Expression of VEGF, EGF and HGF in early- and late-stage colorectal cancer

MAHA-HAMADIEN ABDULLA[1], SHAIK ASMA SULTANA[2], MANSOOR-ALI VAALI-MOHAMMED[1], KHAYAL ABDULMALIK AL KHAYAL[1], THAMER BIN TRAIKI[1], AHMAD MOHAMMED ZUBAIDI[1], TARIQ AL-JOHANI[3], ZAHID SHAKOOR[4] and OMAR ABDULLAH-AL OBEED[1]

[1]Colorectal Research Chair, Department of Surgery, King Khalid University Hospital, College of Medicine; [2]Prince Naif Health Research Centre, King Saud University; [3]Department of Pathology and Laboratory Medicine, College of Medicine; [4]Department of Immunology, King Saud University Medical City, Riyadh 11472, Saudi Arabia

Received August 12, 2020; Accepted March 17, 2021

DOI: 10.3892/mco.2021.2413

Abstract. The heterogenous nature of colorectal cancer (CRC) highlights the need for a better understanding of the growth factors that affect tumour growth and cancer progression. The aim of the present study was to evaluate the role of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in the early (I and II) and late (III and IV) stages of CRC. The serum levels and mRNA expression (n=30) of the aforementioned growth factors were measured and immunohistochemistry (n=20) was performed in patients with CRC. Histological examination revealed comparable distribution of early-stage [I: 8 (26.7%) and II: 7 (23.3%)] and late-stage [III: 8 (26.7%) and IV: 7 (23.3%)] CRC. The mean serum concentrations of VEGF during the early (152.9±14.5 vs. 88.39±3.99 pg/ml; P=0.001) and late (182.7±25.8 vs. 88.39±3.99 pg/ml; P=0.002) stages were significantly higher compared with those in controls. Similarly, the mean serum concentrations of EGF in the early (409.4±7.96 vs. 153.7±13.8 pg/ml; P=0.05) and HGF in the late (90.4±17.4 vs. 56.9±4.97 pg/ml; P=0.05) stages were significantly higher compared with those in controls. The serum concentrations of VEGF, EGF and HGF were comparable between the early and late stages of CRC. Compared to normal tissues, the mRNA expression of both VEGF (P<0.001) and HGF (P<0.01) was upregulated in early-stage and downregulated in late-stage CRC. The expression of EGF remained significantly elevated during both the early and late stages of CRC (P<0.01). Histopathological analyses confirmed increased expression of VEGF in cancerous tissues compared with that in normal tissues. The present study emphasized the need for monitoring the serum levels and tissue expression of growth factors to fully elucidate their role in patients with CRC.

Correspondence to: Dr Maha-Hamadien Abdulla, Colorectal Research Chair, Department of Surgery, King Khalid University Hospital, College of Medicine, King Saud University, Building 4, Exit 2, P.O. Box 7805 (37), Riyadh 11472, Saudi Arabia
E-mail: mabdulla@ksu.edu.sa

Abbreviations: CRC, colorectal cancer; EGF, epidermal growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor

Key words: colorectal cancer, early stage, late stage, VEGF, EGF, HGF, expression

Introduction

Colorectal cancer (CRC) is the third most frequent among all types of cancer, comprising an estimated 8-9% of all cancer cases reported in 2020 worldwide (1). In Saudi Arabia, CRC accounts for 14.1% of all cancer-related deaths (2). The local incidence of CRC is on the rise, affecting men more frequently than women (3) and being more frequently diagnosed in young adults (4).

The heterogenous nature of CRC emphasizes the need for a better understanding the of cellular and molecular factors in the tumour microenvironment that regulate tumour cell proliferation and metastasis (5). Alterations in the expression of growth factors and their receptors profoundly affect the proliferation of cancer cells, development of vasculature (angiogenesis) and spread to other organs (6). Signals generated by growth factors modify the tumour microenvironment at various stages of cancer progression (7,8). Low levels of growth factors in biological fluids during the initial stages of cancer development tend to increase rapidly as cancer progresses (9). Certain growth factors, such as epidermal growth factor (EGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF), exhibit a robust association with cancer progression (10).

