

Introduction: HOXB8 is a protein that was found to promote cancer proliferation and invasion. ILK is a protein kinase which has a role in carcinogenesis. FAT4 is a tumor homologue that has a role in EMT and autophagy regulation.

Aim of the study: To identify expression of Human HOXB8, Integrin-linked kinase (ILK1) and FAT homolog 4 (FAT4) in colorectal cancer (CRC) correlating their expression with pathological, prognostic and clinical parameters of CRC.

Material and methods: We assessed the expression of HOXB8, ILK and FAT4 in fifty CRC patients and ten samples from nearby non-neoplastic colonic mucosa using immunohistochemistry.

Results: The expression of HOXB8 and ILK in CRC was positively associated with high tumor grade, advanced tumor stage, lymph node involvement ($p < 0.001$), occurrence of distant metastases ($p = 0.003$ and 0.024 respectively), higher incidence of tumor recurrence ($p = 0.03$, $p < 0.001$ respectively), worse survival rates ($p = 0.038$ and 0.003 respectively). The expression of FAT4 in CRC was correlated with lower grade, early stage of the tumor, absence of lymph node involvement ($p < 0.001$) and lack of distant metastases ($p = 0.011$). High FAT4 expression was associated with absence of tumor recurrence ($p < 0.001$) and favorable survival rates ($p < 0.001$ and 0.003).

Conclusions: High immunohistochemical expression of HOXB8 and ILK in addition to low immunohistochemical expression of FAT4 was associated with unfavorable prognostic and pathological parameters of CRC.

Key words: HOXB8, ILK and FAT4, colorectal cancer, recurrence, survival.

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Prognostic and clinic-pathological significances of HOXB8, ILK and FAT4 expression in colorectal cancer

Abdulwahab A. Abuderman¹, Ola A. Harb², Loay M. Gertallah³

¹Department of Basic Medical Sciences, College of Medicine, Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia

²Department of Pathology, Faculty of Medicine, Zagazig University, Zagazig, Egypt

³Department of General Surgery, Faculty of Medicine, Zagazig University, Zagazig, Egypt

Introduction

Colorectal cancer (CRC) has been ranked as the 4th cause of cancer related mortalities in the United States [1]. Nearly about 20% of CRC patients were diagnosed late at an advanced stage. Although there is improvement in CRC treatment strategies most CRC patients experienced disease progression and recurrence which results in low survival rates and a poor clinical outcome [2]. Therefore identifying efficient novel biomarkers in addition to discovering the mechanisms of CRC early diagnosis and targeted therapy is needed to improve patients' prognosis [3]. There are many mechanisms which are involved in CRC progression and chemo-resistance and the recently incriminated mechanisms are the cancer stem cells (CSCs) and epithelial-mesenchymal transition (EMT) [4, 5]. EMT is the main mechanisms used by cancer cells for invasion and metastases [6]. Moreover generation of cells with properties of CSC is responsible for CRC invasion, progression, metastasis and resistance to chemotherapy. Thus, targeting CSCs and EMT signaling could be able to provide a novel pathway of treatment of CRC [7]. The gene family homeobox (Hox) encodes proteins which are transcription factors and are found in nearly all eukaryotic cells [8]. There are about 39 identified Human HOX genes which are associated with development, progression and metastasis of many malignant tumors [9]. The HOX family member HOXB8 is located on 17 chromosomes. HOXB8 was found to promote cancer proliferation, invasion and metastasis through activation of EMT [2]. Integrin-linked kinase (ILK) is considered a serine/threonine protein kinase which was discovered in the inter-cellular adhesions and was found to play an essential role in interactions between cell and matrix in carcinogenesis, thus it might represent an important point of research regarding discovering novel anti-cancer targeted therapies [10]. The Fat family gene members were primarily detected in *Drosophila* as members of cadherin family with properties of cancer suppression [11]. FAT homolog 4 (FAT4), was first identified as a tumor suppressor homologue through EMT and autophagy regulation in many cancer cells [12].

In this study we aimed to identify the expression of the novel EMT and CSC markers; HOXB8, ILK and FAT4 in CRC and adjacent non-neoplastic tissues correlating their expression with pathological, prognostic and clinical parameters of CRC and association with progression and chemotherapy resistance.

Material and methods

To perform the current prospective study, we included samples from fifty CRC patients and ten samples from nearby non-neoplastic colonic mucosa. CRC patients were admitted and operated in General Surgery Depart-

ment, Faculty of Medicine Zagazig University hospitals and other private hospitals where left hemi-colectomy, right hemi-colectomy or total colectomy were performed according to site of the tumor and surgically excised specimens were sent to Pathology Department Faculty of Medicine, Zagazig University where they were processed, diagnosed, subtyped, graded and staged [13].

Inclusion criteria: paraffin blocks containing sufficient samples from all CRC patients diagnosed with adenocarcinoma with different grades and stages which were suitable for immunohistochemical staining and evaluations were included.

Exclusion criteria: we excluded paraffin blocks containing insufficient material for staining, extremely necrotic samples or contain other types of cancer colon.

We acquired clinical data as age and sex of the patients before including them in the study. We obtained follow-up data of included patients during period of follow-up in the period from December 2016 to December 2019.

Ethical approval was obtained from the local ethical committee (institutional review board) of Faculty of Medicine, Zagazig University for performance of the present study.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as formerly mentioned [14]. We used primary rabbit poly clonal antibodies; anti-HOXB8 antibody (Cat No. GTX51728; dilution 1 : 100), anti-FAT4 antibody (Cat No. ab130076; dilution 1 : 100) and anti-ILK (Cat No. ab233242 dilution 1 : 100) [2, 10, 12].

