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Long non-coding RNA EGFR-AS1 in colorectal cancer: potential role in tumorigenesis and survival via miRNA-133b sponge and EGFR/STAT3 axis regulation

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ABSTRACT

Background: Colorectal cancer is one of the most common cancers worldwide and a major cause of cancer-related death. Thus molecular biomarkers for colorectal cancer have been proposed. The role of long non-coding RNA EGFR-AS1 in colorectal cancer is still unclear. We aimed to evaluate its expression in different stages of colorectal cancer and determine any possible role in regulating the miR-133b/EGFR/STAT3 signalling pathway.

Materials and Methods: The relative expression of EGFR-AS1 and miR-133b were evaluated by quantitative real-time RT-transcription PCR in 130 colorectal cancer samples and 30 normal tissues. EGFR expression was assessed using immunohistochemistry. Furthermore, levels of p-EGFR, p-STAT3, and apoptotic proteins were determined by ELISA.

Results: Both EGFR-AS1 and EGFR overexpression were positively linked with colorectal cancer status (both $p < 0.01$), grade (both $p < 0.01$), and metastasis ($P < 0.01$ and $p = 0.019$ respectively). EGFR-AS1 and miR-133b were significantly inversely correlated ($P < 0.01$). Low expression of miR-133b was inversely associated with overexpressed EGFR and increased p-STAT3 levels. EGFR-AS1 was an independent prognostic factor for survival of colorectal cancer patients ($P < 0.01$, HR 2.06; 95% CI 1.32–3.19) where low EGFR-AS1 expression was associated with higher survival rate ($p = 0.003$).

Conclusion: EGFR-AS1 may have a role in colorectal cancer by regulation of miR-133b/EGFR/STAT3 signalling. It may be a potential biomarker for early diagnosis and predicting the survival rate of colorectal cancer.

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Colorectal cancer; epidermal growth factor receptor-antisense RNA 1; microRNA-133b; Epidermal growth factor receptor; signal Transducer and Transcription Activator 3

Introduction

Colorectal cancer is the third most common cancer and the fourth most common cause of cancer-related death, with 700,000 deaths per year [1]. The main risk factor is age: past the fifth decade , the risk of developing colorectal cancer is markedly increased [2]. Environmental exposures associated with an increased risk include history of abdominal radiation, smoking, alcohol use, and diet [3]. These risk factors are believed to increase the rate at which genetic mutations occur in various oncogenes and tumour suppressor genes, and/or result in growth-promoting epigenetic modifications [4].

The human epidermal growth factor receptor (EGFR) family consists of four members: HER1 (ErbB-1 or EGFR), HER2 (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). EGFR, a critical receptor tyrosine kinase, is a key factor in epithelial malignancies and one of the most common targets in the treatment of metastatic colorectal cancer [5]. Stimulation of EGFR by its agonists, mediates multiple downstream signalling cascades that control cell proliferation, apoptosis and migration [6]. The overexpression or aberrant kinase activity of EGFR could result in unregulated growth stimulation, tumorigenesis, and metastasis in various tumour types [7].

Signal Transducer and Transcription Activator 3 (STAT3) is one of the most important downstream effectors of EGFR [8]. Inappropriate/persistent activation of STAT3 plays a critical role in multiple neoplasms, such as colorectal, breast and lung cancer [9]. It mediates a variety of cellular functions, including cell differentiation, metastasis, angiogenesis, apoptosis and immune response [10].

miRNAs may function as tumour suppressors or oncogenes that regulate cancer cell proliferation, migration, apoptosis and metastasis [11]. Although several miRNAs have been involved in colorectal cancer, roles in metastatic disease are unclear [12]. miR-133b has been reported as a tumour suppressor in gastric, bladder, prostate and lung cancer [13]. However, the precise molecular mechanisms regulating miR-133b expression in colorectal cancer remain to be elucidated. Recent studies have shown the interactions between miRNAs and other epigenetics

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mechanisms, including long ncRNAs (lncRNAs) [14]. LncRNAs, a class of functional RNAs that transcripts more than 200 nucleotides in length and without protein coding potential have provided an important new perspective in gene regulation [15]. Epidermal growth factor receptor-antisense RNA 1 (EGFR-AS1) is an oncogenic lncRNA present in several types of cancer, such as liver cancer, gastric cancer and renal cell carcinoma [16].

