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RESEARCH ARTICLE

Autophagy-related Protein LC3 in Egyptian Colorectal Cancer: Impact and Possible Relation to STAT3 andmiRNA 101.

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Abstract

Background: - Colorectal cancer (CRC) is a common worldwide cancer and the fourth worldwide cause of cancer death. Autophagy has been highlighted as a promising molecular target in cancer. Its role in carcinogenesis is complex with a reported oncogenic or tumor suppressive role. Detecting LC3 has become a reliable method for monitoring autophagy. Signal transducers and activators of transcription (STAT) proteins are latent cytoplasmic transcription factors. MicroRNAs (miRNAs) are small non-coding RNAs which post-transcriptionally regulate gene expression.

Aim:- To search for the expression of LC3, as a marker of autophagy, STAT3 and miRNA 101 as possible regulators of autophagy and/ or potential molecular prognostic targets in Egyptian CRC patients.

Material and methods:- Twenty five CRC and 25 adjacent normal mucosa specimens were obtained. Immunohistochemical assessment of LC3 expression using anti-LC3 and assessment of STAT3 and microRNA 101 expressions by real time - RT - PCR were done.

Results: - Revealed a statistically significant increase in LC3 (p<0.001) and STAT3 expression in tumor samples (6.31 ± 1.96 folds) than normal mucosa (p<0.001 each). Both of them were directly correlated together (r= 0.833, p <0.001). MicroRNA 101 was inversely correlated to both LC3 and STAT3 (p<0.001) and was significantly reduced in tumor samples (0.37 ± 0.16 folds) (p<0.001) with a possible correlations to tumor characteristics.

Conclusion:- LC3, STAT3 and miRNA 101 may be valuable as biomarkers that may predict cancer colon poor prognosis. The critical role of STAT3 and miRNAs in autophagy would expand our knowledge of the molecular mechanisms of autophagy regulation.

Introduction:-

Colorectal cancer (CRC) is a common worldwide cancer. It is the second most common cause of cancer in women after breast cancer and the third most common in men. Rates are significantly higher in males than in females (Torre et al.,2015). Although CRC is one of the most potentially curable cancers, it is the fourth most common worldwide overall cause of cancer death(Dong et al., 2014).Meanwhile, at the time of diagnosis, surgery can just be performed

on approximately 20% of patients and 5-y survival rates average 25–40 % (Meyerhardtet al., 2005). Therefore, identification of robust molecular prognostic biomarkers could improve the conventional tumor–node–metastasis staging system. This would help to avoid understaging of tumor and to identify patients with early-stage CRC who may benefit from more aggressive treatment (Dong et al., 2014).

Autophagy is an evolutionarily conserved, multistep lysosomal degradation process(Choi et al., 2013), that has a low activity under physiologicalcircumstances (Klionsky et al., 2012). It is stimulated under conditions like amino acid starvation, nutrient limitation, hypoxia, oxidative stress, metabolic demands, etc. (Ryter et al., 2013). Yet, if an excessive autophagy was induced by these cellular stresses, cell death would follow. In that case, autophagy has a death-promoting role as type II programmed cell death (type II PCD), compared with apoptosis (type I PCD) (Maieseet al., 2012).Autophagy has been highlighted as a promising molecular target in cancer (Choi et al., 2013). Its role in carcinogenesis is rather complex with a reported oncogenic or tumor suppressive role for the regulation of core pathways as well as a contribution to therapeutic resistance (Brech et al., 2009 and Schmukleret al., 2013).

Autophagy marker protein light chain 3 (LC3) is a soluble protein that is distributed ubiquitously in mammalian tissues and cultured cells. It is recruited to autophagosomal membranes during autophagy process and its detection has become a reliable method for monitoring autophagy and autophagic cell death (Tanida et al., 2008).Moreover, the increase in LC3B-II was reported to be directly correlated with the number of autophagosomes (Klionsky et al., 2008) and to be a specific marker of the autophagic process (Mizushimaet al., 2010).

Signal transducers and activators of transcription (STAT) proteins are latent cytoplasmic transcription factors that translocate into the nucleus to induce gene transcription(Bromberg, 2002). Notably, STAT3 plays an important role in the tumor response to chemotherapy treatment(Courapiedet al., 2010). It has been considered as an oncogene and has been linked to regulation of cell transformation, apoptosis deregulation, and angiogenesis(Barreet al., 2007). Nevertheless, its effect on the regulation of autophagy largely remains to be elucidated(You et al., 2015).

MicroRNAs (miRNAs) are small non-coding RNAs which post-transcriptionally regulate gene expression, predominantly through imperfect base pairing with the $3^/$-untranslated region ($3^/$ UTR) of target mRNAs. MiRNA-mediated repression of gene expression occurs through complex mechanisms, including translational inhibition and mRNA degradation(Filipowiczet al., 2008).MiRNA expression is often deregulated in cancer where miRNAs could act as oncogenes or tumor suppressors. Further, miRNA expression profiling can be used to predict the clinical outcome of cancer patients (Jiang et al., 2008 and Voliniav et al., 2006). In CRC, deregulated expression of miRNAs that regulate genes of cellular proliferation, differentiation, inflammation, invasiveness, and tumor progression or apoptosis, has been observed(Nagarajuet al., 2015 and Valeriet al., 2010). MiRNA 101 is down regulated in endometrial, hepatocellular carcinomas and prostate cancers(Hiroki et al., 2010, Su et al., 2009 and Varamballyet al., 2008). Additionally, decreased miRNA101 has been found to be involved in cyclooxygenase 2 (COX-2) overexpression in human CRC(Strillacciet al., 2009).