The used positive controls were basal cell carcinoma, breast carcinoma and non-neoplastic gastric mucosa tissues were done for all cases.

Evaluation of HOXB8, ILK and FAT4 immunohistochemical expression

We obtained samples from margins of the tumor or most viable areas avoiding areas of necrosis which mostly located in the center of the tumor.

HOXB8 was found and evaluated in the nuclei of tumor cells while ILK and FAT4 were found and evaluated in the cytoplasm of tumor cells. The intensity of markers expression in stained sample was scored as followed: 0, 1, 2, and 3 points which referred to; no stain, minimal stain,

moderate stain and strong stain respectively. The extent of markers expression was determined as previously described [12]; 0 (0%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%) to reach the final score of markers expression we have multiply intensity and extent score giving scores from 0-12 taking the 4 as cut point above which is high expression and below which is high expression.

Statistical analysis

All data of patients included in the present study were collected, summarized, and statistically analyzed via using Statistical Package for Social Science (SPSS) program version 24. We have calculated differences between qualitative variables through using χ^2 and Fisher exact tests, correlated them using; Spearman's ρ rank correlation test. The performed statistical correlations were two sided and p -value ≤ 0.05 was considered significant, $p < 0.001$ as considered highly significant. We analyzed correlations between HOXB8, ILK and FAT4 and collected data. Kaplan and Meier survival curves was used for estimating overall (OS) and progression free survival (PFS) rates and were stratified according to studied markers.

Results

We have included sixty tissue specimens in our study; 50 of them were retrieved from CRC patients of different histopathological subtypes, different grades and stages and 10 specimens were retrieved from adjacent non-neoplastic colonic mucosa (Table 1).

Demographic characteristics of included CRC patients were detailed in Table 2. The 50 CRC cases included 28 (63.3%) males and 22 (36.7%) females.

HOXB8 was expressed in CRC more than adjacent non-neoplastic colonic mucosa ($p = 0.046$). The expression of HOXB8 in CRC was significantly associated with large tumor size, high tumor grade, advanced tumor stage, presence of lymph node ($p < 0.001$), and distant metastases ($p = 0.003$). No significant correlation was found between HOXB8 expression with initial site of the tumor, age or sex of our patients (Fig. 1).

ILK was expressed in CRC more than adjacent non-neoplastic colonic mucosa (0.009). The expression of ILK in CRC was significantly positively correlated with large tu-

Table 1. HOXB8, ILK and FAT4 expression in all studied samples

Variables	Adenocarcinoma (n = 50) n (%)	Normal mucosa (n = 10) n (%)	Total (n = 60) n (%)	p-value
HOXB8				
Low	26 (52.0)	9 (90.0)	35 (58.3)	0.046
High	24 (48.0)	1 (10.0)	25 (41.7)	
ILK				
Low	26 (52.0)	8 (80.0)	34 (56.7)	0.009
High	24 (48.0)	2 (20.0)	26 (43.3)	
FAT4				
Low	33 (66.0)	0 (0.0)	33 (55.0)	< 0.001
High	17 (34.0)	10 (100.0)	27 (45.0)	

Table 2. Correlations between HOXB8, ILK, FAT4 expression, pathological and clinical parameters in studied colorectal cancer samples

Adenocarcinoma	HOXB8				ILK				FAT4				p-value
	Low (n = 26)		High (n = 24)		Low (n = 26)		High (n = 24)		Low (n = 26)		High (n = 24)		
	n	%	n	%	n	%	n	%	n	%	n	%	
Age group													
< 60 years	14	53.8	12	50.0	16	61.5	10	41.7	16	48.5	10	58.8	0.488
> 60 years	12	46.2	12	50.0	10	38.5	14	58.3	17	51.5	7	41.2	
Sex													
Female	9	34.6	9	37.5	11	42.3	7	29.2	13	39.4	5	29.4	0.486
Male	17	65.4	15	62.5	15	57.7	17	70.8	20	60.6	12	70.6	
Size													
< 5 cm	20	76.9	5	20.8	20	76.9	5	20.8	8	24.2	17	100	< 0.001
> 5 cm	6	23.1	19	79.2	6	23.1	19	79.2	25	75.8	0	0.0	
Initial site													
Ascend	4	15.4	10	41.7	2	7.7	12	50.0	14	42.4	0	0.0	0.002
Rect-sig	17	65.4	10	41.7	18	69.2	9	37.5	12	36.4	15	88.2	
Desc	3	11.5	2	8.3	4	15.4	1	4.2	3	9.1	2	11.8	
Trans	2	7.7	2	8.3	2	7.7	2	8.3	4	12.1	0	0.0	
Duke stage													
A	12	46.2	0	0.0	12	46.2	0	0.0	2	6.1	10	58.8	< 0.001
B	7	26.9	4	16.7	6	23.1	5	20.8	4	12.1	7	41.2	
C	6	23.1	11	45.8	6	23.1	11	45.8	17	51.5	0	0.0	
D	1	3.8	9	37.5	2	7.7	8	33.3	10	30.3	0	0.0	
Stage													
I	12	46.2	0	0.0	12	46.2	0	0.0	2	6.1	10	58.8	< 0.001
II	8	30.8	4	16.7	7	26.9	5	20.8	5	15.2	7	41.2	
III	5	19.2	11	45.8	5	19.2	11	45.8	16	48.5	0	0.0	
IV	1	3.8	9	37.5	2	7.7	8	33.3	10	30.3	0	0.0	
Distant mets													
No	25	96.2	15	62.5	24	92.3	16	66.7	23	69.7	17	100	0.011
Yes	1	3.8	9	37.5	2	7.7	8	33.3	10	30.3	0	0.0	
LN mets													
No	20	76.9	4	16.7	19	73.1	5	20.8	7	21.2	17	100	< 0.001
Yes	6	23.1	20	83.3	7	26.9	19	79.2	26	78.8	0	0.0	
Grade													
I	11	42.3	0	0.0	11	42.3	0	0.0	2	6.1	9	52.9	0.001
II	14	53.8	15	62.5	12	46.2	17	70.8	22	66.7	7	41.2	
III	1	3.8	9	37.5	3	11.5	7	29.2	9	27.3	1	5.9	