Overexpression of EGF receptor (EGFR) in 60-80% of colonic tumours has been associated with poor prognosis (11). The interaction of EGFR with EGF and TGFα promotes tumourigenesis by dysregulation of the cell cycle, resulting in enhanced tumour cell survival (12). Furthermore, TGFα interaction with EGFR has been shown to promote the expression of VEGF (12), and elevated serum levels of VEGF have been associated with shorter disease-free survival among patients

with CRC (13); this may be due to VEGF-mediated angiogenesis through the proliferation, differentiation and migration of vascular endothelial cells (14). Increased serum levels of HGF among patients with cancer, including patients with CRC, have been associated with disease progression (15-17), most likely due to its ability to promote angiogenesis in tumours (18).

The literature indicates that the growth factors EGF, VEGF and HGF may play important roles in determining cancer progression. Since EGF induces VEGF production and HGF regulates EGF expression (11-17), simultaneous assessment of these three growth factors may indicate how they vary at different stages of CRC. Therefore, the present study was performed to assess the serum levels of EGF, VEGF and HGF, along with their mRNA and protein expression, in patients with early- and late-stage CRC.

Materials and methods

Study population. The present study was performed between March 2015 and September 2016 at King Khalid University Hospital, King Saud University (Riyadh, Saudi Arabia). The study protocol was approved by the Institutional Review Board of the College of Medicine, King Saud University. After obtaining written informed consent, blood samples and tissue specimens were collected from a total of 30 patients [14 men (46.7%) and 16 women (53.3%); mean age, 60 years; range, 38-80 years]. Early-stage CRC was defined as stages I and II, whereas late-stage CRC was defined as stages III and IV. Patient demographic and clinical characteristics, including the tumour site and type, were recorded. Tumour staging was performed according to the Union for International Cancer Control (UICC)-TNM staging system; tumour localization, UICC stage and tumour grade were classified as described previously (19). Patients receiving neoadjuvant or adjuvant therapy were excluded from the study.

Blood and tissue samples. A 5-ml venous blood sample was obtained under aseptic conditions from each patient and from age- and sex-matched healthy controls (n=30). Healthy blood samples were obtained from Blood Bank Unit, King Khalid University Hospital (Riyadh, Saudi Arabia). The blood samples were allowed to clot for 1 h at room temperature and were centrifuged at 1,000 x g for 10 min at room temperature to collect the serum. All serum samples were then stored at -80˚C until further use. Cancer and adjacent normal tissue samples (at least 10 cm away from the tumour site) were obtained from the patients, freeze-dried in liquid nitrogen and stored at -80˚C until histological examination.

Measurement of serum concentrations of VEGF, EGF and HGF. The serum concentrations of VEGF, EGF and HGF in patients and controls were quantified using a Human Cytokine multiplex panel (LHC6003, Novex, Invitrogen; Thermo Fisher Scientific, Inc.) and the plates were read using Milliplex™ Luminex xMAP technology (EMD Millipore). The assay was performed in accordance with the manufacturer's instructions. Assessments of standards, internal controls and samples were performed in duplicates and the results are expressed in pg/ml.

Preparation of total RNA and reverse transcription (RT). RNA was extracted from paired tumour and adjacent healthy tissues using PARIS™ kit (Ambion; Thermo Fisher Scientific, Inc.). Reverse transcription was performed as per the manufacturer's instructions using a high-capacity cDNA kit (cat. no. 4368814; Applied Biosystems; Thermo Fisher Scientific, Inc.). The quality of RNA was evaluated by calculating the A260/280 ratio (1.8-2.0).

Quantitative (q)PCR) analysis. qPCR was performed on ViiA™ 7 Real-Time PCR system (Thermo Fisher Scientific, Inc.) using the SYBR Green PCR Master Mix (cat. no. 4385612; Thermo Fisher Scientific, Inc.). The relative mRNA levels of EGF, HGF and VEGF were normalized to GAPDH in order to estimate their expression in tumour and adjacent normal tissues. The qPCR conditions were 95˚C for 15 min, 40 cycles of 94˚C for 15 sec, and 57˚C for 30 sec. The following primers were used: Human EGF, 5'-GGAATTCTACTTGTGTGG GTCCT-3' (sense) and 5'-TCACTGAGACACCAGCATCC-3' (antisense); human HGF, 5'-GACGCAGCTACAAGGGAA CA-3' (sense) and 5'-GCTCGAAGGCAAAAAGCTG-3' (antisense); human VEGF, 5'-TGTGAATGCAGACCAAAGAAA GAT-3' (sense) and 5'-GCTCCAGGGCATTAGACAGC-3' (antisense); and human GAPDH, 5'-ACCCATCACCATCTT CCAGGAG-3' (sense) and 5'-GAAGGGGCGGAGATGATG AC-3' (antisense). The results were normalized to GAPDH levels and the relative expression was calculated with the $2^{-\Delta\Delta Cq}$ method (20).

