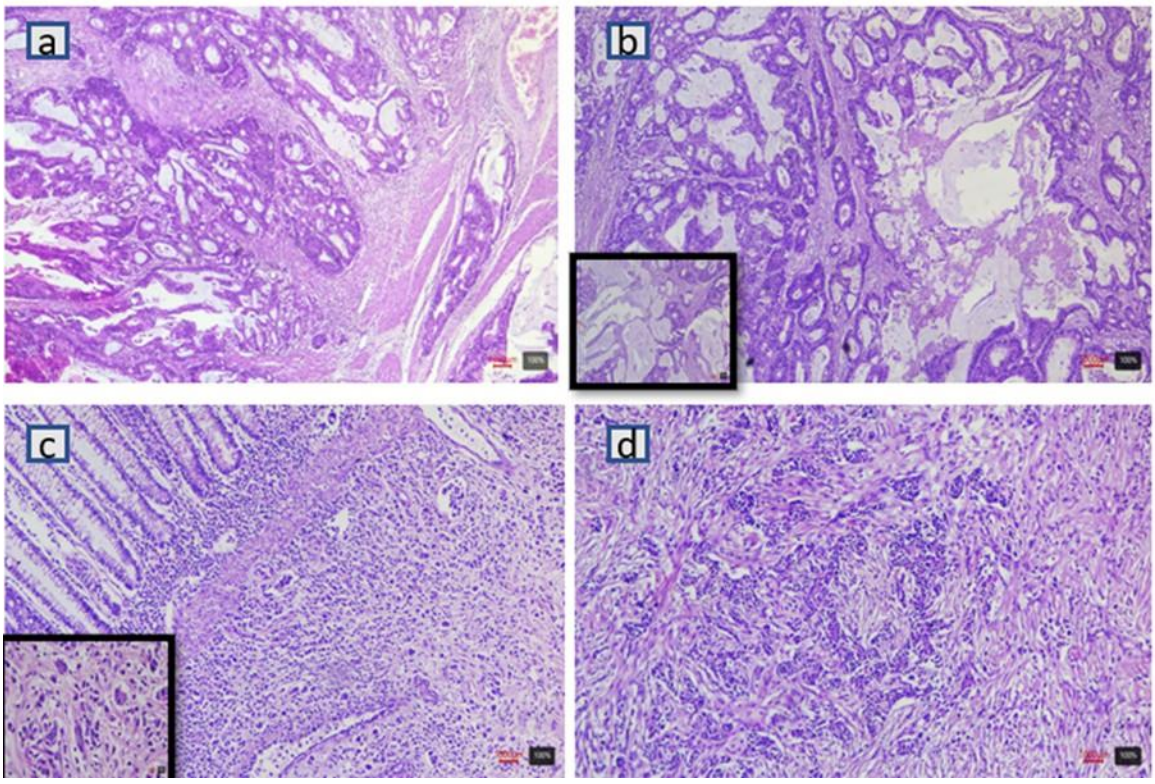

Figure (3): H & E stained sections from adenocarcinoma cases (X200): (a) Case of moderately differentiated adenocarcinoma showing large branching malignant glands invading the muscle. (b) Case of moderately differentiated mucoid adenocarcinoma showing lakes of mucin. (c) Case of signet ring adenocarcinoma showing diffuse submucosal infiltration by malignant signet ring cell. (d) Case of poorly differentiated adenocarcinoma showing diffuse infiltration by malignant cells forming poorly defined sheets.

Immunohistochemical results: Beclin 1 immunostained sections from control cases showed either negative or weak positive cytoplasmic immunostaining. Moderately differentiated adenocarcinoma showed positive beclin 1 immunostaining. While, signet ring adenocarcinoma and poorly differentiated adenocarcinoma showed negative beclin 1 immunostaining as showed in figure (4).