Distant mets – distant metastases, LN mets – lymph node metastases

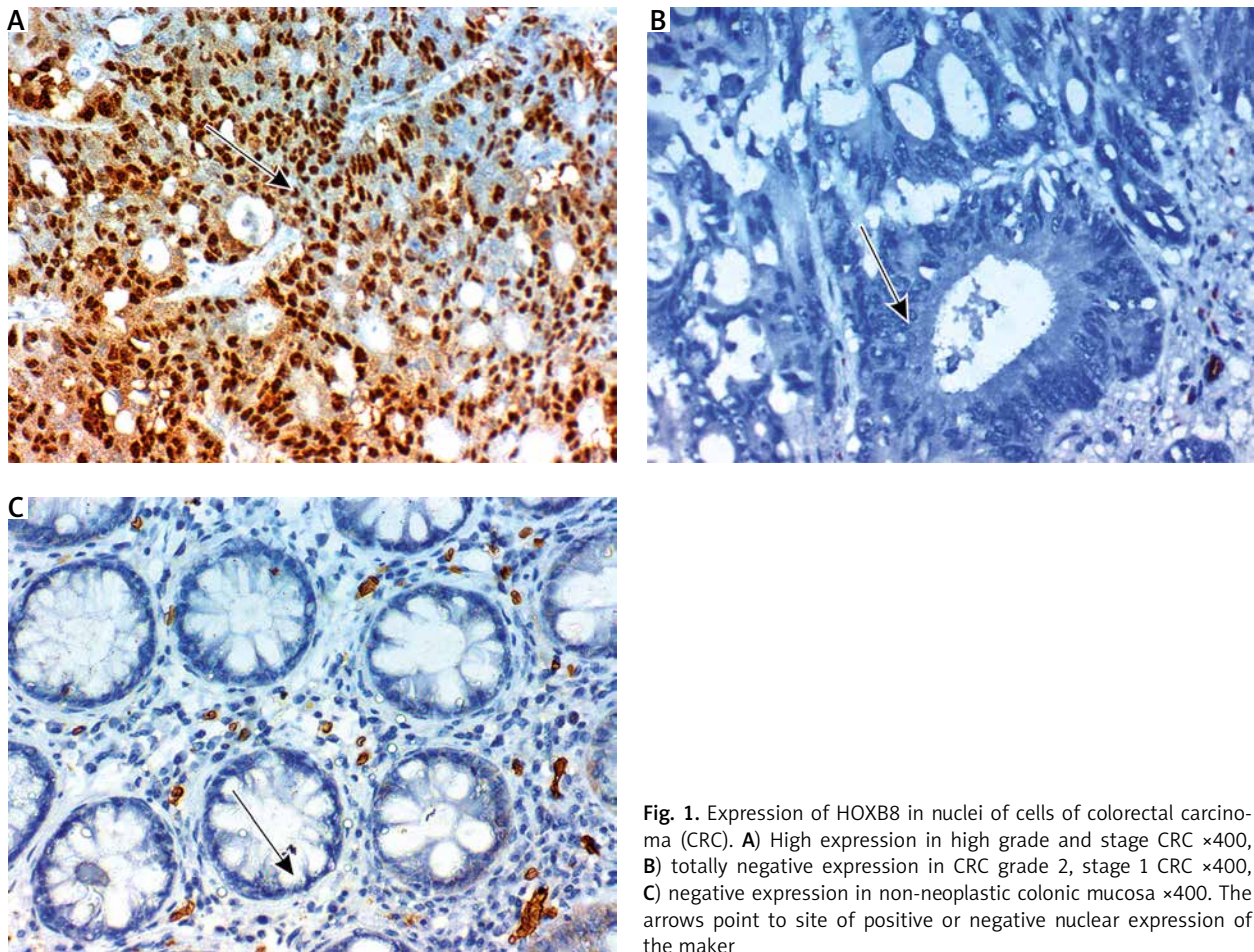


Fig. 1. Expression of HOXB8 in nuclei of cells of colorectal carcinoma (CRC). **A**) High expression in high grade and stage CRC $\times 400$, **B**) totally negative expression in CRC grade 2, stage 1 CRC $\times 400$, **C**) negative expression in non-neoplastic colonic mucosa $\times 400$. The arrows point to site of positive or negative nuclear expression of the maker

mor size, high tumor grade, advanced tumor stage, presence of lymph node ($p < 0.001$) and distant metastases ($p = 0.024$). No significant correlation was found between ILK expression, histopathological subtype of the lesion age or sex of our patients (Fig. 2).

FAT4 was down regulated in CRC more than adjacent non-neoplastic colonic mucosa ($p < 0.001$). The expression of FAT4 in CRC was associated with smaller size of the tumor, lower grade, early stage of the tumor, absence of lymph node ($p < 0.001$) and distant metastases ($p = 0.011$). No significant correlation was found between FAT4 expression and age or sex of our patients (Fig. 3).