Construction of tissue microarrays (TMAs) and immunohistochemistry. TMAs were constructed and immunohistochemistry was performed as described previously (21). VEGF expression was detected using streptavidin-biotinylated horseradish peroxidase (S-ABC) kit cat. no. 65306; NovoLink Max Polymer Detection System; Leica Microsystems, Ltd.) as per the manufacturer's instructions. Negative controls without the primary antibody were also included. The expression of VEGF in tumour and normal tissue was analyzed with the eSlide capture device (ScanScope CS; Aperio Technologies, Inc.). VEGF staining intensity was evaluated as negative, weak or strong and a semi-quantitative analysis was performed based on the categorization of patient samples by staining intensity (22).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.). Data are presented as mean ± standard error. A paired t-test was used to compare growth factor serum and mRNA levels between early- and late-stage CRC. $P<0.05$ was considered to indicate statistically significant differences.

Results

Patients. A total of 30 patients with CRC [14 men (46.7%) and 16 women (53.3%); mean age, 60 years; range, 38-80 years] were included in the study. Histological examination indicated comparable distribution of early-stage [I: n=8 (26.7%) and II: n=7 (23.3%)] and late-stage [III: n=8 (26.7%) and IV: n=7 (23.3%)] CRC. The characteristics of the patients and clinicopathological data are summarised in Table I.

Table I. Clinicopathological characteristics of patients with colorectal cancer.

Variables	Number of patients	Percentage
Mean age (years)		
≤60	17	56.7
>60	13	33.3
Sex		
Male	14	46.7
Female	16	53.3
Primary tumour		
Colon	27	90
Rectum	3	10
Tumour stage (UICC 2010)		
pT1	4	13.3
pT2	8	26.7
pT3	15	50
pT4	3	10
Lymph node status (UICC 2010)		
pN0	15	50
pN1	13	43.3
pN2	2	6.7
Clinical stage (UICC 2010)		
I	8	26.7
II	7	23.3
III	8	26.7
IV	7	23.3
Histological grade (UICC 2010)		
G1	0	0
G2	28	93.3
G3	2	6.7

UICC, Union for International Cancer Control.

Serum levels of VEGF, EGF and HGF. The serum concentrations of VEGF, EGF and HGF were compared between patients with early- and those with late-stage CRC and between patients and controls (Table II). Among patients with CRC, the mean serum concentrations of VEGF during the early (152.9±14.5 vs. 88.39±3.99 pg/ml; P=0.001) and late (182.7±25.8 vs. 88.39±3.99 pg/ml; P=0.002) stages of the disease were significantly higher compared with those in controls. Similarly, the mean serum concentrations of EGF among patients with early-stage (409.4±7.96 vs. 153.7±13.8 pg/ml; P=0.05) and late-stage (669.5±13.1 vs. 153.7±13.8 pg/ml) CRC were higher compared with those controls. The mean serum concentrations of HGF did not differ significantly between patients with early-stage CRC and controls (83.54±14.4 vs. 56.9±4.97 pg/ml; P=0.13); however, the levels of HGF were higher in patients with late-stage CRC compared with controls (90.4±17.4 vs. 56.9±4.97 pg/ml; P=0.05). There was no statistically significant difference in the mean serum concentrations of VEGF, EGF and HGF between early- and late-stage CRC.

Gene expression of VEGF, EGF and HGF in patients with CRC. Relative quantification of growth factor expression in early-stage cancer indicated a substantial upregulation in the levels of VEGF (P<0.001), EGF (P<0.05) and HGF (P<0.01) in early-stage CRC compared to adjacent normal tissues (Fig. 1A). However, decreased expression levels of VEGF and HGF were observed in late-stage CRC compared with the levels observed in the early stages, whereas the levels of EGF remained significantly elevated during both the early and late stages of CRC (P<0.01 vs. control; Fig. 1B).