Despite recent advances in the screening, treatment and prognosis of colorectal cancer, a better understanding of the biological mechanisms and molecular markers underlying colorectal cancer progression is still urgently needed. We hypothesized alterations in the EGFR-AS1/miR-133b/EGFR/STAT3 axis in colorectal cancer, linked to clinical features and survival.

Subjects and methods

All procedures were in accordance with the ethical guidelines of the 2008 Declaration of Helsinki and accepted from the Local Research Ethics Committee of Faculty of Medicine, Tanta University. All subjects signed informed written consent.

We tested our hypotheses on 130 cases with colorectal carcinoma during the period from December 2016 to April 2020. These were 86 males and 44 females with a mean [SD] age of 57.5 [7.9]. Inclusion criteria were diagnosed with colorectal cancer cases through either colonoscopy and/or surgical resection, and colonic biopsy. Exclusion criteria were other concomitant or previous malignancy and serious diseases. All patients were prepared prior to surgery by routine pre-operative investigations and pre-operative colonic preparation. All patients were evaluated for intraoperative tumour size, site, mobility, lymph node (LN) metastasis and vascular invasion. Final diagnosis was achieved by gathering medical history, clinical examination, endoscopic results, intraoperative data and histopathological examination. All patients received adjuvant treatment postoperatively, and routine follow up was applied in the outpatient clinic according to published guidelines.

Normal colorectal tissue specimens were sampled from 30 subjects referred for endoscopy secondary to bowel symptoms. These were 13 males and 17 females aged 56.9 [8.4] years. The control subjects displayed no chronic diseases and reported no family history of cancer. None of the control subjects were on medication at the time of study.

Received colectomy specimens were dissected, washed with ice cold saline, and then divided into pieces. One piece was stored in at – 80 °C for RNA extraction. Another piece was stored for colonic tissue homogenization. The last piece was immersed in 10% formaldehyde solution, processed, embedded in paraffin, cut into 5-µm sections and then stained with Hematoxylin & Eosin (H&E) for confirmation the diagnosis, grading the colonic carcinoma and immunohistochemical evaluation. Tissue samples were homogenized in 10% (w/v) 50 mM phosphate buffer (pH 7.4) for 20 sec. The homogenates were then centrifuged at 5000 × g for 5 min at 4 °C and the supernatants were stored at −80 °C for further processing.

Phosphorylated- epidermal growth factor receptor (p-EGFR) level in colonic tissue homogenates was assessed using a p-EGFR ELISA Kit (MyBiosource, San Diego, USA), phosphorylated-Signal transducer and activator of transcription 3 (p-STAT3) was measured using an RayBio® Human/Mouse/Rat Phospho-STAT 3 (Y705) ELISA Kit (RayBiotech, GA, USA). Survivin and Caspase 3 levels were assayed in colonic tissue samples by ELISA (MyBiosource, San Diego, CA, USA). and total protein concentrations in colonic tissue samples were assessed according to Lowry et al. [17].

Quantitative analysis of EGFR-AS1 and miR-133b expression by quantitative real-time PCR (RT-PCR) was as follows. Total RNA isolation from colonic specimens was performed by Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Assessment of RNA concentration and purity was performed by measuring OD260 and OD260/280 ratio, respectively using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA). RNA was then kept frozen at −80 °C. Extracted RNA was reverse- transcribed using RevertAid H Minus Reverse Transcriptase (Thermo Scientific, USA) producing cDNA which was used as a template, using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA). cDNA was used to detect the relative expression of the EGFR-AS1 (NCBI GenBank Nucleotide accession # NR_047551.1) *and* miR-133b (NCBI GenBank Nucleotide accession # NR_029903.1) genes using StepOnePlus real time PCR system (Applied Biosystem, California, USA). EGFR-AS1 primer sequence was: Forward 5′- AATTACCTGGGGCCTTCTTGGA-3′, Reverse 5′-AGCCATGTTAGACCTCAACAAG-3′. Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control, with primer sequence Forward 5′-ACACTCATGATGGACTCGCTGTCA-3′, Reverse 5′-

Table 1. Biochemical analysis of the studied parameters.