Considering the widespread implications of autophagy, miRNAs and STAT3 in cancer pathobiology and given the lack of enough current evidence linking these rapidly growing fields of research, we were prompted to search for the expression of LC3, a marker of autophagy, in Egyptian CRC patients. Further, to explore the expression of STAT3 and miRNA 101 as possible regulators of autophagy and/ or potential molecular targets in Egyptian CRC patients. Meanwhile, to check the potential prognostic value of the above mentioned biomarkers in such patients.

To the best of our knowledge, this is the first study concerning autophagy and its possible regulators in Egyptian CRC patients.

Subjects and methods:-
Subjects:-
A total of 50 tissue specimens (25tumor and 25 adjacentnormal mucosa) from patients with CRC wereobtained from the Department of experimental and clinical Surgery, Medical Research Institute,Alexandria University. None had received pre-operative chemo therapy. All patients provided written informed consent prior to tissue harvesting. Moreover, the research protocol was approved by the Alexandria University medical ethical committee.

Tissue specimens were quickly removed and rinsed with ice-cold PBS.A part of the tissue was embedded in RNA later (INVITROGEN, USA) andsnap-frozen in liquid nitrogen. The rest was fixed in 10% buffered formalin (FISHER SCIENTIFIC). This was followed by the routine clinical sample preparation protocol of dehydration, clearingand paraffin embedding, using the standard method by the Department of Pathology, Faculty of Medicine, Alexandria University (Shi et al., 1991).

Methods:-
Immunohistochemicalassessment of LC3B:-
Representative paraffin blocks for the tumors and adjacent normal mucosa were selected; routine H&E stained sections were reviewed for the grading of the tumors, pathological staging and any additional prognostic findings as lympho-vascular invasion and LN metastasis. Immunohistochemical assessment of LC3 expression was done on 5μm sections from the selected paraffin blocks mounted on positively charged slides. The slides were baked overnight at 50ºC, deparrafinized in xylene and rehydrated in decreasing grades of alcohol. Endogenous peroxidase activity was blocked by a 10minute treatment with 3% hydrogen peroxide in absolute methanol. The tissue was then preheated in a pressure cooker (20 minutes in citrate buffer at pH 6). Next,rabbit polyclonalAnti-LC3B antibody (ab48394) was added and incubated overnight at 4°C (dilutions: 1:100) in phosphate-buffered saline (pH, 7.2).The bound antibody was detected by the Ultra Vision Detection System [Antipolyvalent, HRP/DAB (Ready-To-Use)] (THERMO SCIENTIFIC, USA). Negative and positive controls were included in all runs(Sato et al., 2007).

Total RNA extraction for STAT3 and miRNA 101 analysis:-
Total RNA, including mRNA, miRNA and other small RNA molecules were extracted from CRC and control tissue samples usingmiRNeasy Mini Kitand following the manufacturer's protocol (QIAGEN, HILDEN, GERMANY). Total RNA preparation and handling steps were performed under strictly sterile and RNAse-free conditions. Assessment of the concentration and purity of the extracted RNA samples was done using NanoDrop 1000 Spectrophotometer (THERMO SCIENTIFIC, USA) by determining the ratios of their spectrophotometric absorbance at 260/280 where pure preparations of RNA should have ratios around 2.0. The isolated RNA was resuspended in RNAse-free DEPC (Diethyl-pyro-carbonate)-treated water and stored at -80ºC until further processing.

Real-timeRT PCR-based detection of STAT3 expression:-
Reverse transcription was performed in 25 μL reaction volume with 100 ng of total RNA, random primer and reverse transcriptase (RT) Superscript II (INVITROGEN, USA). The real-time PCR measurement of STAT3 cDNA was performed using the One Step real-time PCR system (APPLIED BIOSYSTEMS, USA).Amplification of the synthesized cDNAs was performed in duplicates in a 25 μl reaction volume containing 1XSYBR® Green PCR Master Mix (APPLIED BIOSYSTEMS, USA).The specific primer pair for STAT3 was the sense primer 5'-CAT GTG AGG AGC TGA GAA CGG-3' and the antisense primer 5'-AGG CGC CTC AGT CGT ATC TTT-3'(ref|NC_018928.2|). The amplification consisted of one cycle at 95˚C for 30 sec followed by 40 cycles of denaturation at 95˚C for 5 sec, a 65˚C annealing step for 10 sec, and anextension step at 72˚C for 20 sec(Park et al., 2008).

For verification of the correct amplification product, the PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. PCR amplification was followed by a melting curve analysis where the identity of the PCR product was confirmed.A negative control withoutcDNA was run with every PCR to assess the specificity of the reaction. Furthermore, the PCR efficiency was determined by analyzing a diluted series of cDNA solutions (the external standard curve).An analysis of the data was performed using StepOne™ Software v2.3 wherethe level of expression of STAT3 as determined by the comparative CT method for gene expression relative to the housekeeping gene glyceraldehyde 3 phosphate dehydrogenase (GAPDH)(Livaket al., 2001).