High HOXB8 expression was significantly associated with poor response to therapy ($p = 0.026$), higher incidence of recurrence after successful treatment ($p = 0.03$), worse 3-year DFS and OS rates ($p = 0.038$ and 0.003 respectively). High ILK expression was significantly positively correlated with; poor response to therapy ($p = 0.039$), high incidence of recurrence of the tumor after successful treatment ($p < 0.001$), worse 3-year DFS and OS rates ($p < 0.001$ and 0.005 respectively). High FAT4 expression was significantly associated with absence of recurrence of the tumor after successful therapy ($p < 0.001$), good response to therapy ($p = 0.022$), favorable 3-year DFS and OS rates ($p < 0.001$ and $p = 0.003$). HOXB8 and ILK expression are positively correlated with each other $r = 0.439$ ($p < 0.001$)

and both are negatively associated with FAT4 expression $r = -0.521$ ($p < 0.001$) (Figs. 4 and 5, Tables 3 and 4).

Discussion

In the current study we tried to clarify the role of EMT and CSCs markers in CRC progression by evaluation of expression of three markers that were incriminated in EMT induction; HOXB8, ILK and FAT4. HOX genes were recently found to play a role in cancer occurrence, development, invasion and metastasis [8, 15], particularly in CRC of different stages [16]. However, the role of HOXB8; a Hox gene family member is still inconclusive. In our study we have confirmed that up-regulation of HOXB8 is found in CRC tissues more than adjacent mucosa. HOXB8 expression promoted progression and metastases of CRCs, which is similar to results of Wang *et al.* [2], who found the same results in CRC and results of Ding *et al.* [9] in gastric cancer. Similarly Wang *et al.* [2] found that HOXB8 overexpression promotes CRC proliferation and invasion, while its knocking down leads to occurrence of opposite effects. EMT is a major mechanism which is incriminated in cancer cells migration and invasion [17]. Ding *et al.* have showed that HOXB8 increased gastric cancer cells spread by EMT induction [9]. This observation indicated that HOXB8 promoted CRC cells invasion, lymphatic and hematogenous metastasis that subsequently leads to poor patients' prognosis.