	Controls (n = 30)	Colorectal cancer group (n = 130)	p Value
Relative EGFR-AS1 mRNA expression	0.98 ± 0.03	1.96 ± 0.25	0.001
Relative miR-133b mRNA expression	1.03 ± 0.09	0.61 ± 0.50	0.001
p-EGFR (ng/mg protein)	0.48 ± 0.17	2.10 ± 0.66	0.001
p-STAT3 (ng/mg protein)	0.92 ± 0.14	5.94 ± 1.41	0.003
Survivin (ng/mg protein)	87 ± 6	212 ± 36	0.001
Caspase 3 (ng/mg protein)	5.4 ± 0.4	1.5 ± 0.9	0.01

Data are mean ± SD.

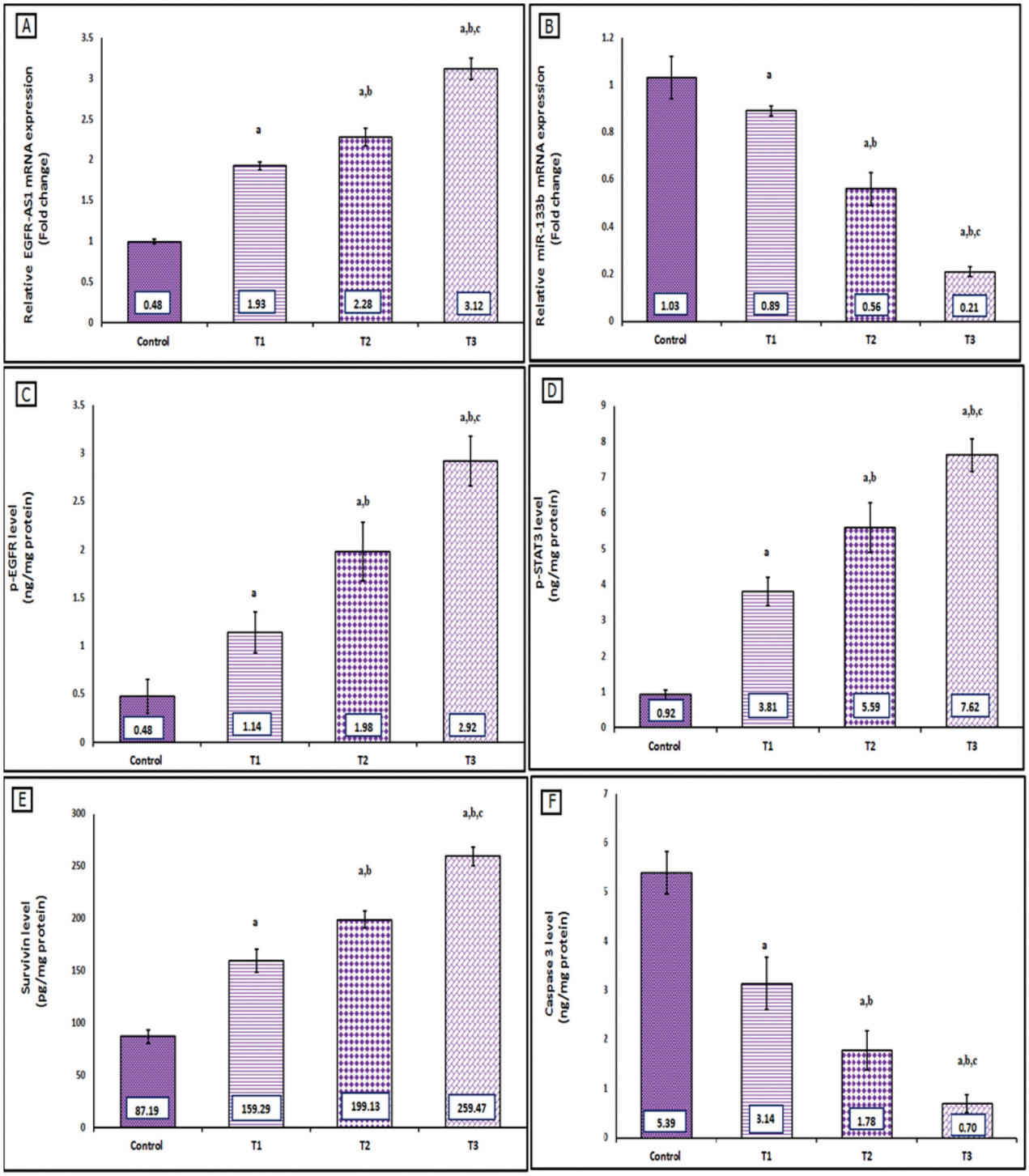

Figure 1. Biochemical analysis of the studied parameters. (A) Relative EGFR-AS1 mRNA expression, (B) Relative miR-133b mRNA expression, (C) pEGFR, (D) pSTAT, (E) Survivin, (F) Caspase 3 levels in colorectal cancer patients and control group. Values are mean ± SD. P value was calculated by one-way ANOVA test followed by Tukey's post hoc test. a Mean difference vs control group ($P < 0.05$). b Mean difference vs T1 ($P < 0.05$). c Mean difference vs T2 ($P < 0.05$).

TAAGCTCTTAGAGGCTCATGT-3′; miR-133b primer sequence was: Forward 5′- CTTTGGTCCCCTTCAACCA-3′, Reverse 5′-GTGCAGGGTCCGAGGT-3′. *U6-snRNA* was used as an endogenous control with primer sequence Forward: 5′-CTCGCTTCGGCAGCACA-3′ and Reverse: 5′-AACGCTTCACGAATTTGCGT-3′. The cycle threshold (Ct) values for target genes and the housekeeping gene were estimated, relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method [18].

Paraffin-embedded sections were immunostained for EGFR. After dewaxing, inactivating endogenous peroxidase activity and blocking cross-reactivity with normal serum, an overnight incubation was performed in a humidity chamber with mouse mAb anti-EGFR