Real-time RT PCR-based detection of miR-101 expression:-
Reverse transcription for miRNAs was performed in a total reaction volume of 15μlusing the TaqManmiRNA Reverse Transcription Kit with specific miRNA 101 primers (APPLIED BIOSYSTEMS, USA). Quantitative RT-PCR analysis for miRNAs was performed in duplicate with a total reaction volume of 20 μlusing TaqMan microRNA assays and TaqMan® Universal PCR Master Mix II (APPLIED BIOSYSTEMS, USA) for relative quantification of the mature miR-101 (hsa-miR-101; 002253) expression level, as described (Ciarapica et al., 2009). A negative control (No-template control) was run with every PCR assay to evaluate the background signal. One Step real-time PCR system (APPLIED BIOSYSTEMS, USA) was used for the measurements. The amplification

consisted of one cycle at 95˚C for 10 min (for enzyme activation) followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/extension step at 60˚C for 60 sec.An analysis of the data was performed using StepOne™ Software v2.3 where the expression fold change of miR-101 was calculated by the $2^{-\Delta\Delta Ct}$method relative to the snoU6 snRNA(001093)(Livakand Schmittgen, 2001).

Statistical analysis:-
Data were analyzed using the Statistical Package for Social Sciences (SPSS ver.20 Chicago, IL, USA).The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test.Data which were normally distributed were described using mean± standard deviation. Meanwhile, datathat were not normally distributed were described using median, range. Moreover, qualitative data were described using number and percent. Comparisons between groups for categorical variables were assessed using Chi-square test and Monte Carlo correction. Additionally, Student t-test was used to compare two groups for normally distributed quantitative variables while ANOVA was used for comparing more than two studied groups. Mann Whitney test was used to compare two groups for abnormally distributed quantitative variables while Kruskal Wallis test was used for comparing more than two studied groups. Spearman coefficient was used to correlate between each two variables. Significance of the obtained results was judged at the 5% level.

Results:-
Distribution of studied parameters:-
Table 1 shows the distribution of different clinicopathological parameters and studied biomarkers in the current study patients.

Immunohistochemical expression of LC3B:-
The cytoplasmic staining of LC3B in colon cancer specimens and adjacent normal mucosa are presented in figures (1-5) showing a marked difference between tissue and mucosa and this difference was statistically significant (P<0.001). (Figure 6)

Furthermore, LC3B immunohistochemical expression was analyzed statistically to determine the relationship of protein expression with clinicopathological parameters of colon carcinoma patients, such as age, gender,tumor site,tumor grade, pathological stage, lymph node status and clinical stage; the results were presented in (Table 2).

Results show increased expression with advanced age and in rectosigmoid site (83.3%) versus colonic site (16.7%). Furthermore, the intensity level of LC3B expression in tumor specimens was significantly correlated with the advanced clinical stage (p=0.013). However, the intensity level of LC3B expression was not correlated with other clinicopathologic factors (Table 2).

Real-time RT-PCR-based detection of STAT3 and miRNA 101 expression:-
Results of Stat3 and miRNA 101 expression in tumor samples and normal mucosa by RT-PCR were displayed in Figure 7.

Stat3showed a statistically significant elevated expression in tumor tissue (6.31 ± 1.96 folds) than corresponding normal mucosa (1.05 ± 0.18 folds) (p<0.0001). Moreover, statistical analysis showed increased expression in higher cancer grades, pathological and clinical stage (p <0.001 for both) and lymph node status (p=0.008), all these relations are summarized in Table 3.

MiRNA 101 expression was significantly lower in tumor tissue (0.37 ± 0.16 folds) than normal mucosa (2.72 ± 0.98), p <0.001. It showed a statistically significant reduced expression in higher tumor grades (p=0.006), pathological stage (p=0.009), lymph node involvement (p=0.017) and advanced clinical stage (p=0.011).

Table 1:- Distribution of the studied cases according to different clinicopathological and molecular parameters (n=25)

	No. (%)
Age	**47.12 ± 14.70**
< 40	6 (24.0%)
≥ 40	19 (76.0%)
Sex	
Male	9 (36.0%)
Female	16 (64.0%)
Tumor site	
Colonic	10 (40.0%)
Rectosigmoid	15 (60%)
Grade	
1	1 (4.0%)
2	21 (84.0%)
3	3 (12.0%)
PT stage	
PT2	4 (16.0%)
PT3	18 (72.0%)
PT4	3 (12.0%)
N stage	
N0	14 (56.0%)
N1	8 (32.0%)
N2	3 (12.0%)
Clinical stage	
I	4 (16.0%)
IIA	8 (32.0%)
IIB	3 (12.0%)
IIIB	7 (28.0%)
IIIC	3 (12.0%)
LC3 tumor	
1	4 (16.0%)
2	9 (36.0%)
3	12 (48.0%)
LC3 mucosa	
0	14 (56.0%)
1	11 (44.0%)
STAT3 tumor (folds)	6.31 ± 1.96
STAT3 mucosa	1.05 ± 0.18
miRNA 101 tumor (folds)	0.33 (0.20 – 0.85)
miRNA 101 mucosa	2.55 (1.04 – 4.82)

Qualitative data were described using number and percent, while normally distributed quantitative data were expressed in mean ± SD and abnormally distributed data were expressed in median (Min. - Max.)

Table 2:- Relation between Tumor LC3 Score and clinicopathological parameters.