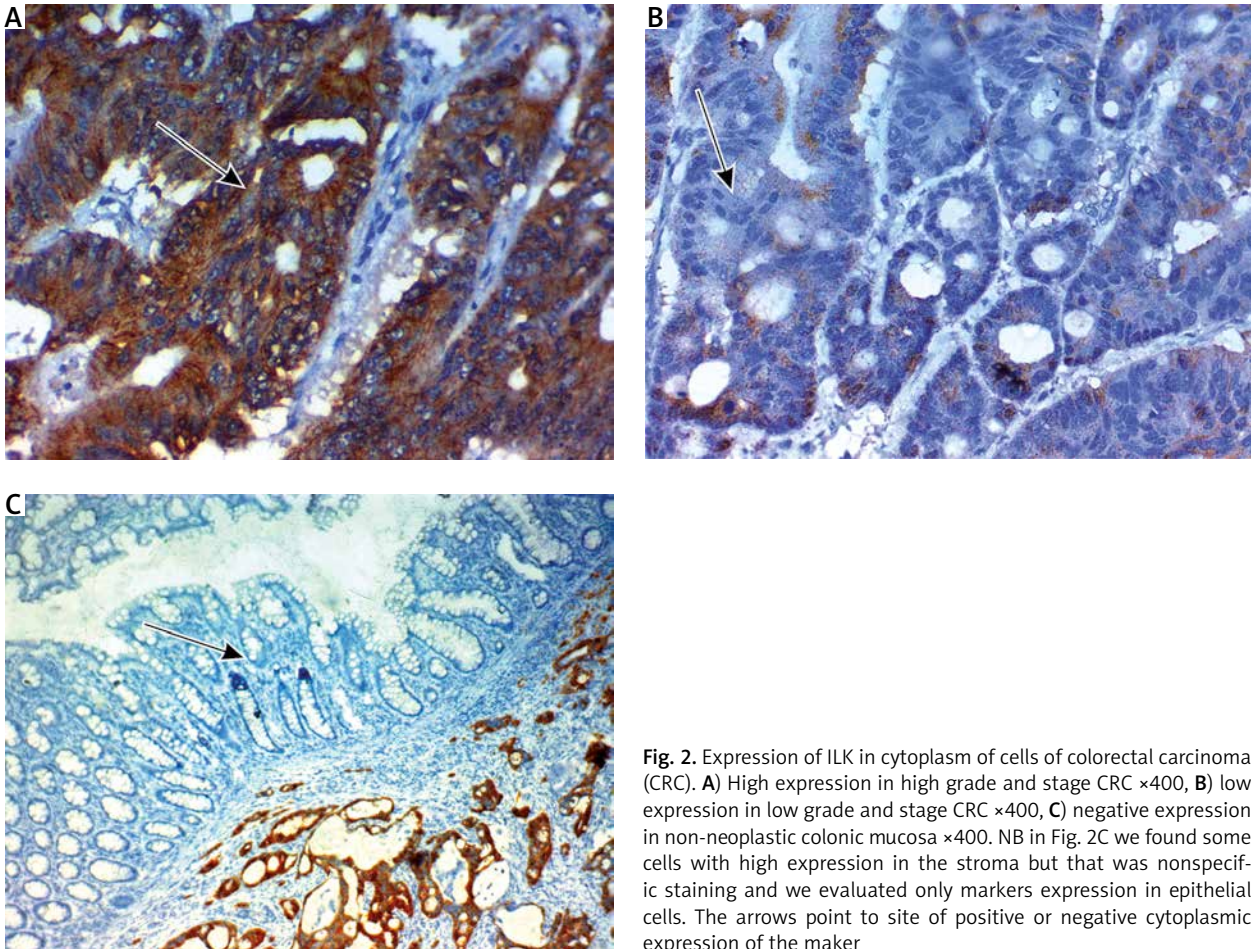


Fig. 2. Expression of ILK in cytoplasm of cells of colorectal carcinoma (CRC). **A)** High expression in high grade and stage CRC $\times 400$, **B)** low expression in low grade and stage CRC $\times 400$, **C)** negative expression in non-neoplastic colonic mucosa $\times 400$. NB in Fig. 2C we found some cells with high expression in the stroma but that was nonspecific staining and we evaluated only markers expression in epithelial cells. The arrows point to site of positive or negative cytoplasmic expression of the marker

A possible mechanism of action was showed by Wang *et al.* [2], that increased HOXB8 expression significantly activate the phosphorylated-STAT3 and STAT3 which cooperated with Twist to induce malignant invasion and metastasis through EMT induction. HOXB8 could lead to induction of EMT via STAT3 pathway activation in CRC cells denoting the essential connection between EMT and HOXB8 [18]. We showed that HOXB8 overexpression is related to chemo-resistance which is similar to results of Lu *et al.* who established that HOXB8 is accurate in predicting FOLFOX4 chemo-sensitivity in CRC patients with liver metastases [19]. Advanced CRC patients with increased HOXB8 expression have stable or progressive disease, while patients with low expression have partial response [19]. Ding *et al.* [9] showed that HOXB8 played a major role in EMT induction in gastric cancer and distant metastasis, additionally, the expression of HOXB8 in gastric cancer cells was more than non-neoplastic mucosa [9]. A previous study has shown that overexpression of HOXB8 increased tumor cell proliferation [20]. Ding *et al.* [9] pointed to that HOXB8 not only induce EMT, but its inhibition leads to MET. Due to few studies regarding roles of HOXB8 in CRC we assessed the expression of other biomarkers to prove its roles. We have assessed the expression of ILK as previous studies showed that ILK is implicated in CRC carcinogenesis and represented an important Wnt/ β -catenin pathway regulation [10]. ILK's was previously found to be

involved in CRC carcinogenesis through induction of EMT and causing chemo-resistance in many cancers, so we hypothesized that ILK might have a role in CRC progression and chemo-resistance [20, 21].

In the present study we stated that overexpression of ILK in CRC was related to poor prognosis, dismal outcome, cancer progression and chemo-resistance which was similar to Tsoumas *et al.* [10]. We showed that ILK overexpression in CRC is related to high grade, advanced stage and it is considered a predictor of lymph node metastasis proving its role in CRC progression [10, 22]. Moreover, we showed that ILK expression in our samples is positively correlated with HOXB8 expression that is an EMT biomarker, similarly Tsoumas *et al.* [10] showed that ILK is associated with EMT and CSCs biomarkers in CRC cells and that expression of ILK was related to altered expression of β -catenin and E-cadherin. Collectively these results suggested that high expression of ILK induced CSC and EMT phenotype in CRC cells and related to tumor progression as was previously stated [23]. Moreover, Tsoumas *et al.* found that inhibition of ILK in CRC cells inhibit the main EMT regulator Snail [10]. ILK was found to stimulate stem cell characteristics in cancer cells [24]. We showed that ILK overexpression is related to poor response to chemotherapy. In agreement with our results, ILK has been incriminated in chemo-resistance in other malignancies as cancer lung and glioma [21, 22]. Our data and results of previous studies pointed to that