Table 2. EGFR-AS1 and miR-133b mRNA expressions in relation to patient and tumour characteristics.

		EGFR-AS1 mRNA Expression					miR-133b mRNA Expression				
		High expression		Low Expression			High expression		Low Expression		
		N = 100	76.9%	N = 30	23.1%	p-value	N = 36	27.69%	N = 94	72.31%	p-value
Age	<60	46	46%	18	60%	0.442	16	44.4%	48	51.1%	0.865
	≥60	54	54%	12	40%		20	55.6%	46	48.9%	
Sex	Male	62	62%	24	80%	0.503	22	61.1%	64	68.1%	0.602
	Female	38	38%	6	20%		14	38.9%	30	31.9%	
Vascular invasion	No	36	36%	26	86.7%	0.001	36	100%	26	27.6%	0.001
	Yes	64	64%	4	13.3%		0	0%	68	72.4%	
Tumour Status	T1	6	6%	14	46.7%	0.001	10	27.8%	10	10.6%	0.001
	T2	54	54%	16	53.3%		26	72.2%	44	46.1%	
	T3	40	40%	0	0%		0	0%	40	42.3%	
Tumour Grade	I	0	0%	10	33.3%	0.004	8	22.2%	2	2.1%	0.001
	II	70	70%	20	66.7%		28	77.8%	62	66.0%	
	III	30	30%	0	0%		0	0%	30	31.9%	
Nodal Status	Negative	34	34%	26	86.7%	0.001	34	94.4%	26	27.6%	0.001
	Positive	66	66%	4	13.3%		2	5.6%	68	72.4%	

Table 3A. EGFR expression in relation to patient and tumour characteristics.

		EGFR Expression		
		Negative	Positive	
		N = 34 (26%)	N = 96 (74%)	p-value
Age (years)	<60	20 (59%)	44 (46%)	0.357
	≥60	14 (41%)	52 (54%)	
Sex	Male	22 (65%)	64 (67%)	0.883
	Female	12 (35%)	32 (33%)	
Vascular invasion	No	16 (47%)	46 (48%)	0.951
	Yes	18 (53%)	50 (52%)	
Tumour Status	T1	14 (41%)	6 (6%)	0.01
	T2	16 (47%)	54 (56%)	
	T3	4 (12%)	36 (38%)	
Tumour Grade	I	6 (18%)	4 (4%)	0.005
	II	28 (82%)	62 (65%)	
	III	0	30 (31%)	
Nodal Status	Negative	24 (71%)	36 (37.5%)	0.019
	Positive	10 (29%)	60 (62.5%)	

Table 3B. Relation of EGFR staining scores to grades and lymph node status of colorectal cancer cases.