	Tumor LC3 Score			p
	I **(n = 4)**	**II** **(n = 9)**	**III** **(n = 12)**	
Age				
< 40	4(100.0%)	2(22.2%)	0(0.0%)	<0.001[*]
≥ 40	0(0.0%)	7(77.8%)	12(100.0%)	
Sex				
Male	2(50.0%)	2(22.2%)	5(41.7%)	0.625
Female	2(50.0%)	7(77.8%)	7(58.3%)	
Tumor site				
Colonic	4(100.0%)	4(44.4%)	2(16.7%)	0.013[*]
Rectosigmoid	0(0.0%)	5(55.6%)	10(83.3%)	
Grade				
1	1(25.0%)	0(0.0%)	0(0.0%)	0.120
2	3(75.0%)	9(100.0%)	9(75.0%)	
3	0(0.0%)	0(0.0%)	3(25.0%)	
PT stage				
PT 2	2(50.0%)	2(22.2%)	0(0.0%)	0.061
PT 3	2(50.0%)	7(77.8)	9(75.0%)	
PT 4	0(0.0%)	0(0.0%)	3(25.0)	
N stage				
N0	4(100.0%)	5(55.6%)	5(41.7%)	0.204
N1	0(0.0%)	4(44.4%)	4(33.3%)	
N2	0(0.0%)	0(0.0%)	3(25.0%)	
Clinical stage				
I	3(75. 0%)	1(11.1%)	0(0.0%)	0.013[*]
IIA	1(25.0%)	3(33.3%)	4(33.3%)	
IIB	0(0.0%)	3(33.3%)	0(0.0%)	
IIIB	0(0.0%)	2(22.2%)	5(41.7%)	
IIIC	0(0.0%)	0(0.0%)	3(25.0%)	

Qualitative data were described using number and percent and were compared using Chi square test
*: Statistically significant at $p \leq 0.05$

Table 3:- Relation between each of Tumor STAT3 and Tumor miRNA 101 relative expressions and clinicopathologicalparameters

	STAT3 (folds)	p	miRNA 101(folds)	P
Age				
< 40	4.14 ± 1.62	0.001*	0.61(0.36 – 0.85)	0.001*
≥ 40	6.99 ± 1.53		0.29(0.20 – 0.45)	
Sex				
Male	6.58 ± 2.16	0.611	0.36(0.20 – 0.85)	0.496
Female	6.15 ± 1.90		0.31(0.22 – 0.66)	
Tumor site				
Colonic	4.75 ± 1.85	0.002*	0.39(0.27 – 0.85)	0.021*
Rectosigmoid	7.34 ± 1.24		0.29(0.20 – 0.45)	
Grade				
1	3.02	<0.001*	0.85(0.85 – 0.85)	0.006*
2	6.14 ± 1.81		0.34(0.25 – 0.75)	
3	8.57 ± 0.07		0.22(0.20 – 0.23)	
PT stage				
PT2	3.57 ± 0.63	<0.001*	0.51(0.27 – 0.85)	0.009*
PT3	6.54 ± 1.63		0.34(0.25 – 0.75)	
PT4	8.57 ±0.07		0.22(0.20 – 0.23)	
N stage				
N0	5.37 ± 2.04	0.008*	0.35(0.25 – 0.85)	0.017*
N1	7.09 ± 0.90		0.32(0.26 – 0.44)	
N2	8.57 ± 0.07		0.22(0.20 – 0.23)	
Clinical stage				
I	3.36 ± 0.49	<0.001*	0.61(0.31 – 0.85)	0.011*
IIA	5.89 ± 1.89		0.35(0.26 – 0.75)	
IIB	6.21 ± 0.45		0.36(0.33 – 0.44)	
IIIB	7.54 ± 0.82		0.29(0.25 – 0.40)	
IIIC	8.57 ± 0.07		0.22(0.20 – 0.23)	

Normally distributed quantitative data were expressed as Mean ± SD and compared using student t-test or F: F test (ANOVA). Moreover, abnormally distributed data were expressed using Median (Min. – Max.) and were compared using Mann Whitney test or Kruskal Wallis test. *: Statistically significant at $p \leq 0.05$