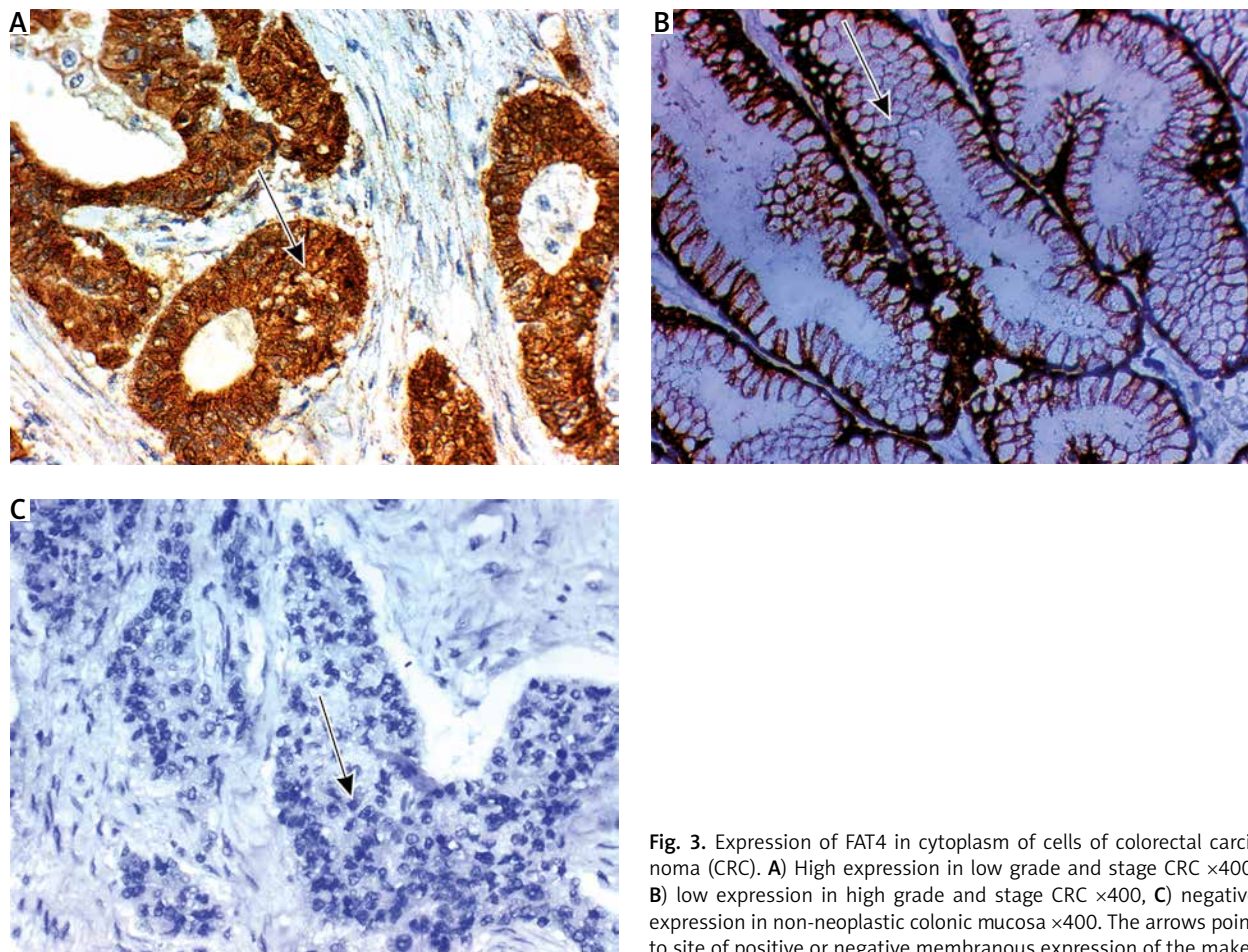


Fig. 3. Expression of FAT4 in cytoplasm of cells of colorectal carcinoma (CRC). **A)** High expression in low grade and stage CRC $\times 400$, **B)** low expression in high grade and stage CRC $\times 400$, **C)** negative expression in non-neoplastic colonic mucosa $\times 400$. The arrows point to site of positive or negative membranous expression of the marker

ILK targeting ILK could have a synergistic effect with currently used chemotherapeutic agents in many cancers and might be able to reduce chemo-resistance in gastric cancer [25, 26].

Autophagy is a homeostatic mechanism that occurs in response to cellular stress and it involved engulfment and recycling of unneeded cellular organelles and cytoplasmic components via lysosomal degradation pathways to maintain cellular metabolism [27]. Effect of autophagy in malignant cells in cancer progression remains controversial [28]. Autophagy inhibition could exert many effects on EMT, and FAT4 roles in autophagy in CRCs require clarification.

We assessed the expression of FAT4 in CRC and adjacent non-neoplastic tissues due its role in autophagy and correlating its expression with studied EMT and CSCs biomarkers; HOXB8 and ILK. We found that FAT4 was down-regulated in CRC cells than adjacent non-neoplastic colonic mucosa and loss of its expression was related to CRC progression which is similar to Wei *et al.* [12] in CRC and Jiang *et al.* [11] in gastric cancer. In the Jiang *et al.* [11], study, loss of FAT4 expression was related to poor prognosis, larger tumor size, deeper invasion, and presence of vascular and lymphatic invasion, lymph node and distant metastases of gastric cancer and they stated that levels of FAT4 mRNA were lower in cancer tissues than adjacent

non neoplastic gastric tissues which was near our results in CRC.

Our data is in line with former reports studies which showed the tumor suppressive role of FAT4 in many malignancies [29, 30].

Our results were explained by those Fat gene family members particularly FAT4 were considered cadherin superfamily members that controls cellular adhesion, proliferation and Hippo signaling pathway [29, 31]. Disturbances in Hippo pathway are related to development and progression of cancer [32]. Collectively we found that low expression of FAT4 in CRC is associated with its progression which resulted in poor prognosis and that FAT4 is a novel prognostic biomarker and therapeutic target for patients with CRC [33].

FAT4 was previously found to increase expression of E-cadherin and decrease N-cadherin and Twist1 expression which leads to EMT inhibition [12, 33]. These results were near our results that FAT4 expression was inversely associated with HOXB8 and ILK expression which were EMT and CSCs biomarkers [12, 33]. Thus, our study highlighted the inverse association between autophagy, EMT and CSCs in CRC tissues. Autophagy is pathway which is responsible for engulfment, digestion in addition to recycling intracellular organelles to produce energy through lysosomal degradation [34], additionally it could reduce cell damage and maintain cellular viability under stressful