	Grade I		Grade II		Grade III		
EGFR scores	N	%	N	%	N	%	p-value
0	4	40	20	22.2	0	0	0.011
+1	2	20	8	8.8	0	0	
+2	4	40	40	44.5	10	33.3	
+3	0	0	22	24.5	20	66.7	
	Without LN metastasis		With LN metastasis				
EGFR scores	N	%	N	%			p-value
0	14	23.3	4	5.7			0.014
+1	10	16.7	6	8.7			
+2	24	40	20	28.6			
+3	12	20	40	57.0			
Total	60	100	70	100			

Abbreviations: EGFR: Epidermal growth factor receptor, LN: lymph node.

(1:200, Clone H11; Daco, USA), followed by washing in PBS and then covered with 2–3 drops of secondary antibody, incubated for 10 min at room temperature, then washed in PBS and counterstained with haematoxylin. As positive controls, sections from human oesophageal tissue were used. Negative controls were prepared by primary antibody with PBS and normal mouse serum. Cytoplasmic and cytoplasmic membrane brownish staining was considered positive. Immunoreactivity was scored as negative (0: no staining or 1+: Weak staining of more than 10%of tumour cells) and positive. Positive immunoreactivity was graded as 2+: Moderate staining of more than 10% of tumour cells and 3+: Strong staining of more than 10% of tumour cells [19].

Statistical analysis used SPSS v 21. Continuous quantitative variables are expressed as mean ± SD. Degree of gene expression with tumour characteristics was performed by the chi-square test. Student's t-test was used for comparing the results of the two groups. Analysis of variance (ANOVA) was used for multiple comparisons between different groups. Kaplan–Meier method and the log-rank test were used to analyse survival rate and compare the survival distribution between patients with high and low EGFR-AS1 expression. Survival data were evaluated using multivariate Cox's regression analysis based on studied biochemical variables. Receiver Operating Characteristic (ROC) curve was done for the optimized cut-off point detection for relative EGFR-AS1 and miR-133b mRNA expression. P-values <0.05 was considered statistically significant.

Results

The 130 cases and 30 controls were matched for age ($p = 0.25$) but not sex ($p = 0.02$). The tumour size was (4.7 cm ±1.6). The majority of cases were T2 (70 cases). There were 60 colorectal cancers without, and 70 with regional lymph node metastasis. Colorectal cancer cases were 10 at grade I, 90 at grade II and 30 at grade III.

There was marked up-regulation of EGFR-AS1 mRNA expression with down-regulation of miR-133b in patients compared to the control group. Colorectal cancer patients also showed significantly higher levels of p-EGFR, p-STAT3 and surviving, but lower levels of caspase 3 (Table 1). These markers were strongly linked to stage of the disease (Figure 1).