Autophagy related protein LC3B expression correlation to Stat3 and miRNA:-
Statistical analysis using Spearman coefficient test, revealed a highly significant direct correlation between LC3B expression and Stat3expression (r= 0.833, p <0.001). Moreover, a highly significant inverse correlation was found betweenLC3B expression and miRNA 101 expression (r=-0.759, p<0.001). There was also an inverse correlation between Stat3 and miRNA expression and this correlation was statistically significant (r= -0.783, p<0.001).
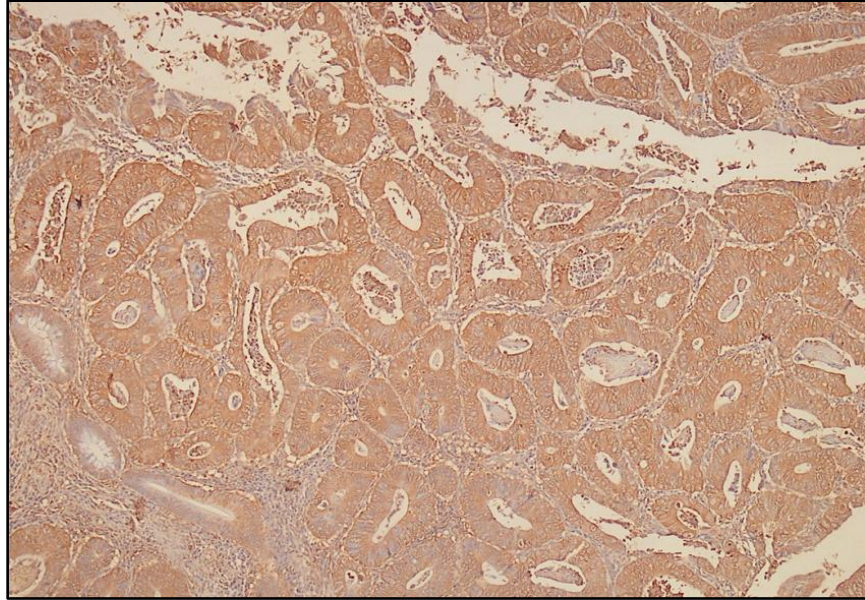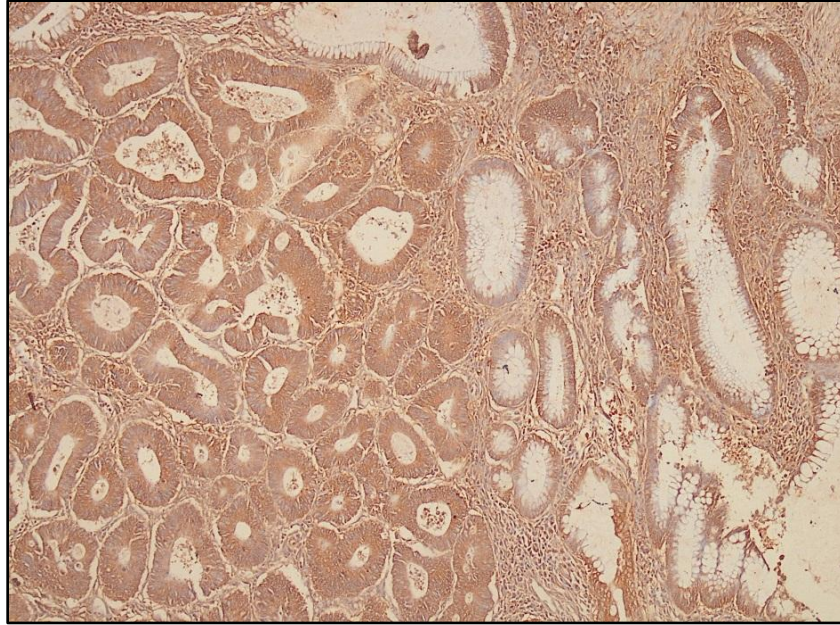
Figure 1:-Strong positive LC3 immunohistochemical staining in tumor tissue:
Strong LC3B cytoplasmic positivity in well differentiated adenocarcinoma colonscore 3+(ABC x100)
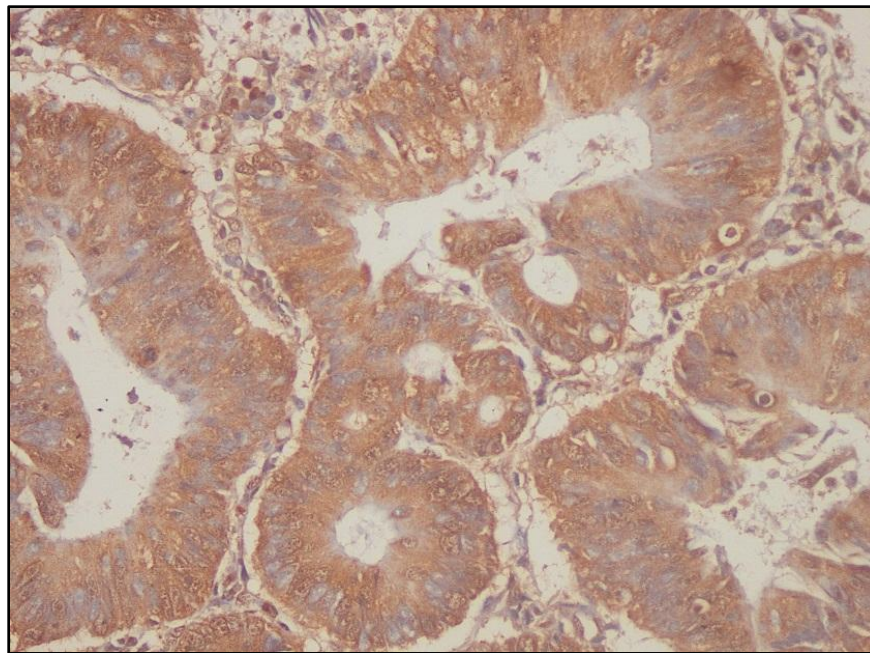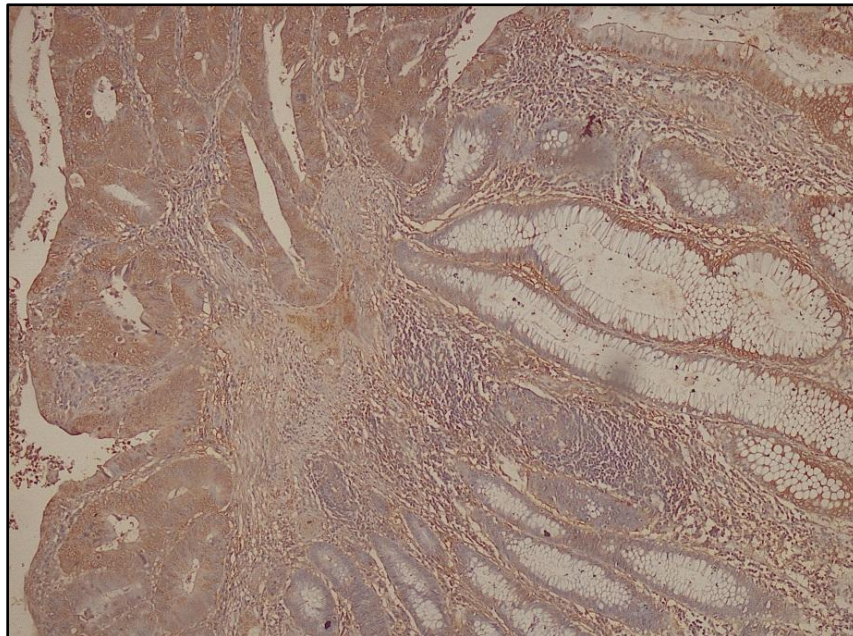