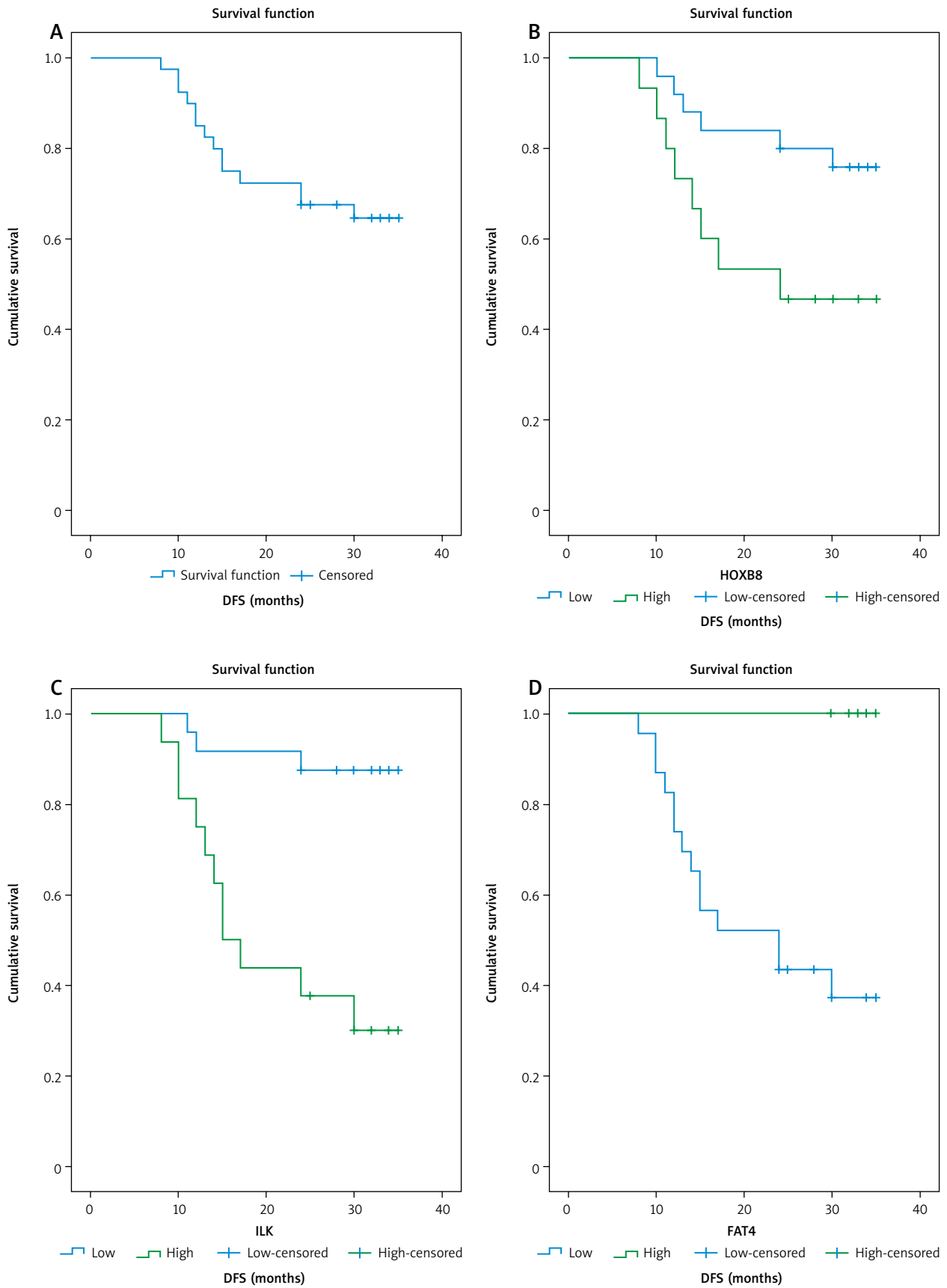


Fig. 4. Kaplan Meir survival curves of disease-free survival (DFS) of the studied colorectal carcinoma (CRC) patients. **A)** DFS rate of all the studied CRC cases, **B)** DFS rate stratified according to HOXB8 expression, **C)** DFS rate stratified according to ILK expression, **D)** DFS rate stratified according to FAT4 expression