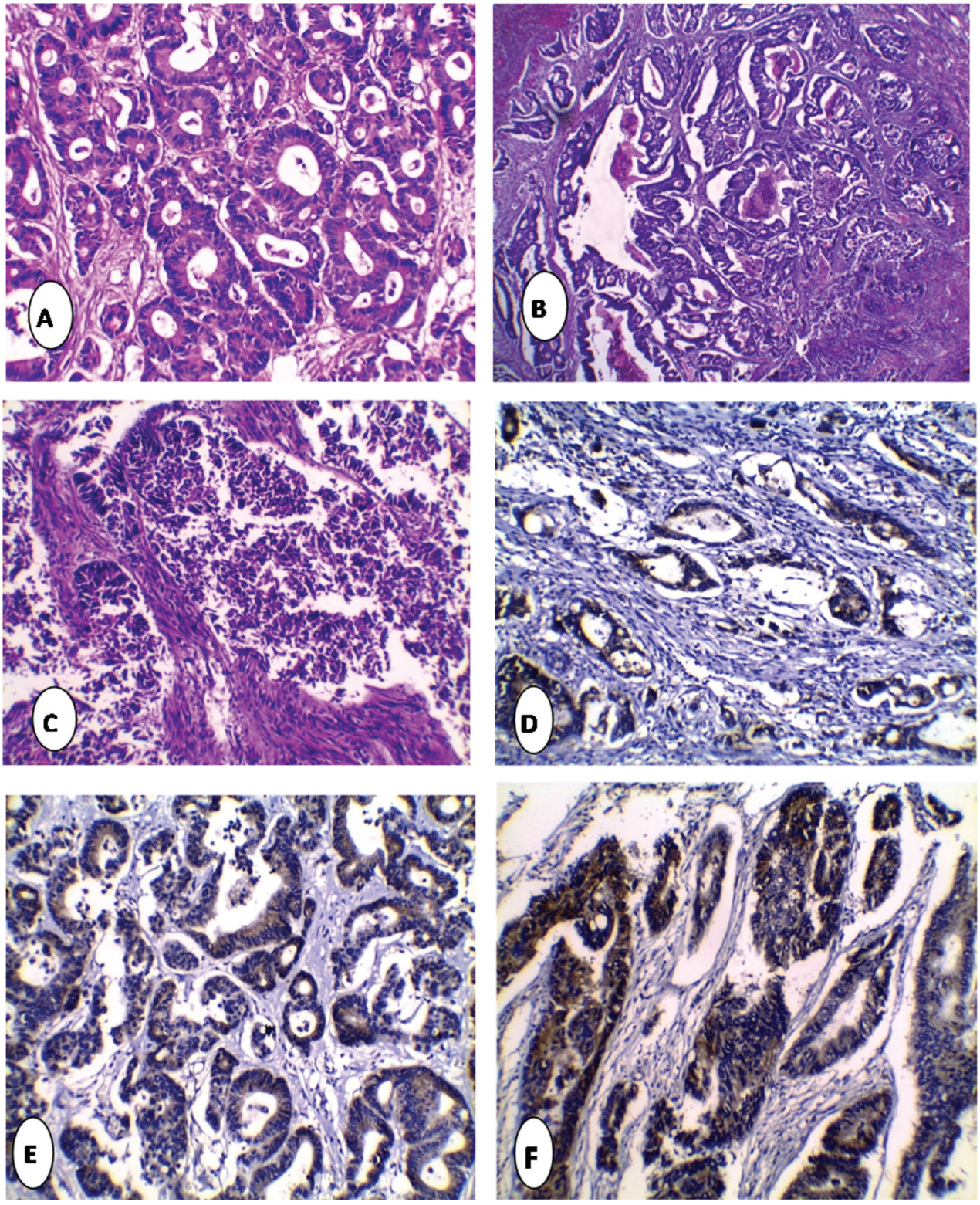

Figure 2. Histological and immunohistological staining of tumours. Grading of colorectal cancer into: (A)-grade I (B)-grade II (C)-grade III H&E; X200 and Immunohistochemical expression of EGFR into different colorectal cancer grades (D)-weak (E)-moderate (F) strong immunostaining; X200. EGFR: Epidermal growth factor receptor.

To assess the clinicopathological relevance of EGFR-AS1 and miR-133b expression, the median control group expression level was used to categorize the patients into low expression and high expression groups. As summarized in Table 2, up-regulation of EGFR-AS1 with concomitant down-regulation miR-133b was linked with tumour grade, tumour status (TNM stage), lymph node metastasis and vascular invasion but not with age and sex.