Protein expression of VEGF in patients with CRC. Immunohistochemical analysis of VEGF expression was performed on TMAs of paired tumour and adjacent normal specimens (n=20). Increased expression of VEGF (brown staining) was confirmed in cancerous tissues compared with that in normal tissues (Fig. 2A-C). Early-stage (I and II) tumour tissues exhibited a higher number of VEGF-positive tumour cells (Fig. 2A) compared with late-stage (III and IV) tumour tissues (Fig. 2B). A semi-quantitative analysis was also performed based on the staining intensity of VEGF as negative, weak or strong. Strong staining was observed in 47% of late-stage CRC samples, whereas 53% of the early-stage samples exhibited strong staining. While all tumours displayed VEGF positivity, the intensity of staining varied from weak to strong between early and late cancer stages, and also within the same stage, demonstrating heterogeneous expression.

Discussion

Growth factors in cancer are well known to stimulate cellular proliferation, tumour progression, angiogenesis and subsequent metastasis (6). Increased expression of growth factors is frequently encountered in neoplastic/cancerous cells (23). This may have potential clinical implications in the management and outcome of CRC, as a number of growth factors have been shown to serve as prognostic markers in monitoring the response to therapeutic interventions (24). Elevated serum levels of tumour growth factors, such as EGF, VEGF and HGF, have been reported among patients with CRC, whereas these levels tend to decline following effective treatment (25). Moreover, higher preoperative levels of VEGF and HGF have been reported to be associated with recurrence of CRC after therapy (26). Targeting these angiogenic factors with biological agents may counteract the tumour-promoting effects of these circulating factors. In the present study, significantly higher serum concentrations of EGF, VEGF and HGF were found among patients with early- and late-stage CRC compared to controls.

Owing to its angiogenic potential, the role of VEGF is considered to be critical in CRC. Significantly higher expression of VEGF has been reported in 50% of patients with CRC compared to its expression in healthy tissues (27). Enhanced expression of VEGF is considered as a poor prognostic marker in CRC (28,29) and is also associated with poor overall survival among affected patients (30). Furthermore, elevated serum levels of VEGF are positively correlated with tumour stage, with significantly higher levels of circulating VEGF observed in advanced stages of CRC compared to early stages (31,32). The results of the present study were consistent

Table II. Comparison of serum levels of growth factors between patients with CRC and healthy controls.

Growth factors	Controls	CRC		P-value		
		Early-stage	Late-stage	Control vs. early-stage CRC	Control vs. late-stage CRC	Early- vs. late-stage CRC
VEGF	88.39±3.99	152.9±14.5	182.7±25.8	0.0012[c]	0.002[b]	0.291
EGF	153.7±13.8	409.4±7.96	669.5±13.1	0.05[a]	0.01[b]	0.08
HGF	56.9±4.97	83.54±14.4	90.4±17.4	0.13	0.05[a]	0.762

Values are provided in pg/ml and are presented as mean ± SD. [a]P≤0.05; [b]P≤0.01; [c]P≤0.001. EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; CRC, colorectal cancer.