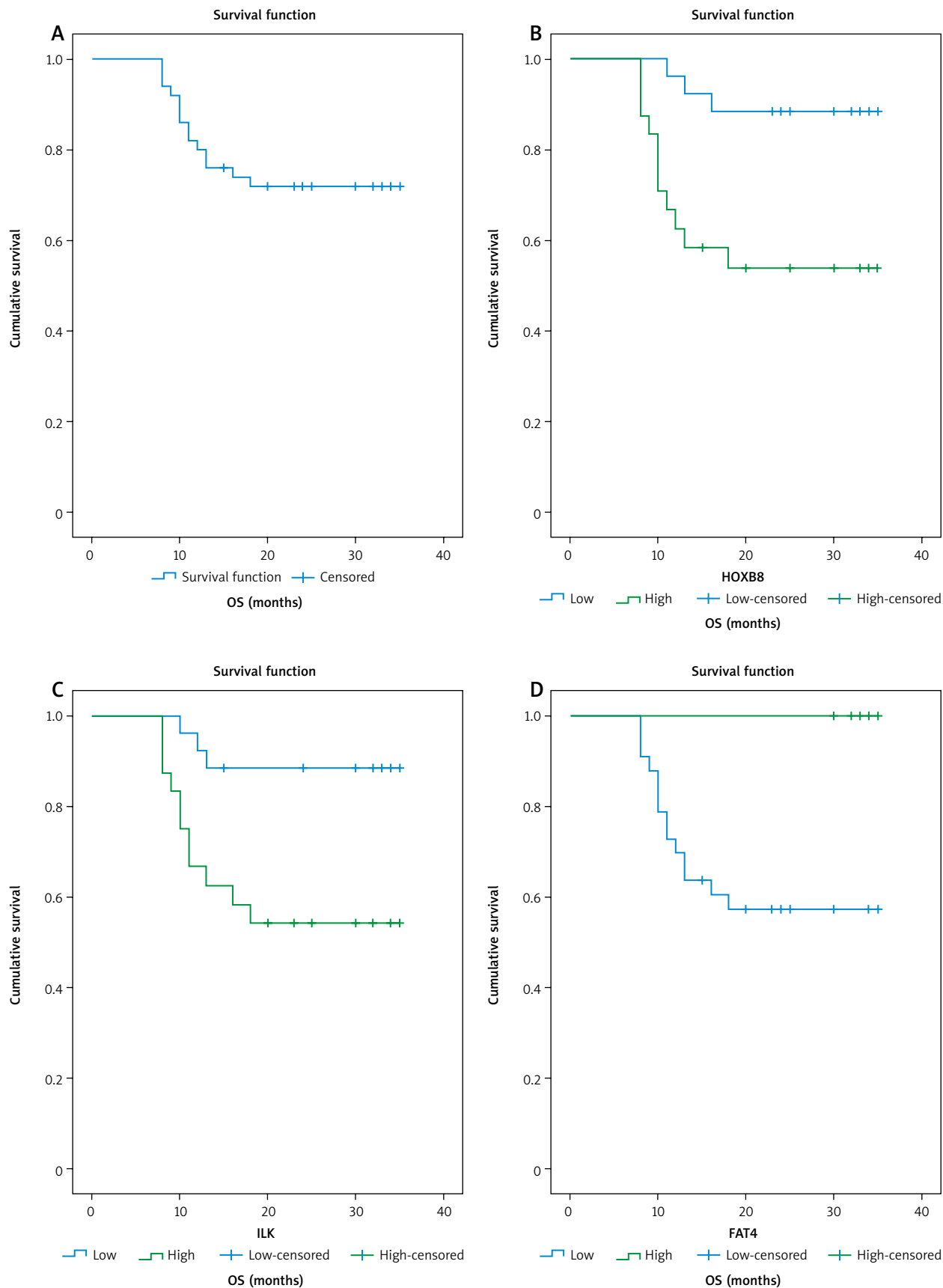


Fig. 5. Kaplan Meir survival curves of overall survival rate (OS) of the of the studied colorectal carcinoma (CRC) patients. **A)** OS rate of all the studied CRC cases, **B)** OS rate stratified according to HOXB8 expression, **C)** OS rate stratified according to ILK expression, **D)** OS rate stratified according to FAT4 expression

Table 3. Correlations between HOXB8, ILK, FAT4 expression and outcome of colorectal cancer colorectal cancer patients

Adenocarcinoma	HOXB8		p-value		ILK		p-value		FAT4		p-value				
	Low (n = 26)		High (n = 24)		Low (n = 26)		High (n = 24)		Low (n = 26)		High (n = 24)				
	n	%	n	%	n	%	n	%	n	%	n	%			
Response to treatment															
PD	1	3.8	4	16.7	0.046	4	15.4	8	33.3	0.039	10	30.3	2	11.8	0.022
SD	0	0.0	3	12.5		5	19.2	9	37.5		11	33.3	3	17.6	
PR	0	0.0	2	8.3		6	23.1	10	41.7		12	36.4	4	23.5	
CR	25	96.2	15	62.5		7	26.9	11	45.8		13	39.4	5	29.4	
Relapse															
No	19	73.1	7	29.2	0.03	8	30.8	12	50.0	< 0.001	14	42.4	6	35.3	< 0.001
Yes	6	23.1	8	33.3		9	34.6	13	54.2		15	45.5	7	41.2	
Death															
No	23	88.5	13	54.2	0.005	10	38.5	14	58.3	0.007	16	48.5	8	47.1	0.002
Yes	3	11.5	11	45.8		11	42.3	15	62.5		17	51.5	9	52.9	

PD – progressive disease, SD – stable disease, PR – partial response, CR – complete response

Table 4. Correlations between HOXB8, ILK, FAT4 expression disease-free survival and overall survival in colorectal cancer patients

Variables	3-year DFS (%)	p-value	3-year OS (%)	p-value
HOXB8				
Low	75.8	0.038	88.5	0.003
High	46.7		53.8	
ILK				
Low	87.5	< 0.001	88.5	0.005
High	30		54.2	
FAT4				
Low	37.3	< 0.001	57.3	0.003
High	100		100	

DFS – disease-free survival rate, OS – overall survival rate

conditions. The normal cells in comparison to cancer cells might face many stresses and were more dependent on autophagy so they express more autophagy biomarkers [35]. FAT4 promoted autophagy in CRC cells so reduced its progression. In Wei *et al.* [12] study, they observed that FAT4 promoted autophagy in cancer cells. Egan *et al.* [33] and Byers *et al.* [36] showed that FAT4 has a role in inhibition of EMT. Moreover inhibition of FAT4 could be able to activate Yes-associated protein (YAP) and WNT signaling pathways so it is responsible for cancer progression [11].

Up to our knowledge we are the first study that tried to identify roles of the expression of HOXB8, ILK and FAT4 in CRC. However, there are several limitations. The study included few samples of studied CRC and normal mucosa samples, with evaluation of the markers expression using immunohistochemistry only. Selection bias was existed during inclusion of cases due to little number of cases and design of the study to perform assessment of the markers semi-quantitatively by immunohistochemistry and not by other methods like genetic evaluation.

Conclusions

We found that up-regulation of HOXB8 and ILK in addition to down-regulation of FAT4 was found in CRC tissues more than adjacent non-neoplastic colonic mucosa and was associated with unfavorable prognostic and pathological parameters pointing to the possibility of using these markers as novel prognostic biomarkers and therapeutic targets for CRC to improve its prognosis and management strategies.

The authors declare no conflict of interest.

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Address for correspondence

Ola A. Harb

Department of Pathology

Faculty of Medicine

Zagazig University

11111 Zagazig, Egypt

e-mail: olaharb2015@gmail.com

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