Figure 2:- Strong positive LC3 immunohistochemical staining in tumor tissue:
Strong LC3B cytoplasmic positivity in well differentiated adenocarcinoma score3+ (ABC x400)
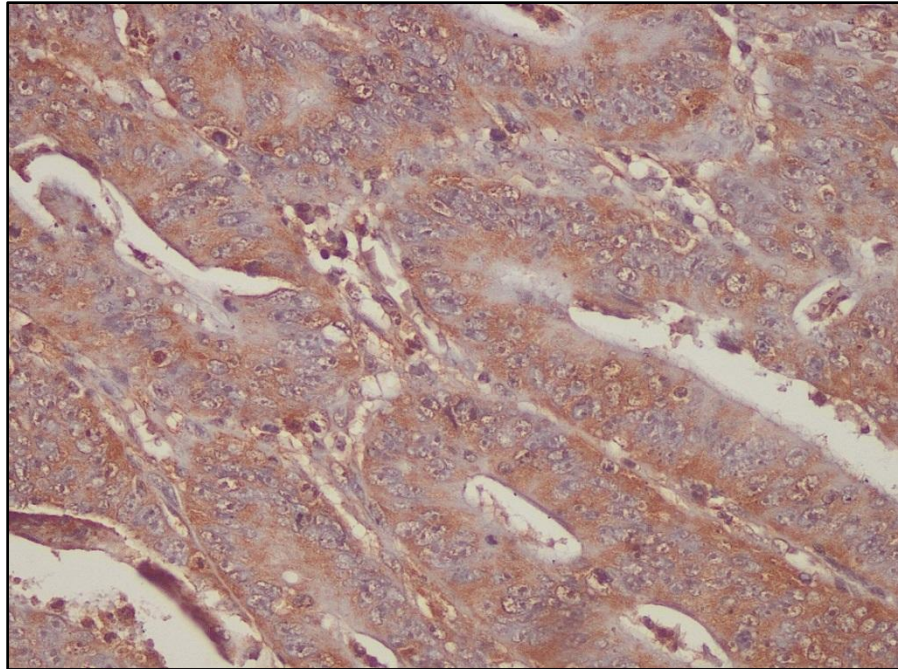
Figure 3:-Weak positive LC3 immunohistochemical staining in adjacent tissue:
Strong LC3B positivity in tumor tissue and weak positivity in adjacent tumor tissue (ABC x100)


Figure 4:- Weak positive LC3 immunohistochemical staining in adjacent tissue:
Strong LC3B positivity in tumor tissue and weak positivity in adjacent tumor tissue (ABC x400)

Figure 5:- Moderate positive LC3 immunohistochemical stainingin tumor tissue:
Moderate cytoplasmic positivity for LC3 antibody score 2+ (ABC x400)
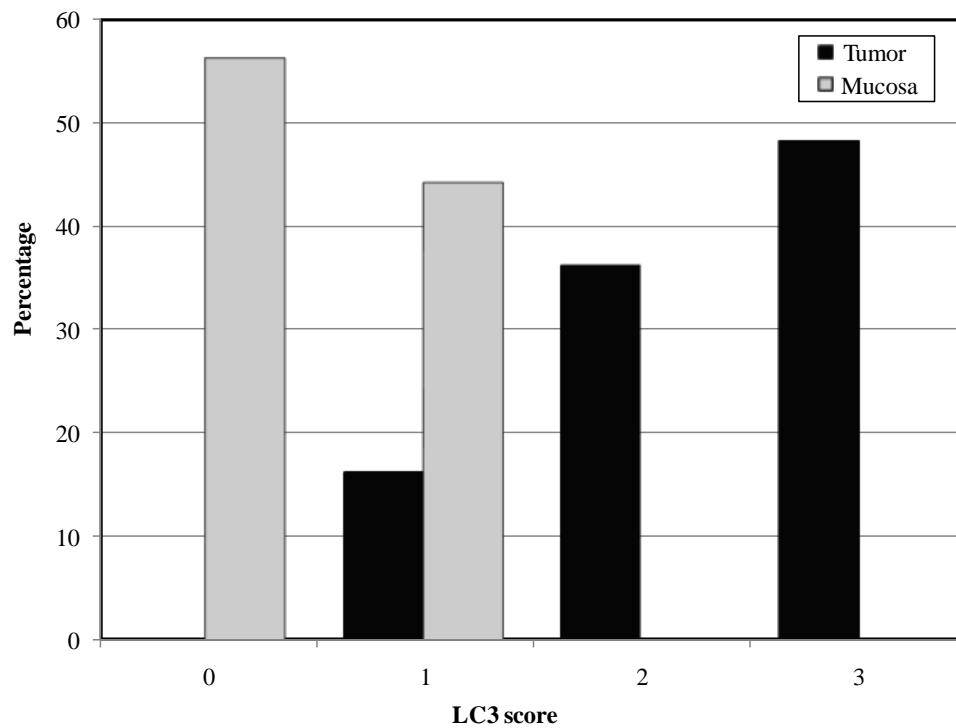
Figure 6:- LC3 score in tumor vs normal mucosal tissue.
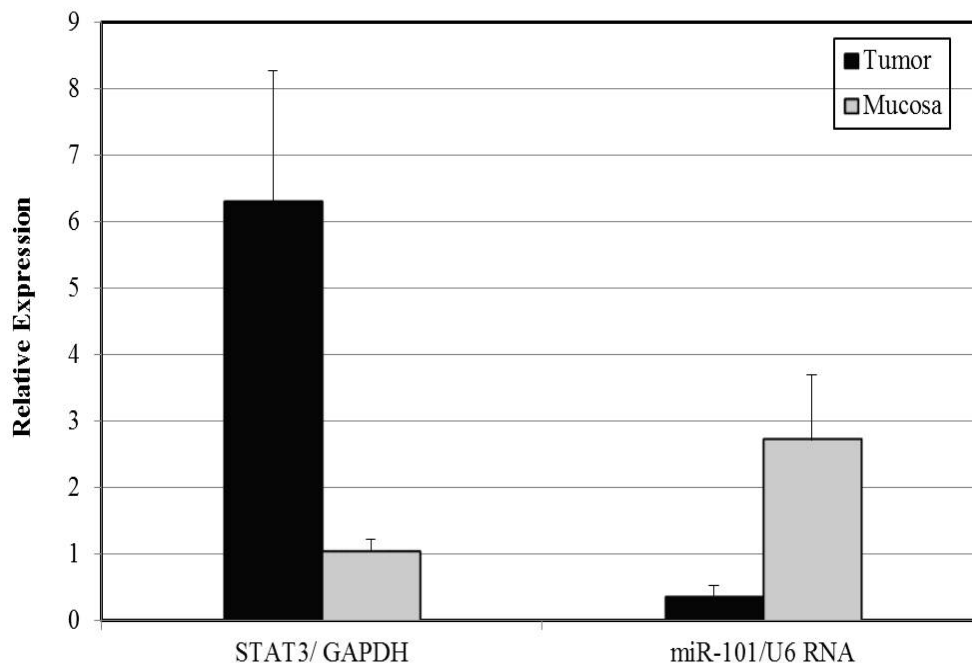
Figure 7:-STAT3 and miRNA 101 relative expression in tumor vs normal mucosal tissue.