The immunohistochemical analysis revealed that EGFR expression showed a highly significant association with high grade colonic carcinoma, high tumour status and cases with lymph node metastasis as presented in Figure 2. Table 3 shows the relation of EGFR

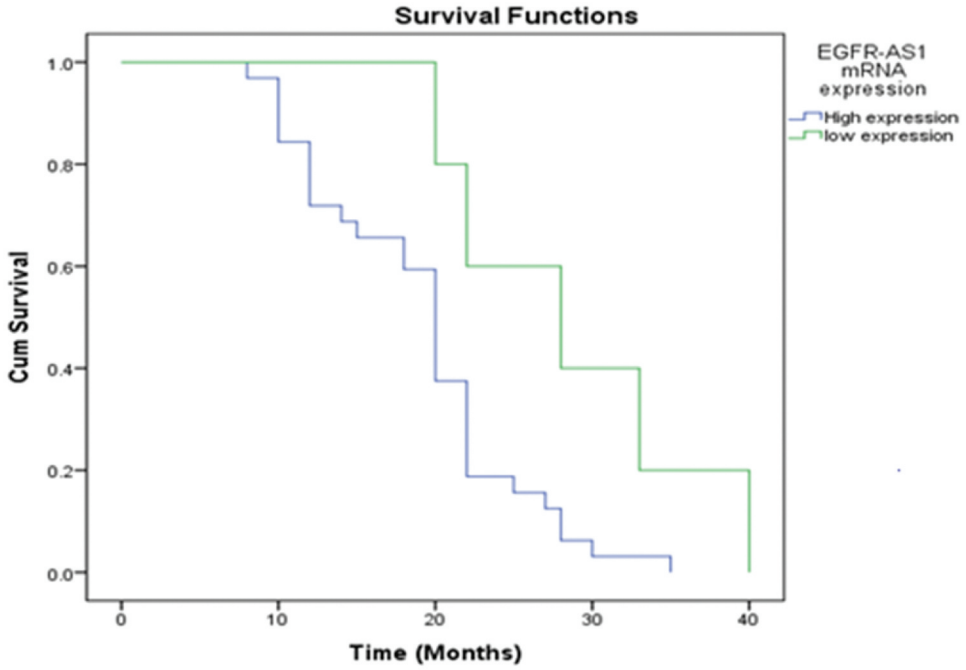

Figure 3. All survival of colorectal cancer cases based on high (>1.85) and low (<1.85) relative expression of mRNA levels.

Table 4. Multivariate analysis of prognostic factors in colorectal cancer patients.

	HR (95% CI)	p Value
EGFR-AS1 mRNA expression	2.06 (1.32–3.19)	0.001
miR-133b mRNA expression	0.08 (0.01–0.75)	0.027
Tumour Grade	3.68 (1.59–8.49)	0.002
Tumour Status	0.23 (0.07–0.75)	0.015
p-EGFR level	1.04 (0.68–1.60)	0.857
p-STAT3 level	0.96 (0.68–1.60)	0.775
Survivin level	1.00 (0.99–1.09)	0.978
Caspase 3 level	0.95 (0.74–1.23)	0.710

CI: Confidence interval, HR: Hazard Ratio

expression to clinicopathological features. EGFR showed positivity (+2 and +3) in 96 cases (73.8%). There was no significant association between EGFR expression and age (p=0.357), sex (p=0.883), or vascular invasion (p=0.951).

Patients were divided by median EGFR-AS1 mRNA expression level (1.85) into high and low expression groups. High mRNA expression exhibited a poorer outcome survival (P = 0.003), compared with low expression (Figure 3).

Multivariate analysis using Cox's proportional hazard model showed that EGFR-AS1 and miR-133b mRNA expression levels, tumour histological grade and tumour status were all independent predictors of prognosis (Table 4).

The sensitivity and specificity of relative EGFR-AS1 and miR-133b mRNA expression was evaluated using ROC curve analysis. The optimal cut-off point for EGFR-AS1 mRNA expression was 1.92 with sensitivity 98% and specificity 91%. In miR-133b mRNA expression, the optimal cut-off point was 0.89 with sensitivity 91% and specificity 92% for discriminating colorectal cancer from healthy controls. Both AUCs were 0.98, P < 0.001.

Discussion

Colorectal cancer is a global burden; its prevalence has greatly increased significantly over the last decade and ranks third among all cancer types [20]. It is widely believed that colorectal cancer develops over an extended period of time in multi-step process [21]. Obtaining a colorectal cancer tumour biopsy is a routine procedure and that transforms this type of malignancy into an ideal model for the study of pathogenesis of cancer [22].