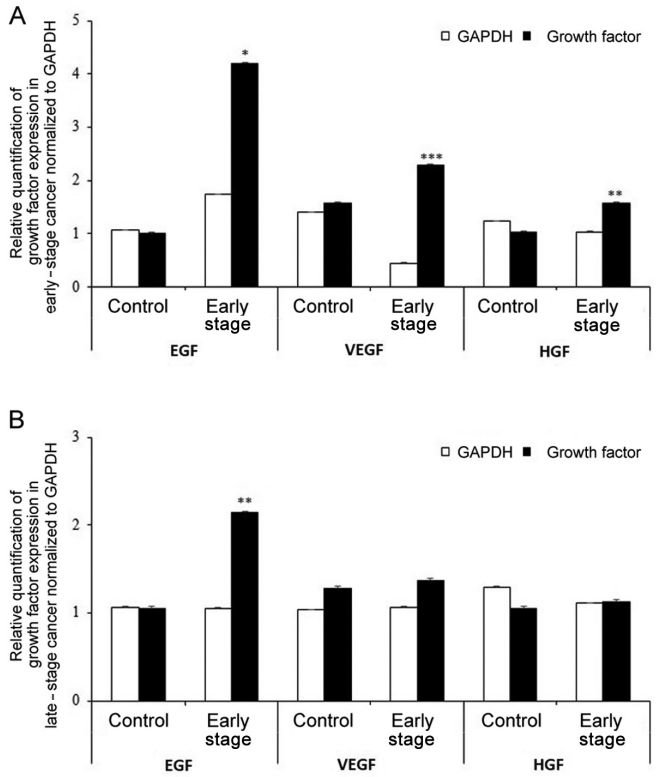

Figure 1. Relative quantification of growth factor expression normalized to GAPDH in (A) early-stage and (B) late-stage colorectal cancer. *P<0.05, **P<0.01 and ***P<0.001 vs. control. EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor.

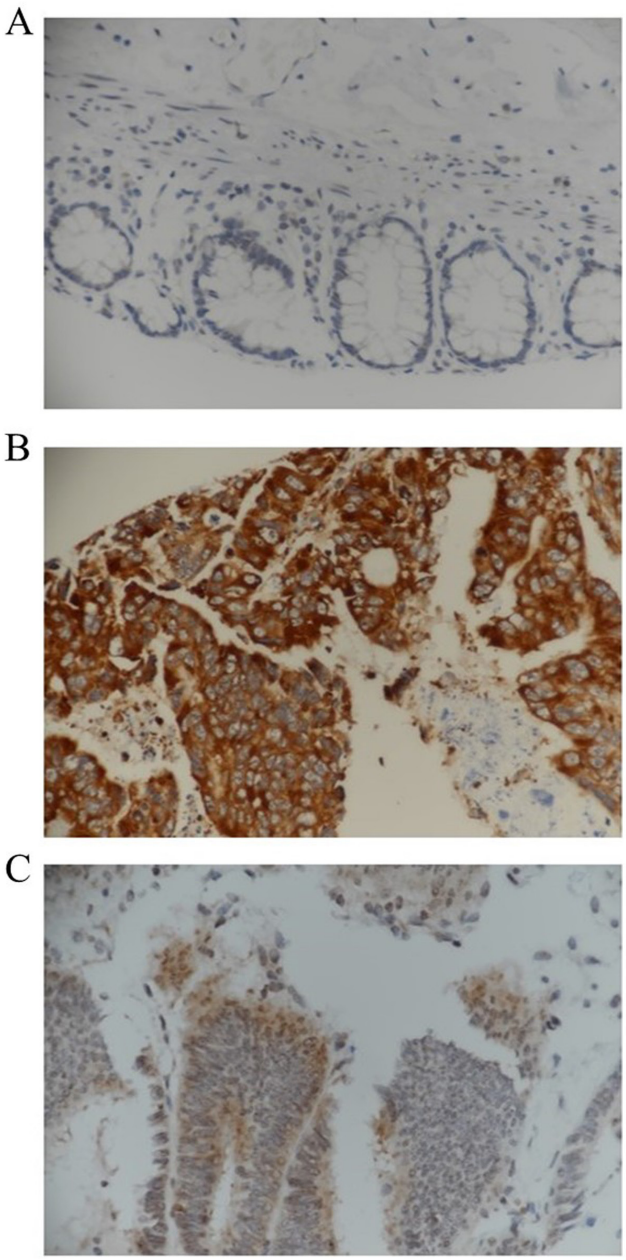

Figure 2. Immunohistochemistry staining for VEGF. (A) Photomicrograph showing normal mucosa with negative staining for VEGF. (B) Colonic adenocarcinoma exhibiting strong positive epithelial neoplastic luminal staining for VEGF in a patient with early-stage disease. (C) Colonic adenocarcinoma exhibiting weak staining for VEGF in a patient with late-stage disease. Magnification, x600.

with these observations, and the intense expression of VEGF in the later stages of the disease suggested a possible role of VEGF in disease progression. Despite the convincing data linking VEGF with disease progression among patients with CRC, there is however evidence refuting these claims and stating that VEGF has no prognostic significance as a risk factor for CRC progression (33). Moreover, no difference in the serum concentrations of VEGF between patients with CRC and healthy controls has been reported (34). Due to the conflicting reports, investigations of factors, such as the involvement of other members of the VEGF family of growth factors (29,35), the extent of inflammation and the number of tumour-infiltrating lymphocytes in CRC, may be useful in gaining a better understanding of the pathogenesis of CRC.