Discussion:-

Immunohistochemical staining is a convenient method for evaluating autophagic activity in surgically resected cancer specimens, and it has also been adopted by many studies(Sato et al., 2007, Fujiiet al., 2008 and Yoshioka et al., 2008). In the present study, a low level of LC3B expression was observed (score I) in 44.0% of noncancerous cells, consistent with the basal function of autophagy. None of the normal mucosal specimens exhibited higher expression (scores II and III). In normal cells, autophagy functions as a surveillance mechanism to eliminate damaged organelles and aggregated proteins, reducing DNA damage, reactive oxygen species (ROS), and mitochondrial abnormality, which likely protects normal cells from transforming to tumor cells(Yang et al., 2011).

LC3B was expressed in all 25 studied CRC specimens (100%), consistent with the results of other studies(Sato et al., 2007, Yoshioka et al., 2008 and Zhenget al., 2012).About 48.0% of them expressed a very high level of the protein (score III) indicating a suggested link between autophagy induction and tumor development in CRC. In established tumor cells, autophagy takes over to serve as a cell survival mechanism that plays a vital role in facilitating tumor cell growth. A number of potential mechanisms have been suggested: including promotion of metabolite turnover in tumor cells and inhibition of apoptosis by autophagy. Now autophagy has been recognized as an important regulator of cancer development and a key factor in determining tumor cell sensitivity to anticancer therapy (Yang et al., 2011 and Gong et al., 2012).However, its role is complex with a dual role in cancer(Vinodet al., 2014),being tumor-suppressing during the early stages of tumorigenesisand tumor-promoting in established tumors(Choi, 2012, Kenificand Debnath, 2014).

A significant correlation between LC3B expression and tumor aggressiveness was found, signifying its role in deteriorating CRC conditions (Guoet al., 2011). Furthermore, autophagy has been reported to be involved in tumor recurrence as metastatic tumor cell depends on it to survive against metabolic stress(Aguirre-Ghiso, 2006).Thus, targeting autophagy may be a great innovation for improving prognosis (Li et al., 2011). A number of cancer-promoting signaling pathways could actually impact tumor development through alterations in autophagy(Kenificet al., 2014), which is regulated by a complex network of signaling pathways. The interplay between autophagy and STAT3 signaling may have an influence on the survival or death of a cell (Pietrocola et al., 2013 and Maycotteet al., 2014).

Our results showed a significant direct correlation between STAT3 and LC3B expression in tumor specimens. In fact, reports showed the requirement of autophagy for activation of interleukin 6 (IL-6) - STAT3 signaling in pancreatic carcinogenesis throughreceptor for advanced glycation end products (RAGE) (Kanget al., 2012).Also,

STAT3 knockdown or pharmacological inhibition significantly reduce LC3 expression, suggesting that STAT3 transcriptionally regulates autophagy through LC3 (Gong et al., 2014).STAT3 is an important link between oxidative stress and autophagy (Wei et al., 2003 and Niuet al., 2002).It has a substantial role in the assembly of autophagosomes to their maturation. It also up-regulates the hypoxic expression of hypoxia inducible factor 1 (HIF1A)(Junget al., 2005), that activate the transcription of genes encoding 2 BH3-only proteins in favor of autophagy induction(Mazureand Pouyssegur, 2010). It also stabilizes HIF1A from ubiquitination and up-regulates autophagy via increasing BNIP3 expression (Jung et al., 2008) (Prattand Annabi, 2014).