An emerging topic in the field of EGFR signalling is the role of the upstream regulatory proteins that mitigate signalling following EGFR activation [23]. Recent work has focused on lncRNAs as newly discovered key players involved in the development of various human diseases, especially cancer [11]. We add to the literature, showing that that EGFR-AS1 is significantly up-regulated in colorectal cancer patients and positively associated with advanced TNM stage and lower survival rate. We speculate that EGFR-AS1 may promote colorectal cancer development and metastasis and affects its prognosis. In addition we demonstrate that both EGFR expression the levels of phosphorylated EGFR were also increased significantly in late stages of colorectal cancer with lymph node metastasis. The positive correlation between EGFR-AS1 and EGFR suggests an oncogenic role for EGFR-AS1 , in agreement with previous studies [24].

Growing evidence has indicated that lncRNAs modulate cancer cell proliferation, migration, and invasion [25]. EGFR-AS1, which is transcribed on the antisense strand of EGFR and shares a complementary sequence

with EGFR, has been shown to have different functions depending on the type of cancer [26]. EGFR-AS1-modulated malignancy was accomplished by up-regulation of EGFR by increasing the stability of EGFR mRNA, thus promoting the proliferation of cancer cell [27].

In addition, our data point to a molecular mechanism of EGFR-AS1 up-regulated EGFR expression. LncRNAs may act as 'miRNA sponges' and thus regulate the expression of miRNA target genes [28]. We revealed a negative association between the expression EGFR-AS1 and miR-133b and speculate that EGFR-AS1 may form base pairing with miR-133b to act as a sponge to miR-133b, resulting in up-regulation of EGFR. Notably, Zeng et al., revealed that EGFR was a direct functional target of miR-133b in oesophageal squamous cell carcinoma (ESCC), confirming the tumour suppressor role of miR- 133b in the progression of ESCC by EGFR down-regulation [29].

Activated EGFR stimulates multiple downstream signalling pathways, resulting in tumour growth and metastasis [30]. As one of the most important downstream effectors, STAT3 can be phosphorylated by activated EGFR, and can then translocate into the nucleus to exert transcriptional regulations, primarily contributing to cell proliferation, apoptosis resistance and angiogenesis [31]. This is in agreement with our study that revealed significant increase in pSTAT3 levels in patients with advanced cancer.

The STAT family, particularly STAT3, maintains a pro-carcinogenic microenvironment during malignant transformation and cancer progression [32]. Song et al., highlighted the relationship between EGFR and STAT3 and illustrated that EGFR was a positive STAT3 regulator and regulated cell proliferation in triple negative breast cancers (TNBC) and revealed that EGFR/STAT3 axis as a target for TNBC treatment [33].

In agreement with previous studies [34], our study showed that increased pSTAT3 is associated with increased survivin level and inversely associated with caspase 3 level in progressive stages of colorectal cancer suggesting a role of pSTAT3 activation in tumour development and progression.

Several studies have delineated the impact of EGFR-AS1 and miR-133b on apoptosis. Dong et al. revealed that EGFR-AS1 knockdown significantly induced apoptosis in glioma cells [35]. Zhou et al. demonstrated that miR-133b overexpression induced apoptosis in renal cell carcinoma by suppression of pSTAT and down-regulation of Bcl-2 [36].

We are unaware of other data demonstrating the role of EGFR-AS1 in pathogenesis, development, and prognosis of colorectal cancer. Overall, we speculate that overexpression of EGFR-AS1 with subsequent sponge of miR- 133b may contribute to the initiation and progression of colorectal cancer. This may operate through up-regulation and phosphorylation of EGFR with further activation of STAT3 signalling.

Our data represents an advance in biomedical science as it provides a framework for the potential of EGFR-AS1 as an oncogene in colorectal cancer pathogenesis and predicting its survival rate, and so may be a novel therapeutic strategy.

Summary table

What is known about this subject:
- Colorectal cancer is one of the most common cancers worldwide and the fourth most common cause of cancer mortality.
- EGFR is a key factor in epithelial malignancies and a common target in the treatment of metastatic colorectal cancer.

What this study adds:
- EGFR-AS1 contributes to colorectal cancer via regulation of miR–133b /EGFR/STAT3 signaling.
- Low EGFR–AS1 expression is associated with higher survival rate.

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Disclosure statement

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