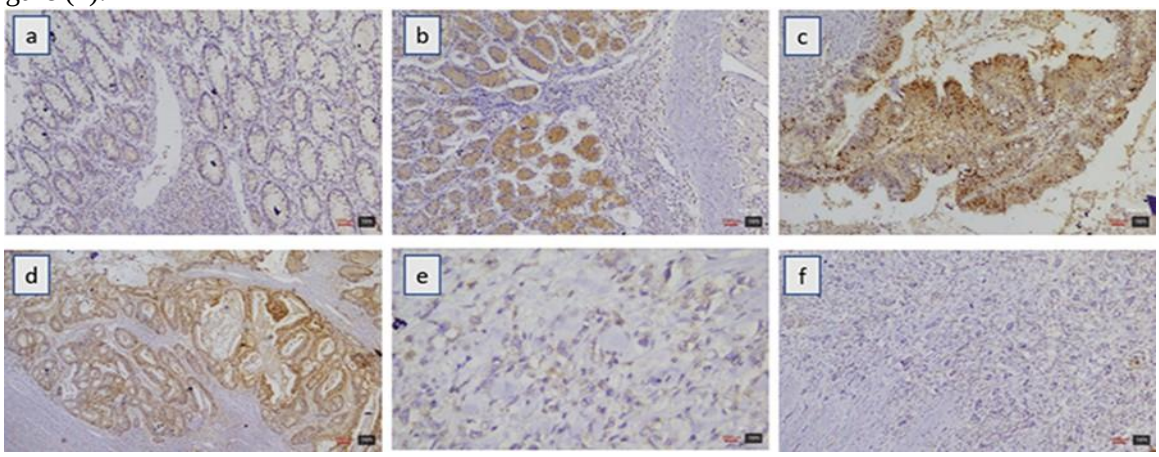

Figure (4): Beclin 1 immunostained sections from control and adenocarcinoma cases (X200). (a) Case of normal mucosa showing negative beclin 1 immunostaining. (b) Case of normal mucosa showing positive cytoplasmic beclin 1 immunostaining. (c) Case of moderately differentiated adenocarcinoma showing positive beclin 1 immunostaining. (d) Another case of moderately differentiated mucoid adenocarcinoma showing positive beclin 1 immunostaining. (e) Case of signet ring adenocarcinoma showing negative beclin 1 immunostaining x400. (f) Case of poorly differentiated adenocarcinoma showing negative beclin 1 immunostaining.

DISCUSSION

Although, there is great progress in lipophagy understanding. The mechanism of lipophagy, and its role in CRC pathogenesis is still unclear. The current research aimed to elucidate the interplay between SREBP-1 and the lipophagy receptor ORP8 in CRC in order to clarify their potential contribution in disease pathogenesis. The synthesis and metabolism of cholesterol is regulated by SREBP-1 [2]. Colorectal carcinoma tissue displayed upregulation of SREBP-1 gene expression level relative to control group, in the current study. This aligns with the results of **Xu *et al.*** [20]. **Hartal-Benishay *et al.*** **[2]** documented that downregulation of SREBPs inhibits colon cancer tumor growth.

The growth of malignant cells requires abundant cholesterol for building new cell membranes, so decline of cholesterol level results in cell cycle arrest [21]. Furthermore, hypercholesterolemia is involved in cancer development as, exogenous cholesterol triggers the oncogenic hedgehog pathway [22] .

The lipid transport activity of ORP8 is mediated by the interaction with LC3/GABARAPs. Accumulation of LDs results from interruption or deletion of ORP8-LC3/GABARAP genes [10]. This supports the suggested role of ORP8 as lipophagy receptor [11]. The current research showed significant decrement in the gene expression levels of ORP8 in CRC tissue compared to control tissue. According to **Guo *et al.*** **[23]** ORP8 suppresses gastric cancer via induction of endoplasmic reticulum stress and tumor cell apoptosis [23].

Regarding GABARAP, its gene expression displayed decline in CRC, relative to adjacent non-tumor tissue, in the current study. This aligns with the findings of **Gurwara *et al.*** **[24]** and **Liu *et al.*** [25]. The current study displayed impaired lipophagy in CRC indicated by decline of ORP8 and GABARAP gene expression levels. It is reported that lipophagy has anti-cancer role through accumulating free fatty acids, which triggers endoplasmic reticulum stress to induce cancer cell apoptosis [6]. However, certain studies declared that lipophagy has dual pro- and anti-cancer role, suggesting that the role of lipophagy differs according to the type and stage of tumor, where lipophagy via lipids degradation might support the survival of proliferating cancer cells through providing energy substrates and intermediates for biomolecules synthesis [5].

Both LC3 and beclin 1 mediate autophagy [8, 9]. The present study showed significant increase in the level of LC 3 in CRC tissue, compared to control tissue. This can be reinforced with the study of **Mahgoub *et al.*** [26].

Concerning beclin 1, the tumor tissue demonstrated significant upregulation of its immunohistochemical expression, relative to adjacent non tumor tissue, in the current study. This aligns with the findings of **Wu *et al.*** **[27]** and **Li *et al.*** [28]. A possible explanation is that autophagy is activated in cancer associated hypoxia and starvation [28] where degradation of damaged peroxisomes and mitochondria act as potential source for reactive oxygen species [5]. Elevated levels of reactive oxygen species in CRC promote cellular proliferation [29],

metabolic reprogramming, immune escape and metastasis [30].

The current study showed elevated levels of MDA, and decreased catalase activity in tumor tissue relative to adjacent non-tumor tissue. This is in harmony with the findings of **Salehi S *et al.*** [13].

The current study showed uprise of the levels of IL-4, and IL-6 in CRC compared to normal tissue specimens. This aligns with the results of **Zhou *et al.*** **[31]** and **Lin Y *et al.*** [32]. It is reported that IL-4 and IL-6 can mediate tumor cellular proliferation and metastasis in colon cancer [16]. IL-6 can also stimulate tumor invasion, angiogenesis, and drug resistance [32]. It is reported that the ablation of murine GABARAP results in an enhancement of the immune response through increased secretion of IL-6 [33].

Colorectal carcinoma tissue, in the present research showed upregulation of SREBP-1, which is accompanied by suppressed lipophagy through down regulation of ORP8 and GABARAP. This is in harmony with the findings of **Li *et al.*** **[34]** who declared that ORP8 acts as a negative regulator of intracellular cholesterol in hepatic cells. According to **Li *et al.*** **[35]** ORP8 modulated SREBP activity in hepatic cells [35].

CONCLUSION

Collectively, the current research displayed elevated gene expression levels of SREBP-1, and decline of the gene expression levels of ORP8 and GABARAP, which indicated their potential contribution in disease pathogenesis. This study paves the way for further research about targeting SREBP-1 in potential therapeutic interventions.

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