On the other hand, a report by Kroemer group unraveled a possible role of cytoplasmic STAT3 inhibition of autophagy by inhibiting eukaryotic translation initiation factor 2-a kinase 2 (EIF2AK2)(Shen et al., 2012). So, there was a need for further investigation to describe the relation of STAT3 to autophagy in CRC.

Our results revealed an increase in STAT3 expression in CRC and this could be ascribed to its participation in tumorigenesis, and its activation by oncoproteins(Yu et al., 2009). It might therefore, constitute a valid oncogene(Bromberg et al., 1999).STAT3 amplified expression in the tumor samples may be explained bySTAT3 activation that enhances transformation of cells, it is also associated with the up-regulation of anti-apoptotic genes as bcl-xL, myeloid cell leukaemia sequence- 1 (mcl-1) and surviving (Epling-Burnetteet al., 2001), and with other factors accelerating cell cycle progression, such as cyclin D1 and c-myc(Gong et al., 2014).

A significant association of STAT3 expression with clinical stage and pathological grade and stage was found. Thus, its involvement in tumor advancement was suggested. Also, it correlated significantly to lymph node involvement consistent with a previous study(Kusabaet al., 2005). This could be explained by its role in increasing the expression of matrix metalloproteinase 2, which is involved in cancer metastasis and invasion (Xie et al., 2004). In many cancers, STAT3 correlated well to poor prognosis(Lassmannet al., 2007), as activated STAT3 directly induces the transcription of vascular endothelial growth factor (VEGF) (Wei et al., 2003). STAT3 assists the expression of genes related to angiogenesis and tumor invasion, thus regulating tumor growth and metastasis. Accordingly, it could be considered as a valued biomarker in predicting poor prognosis and therapeutic resistance (Lassmann et al., 2007).

Besides, another possible mechanism for STAT3 regulation of autophagy may be related to expression of microRNAs that target autophagy-related genes (Brock et al., 2009). A number of miRNAs have been transcriptionally controlled by STAT3 to be able to target autophagy pathways (Brock et al., 2009 and Mikhaylovaet al., 2012).The interaction between autophagy and miRNA is important and complicated. On the other hand, autophagy is also important to maintain miRNA homeostasis. However, in CRC, the role of miRNAs in the regulation of STAT3-mediated autophagy has not been well established.In our study, a statistically significant inverse correlation was found between miRNA 101 expression and LC3B expression and this could be explained by the ability of miRNA-101 to target RAB5A to inhibit autophagyat the step of vesicle nucleation (Frankel et al., 2011 and Ravikumar et al., 2008). In hepatocellular carcinoma (HCC), the inhibition of autophagy by miR-101 sensitized HCC cells to doxorubicin, fluorouracil and cisplatin treatment(Xuet al., 2013 and Xu et al., 2014). It effectively reversed tamoxifen-induced autophagy and sensitized breast cancer cells to tamoxifen(Frankel et al., 2011). Thus, modulation of autophagy by miR-101 represents a potential novel therapeutic target.

MiR-101 reduced expression in our tumor samples goes in accordance to its proposed function as a tumor suppressor that is lost during tumorigenesis(Schee et al., 2012).MiRNAs are located on the genome fragile sites, which may undergo deletion during carcinogenesis (Calinet al., 2004). In addition, miR-101 expression is reduced in advanced cancer grades and stages. Its profiling can be used to predict the clinical outcome of cancer patients(Jiang et al., 2008,Volinia et al., 2006 and Lu et al., 2005).This goes with previous evidence including liver, prostate and breast cancer (Buechneret al., 2011).It induces apoptosis and suppresses tumorigenicity in vitro and in vivo, and was recently reported to inhibit migration and invasion of gastric cancer cells (Su et al., 2009, Varambally et al., 2008 and Wang et al., 2010). On the other hand, Scheeet al (2012) found only a few associations with clinicopathological parameters in their CRC cohort.

Interesting recent findings in pancreatic cancer suggest miR-101 as a key regulator of stem cell protein markers; its loss favoring the stem cell phenotype and its re-expression constituting a possible therapeutic strategy (Bao et al., 2012). The mechanism for miR-101's anti-tumourigenic potential is obviously complex, mediated by a diverse range of targets that are likely to vary depending on the cell type and environmental setting. It is interesting to speculate that loss of miR-101 contributes to colorectal cancer progression at least in part by increased EP4

expression (Chandramouli et al., 2012). However, since miR-101 has multiple targets that play role in cancer (i.e., fos, zeste homologue 2 (EZH2) cyclooxygenase-2 (Cox-2), N-Myc, Mcl-1), and it is likely that other post-trancriptional targets of miR-101 also play a role in colorectal carcinogenesis (Bao et al., 2012).

Conclusions:-
These results showed little doubt that aberrant expressionof LC3, STAT3 and miRNA 101 might be involved in tumor progression of Egyptian colorectal cancer and to be further related to the outcome of CRCpatients.Further, STAT3 and miRNA 101 are potential regulators of autophagy in such patients. Consequently, LC3, STAT3 and miRNA 101 may be valuable diagnostic/prognostic biomarkers in Egyptian CRC patients. Hence, when verified by large-scale studies, these markers could represent new therapeutic targets for treatment of CRC.

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Conflicts of interests:-
The authors disclose no conflicts of interests to show.

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