The serum levels of EGF were found to be significantly elevated in patients with early- and late-stage CRC compared to healthy controls in the present study. Data for EGF among patients with CRC are scarce and, to the best of our knowledge, no previous study has compared EGF levels between the early and late stages of CRC. Elevated serum levels of EGF have been shown to exhibit 80% sensitivity and 65% specificity in the detection of ovarian cancers (36). Increased expression of EGF has not only been associated with various types of cancer, but is also considered to be a poor prognostic marker in terms of adverse clinical outcomes (37-41). By contrast, low levels of EGF have been reported among patients with non-small cell lung cancer and head and neck cancer compared with healthy controls (42). In the present study, however, EGF was found to be markedly elevated in both the early and late stages of CRC, indicating the potential clinical role of this marker. Although studies ascertaining the clinical significance of this finding in CRC are currently lacking, the evidence reported by studies performed on patients with ovarian (36), lung, and head and neck cancers (42) indicate that EGF expression may be of substantial diagnostic value. Further studies are therefore needed to evaluate the diagnostic and/or prognostic significance of this marker in CRC.

HGF and its receptor, c-Met (tyrosine-protein kinase Met or hepatocyte growth factor receptor), are involved in a number of important biological processes (43). HGF/c-Met interaction has been implicated in HGF-activated colonic fibroblast-mediated carcinogenesis of colonic epithelial cancer cells (44,45). The higher levels of c-Met mRNA and protein in CRC liver metastasis, the positive correlation between c-Met expression and liver metastasis, and the association of the downregulation of HGF/c-Met signalling with the reduction in cell proliferation, invasion and metastasis of liver cancer, indicate a potentially crucial role of HGF in CRC (46). Moreover, increased expression of HGF among patients with CRC has been shown to exhibit a positive correlation with disease progression (25). The serum concentration of HGF was found to be higher in the early stages of CRC in the present study, whereas its levels normalized in the late stages. These observations suggest an involvement of HGF in disease pathogenesis in the early stages of CRC, thus highlighting it as a promising target for therapeutic interventions. Furthermore, there appears to be a need for further investigations to elucidate the role of HGF in cancer development, lymphatic invasion and survival in CRC (17).

It is evident that tumour progression from early to late stage involves a number of cellular, biochemical and molecular events, including altered expression of several growth factors (46). However, mRNA expression levels may not be reflective of serum concentrations, possibly owing to post-transcriptional, translational and post-translational modifications, which may determine protein degradation and its regulation (47). Further studies are needed to understand this perspective. Moreover, an increase in the concentrations of growth factors may be associated with an increase in the numbers of cancer niche cells during carcinogenesis, which also needs additional supportive evidence (48). The growth factor expression profile observed in the present study during the early and late stages of CRC offers an opportunity for further investigations to identify targets for predicting the prognosis and establishing therapeutic interventions for the containment of CRC progression.

In conclusion, the elevated levels of growth factors during the early and late stages of CRC observed in the present study highlight the importance of growth factor expression in CRC. However, a major limitation of the present study was its small sample size. Large scale studies are recommended to validate the findings of the present study and to gain a better understanding of the exact role of growth factors in the pathogenesis of CRC.

Acknowledgements

Not applicable.

Funding

The present study was supported and funded by Researchers Supporting Project number (grant no. RSP-2021/344), King Saud University, Riyadh, Saudi Arabia.

Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

MHA, OAAO and MAVM conceived and designed the study, and drafted the manuscript; MHA, SAS and MAVM performed the statistical data analysis; MAVM, MHA, SAS and TBT have seen and can confirm the authenticity of the raw data; KAAK, TBT, TAJ, ZS and AMZ were involved in data acquisition and interpretation, and critically revised the manuscript for important intellectual content. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Institutional Review Board of College of Medicine (approval no. 15/0482), King Saud University, and all the participants provided their written informed consent prior to enrolment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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