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Apelin Association with Hepatic Fibrosis and Esophageal Varices in Patients with Chronic Hepatitis C Virus

Lamyaa Abdellatif Soliman,[1] Rania A. Zayed,[1]* Dalia Omran,[2] Fadwa Said,[1] Samar Kamal Darweesh,[2] Doaa Mohamed Ghaith,[1] Rasha Eletreby,[2] Mahmoud Salama Barakat,[2] Mahmoud M. Bendary,[3] Doaa Zakaria Zaky,[4] Eman Amer,[5] and Iman Rifaat Elmahgoub[1]

[1]*Clinical and Chemical Pathology Department, Faculty of Medicine, Cairo University, Cairo, Egypt;* [2]*Department of Endemic Medicine and Hepatogastroenterology, Faculty of Medicine, Cairo University, Cairo, Egypt;* [3]*Department of Microbiology and Immunology, Faculty of Pharmacy, Port Said University, Port Said, Egypt;* [4]*Department of Tropical Medicine, Ain Shams University, Cairo, Egypt;* [5]*Department of Biochemistry, Faculty of Pharmacy, Ahram Canadian University, Cairo, Egypt*

Abstract. Portal hypertension and esophageal varices complicating hepatitis C virus (HCV)-related chronic liver diseases are some of the most devastating sequelae. Angiogenesis is the hallmark of their pathogenesis. Apelin is one of the recently identified angiogenic and fibrogenic peptides. We studied apelin gene expression, apelin (rs3761581) single-nucleotide polymorphism (SNP), and serum apelin level in patients with chronic HCV, and their association with liver fibrosis and esophageal varices in 112 patients with HCV-related chronic liver disease (40 with liver cirrhosis [LC]/low-grade varices, 33 with LC/high-grade varices, and 39 with fibrotic non-cirrhotic liver/no varices) and 80 healthy control subjects. Real-time polymerase chain reaction was used for apelin gene expression assay and apelin rs3761581 SNP analysis in peripheral blood samples. The serum apelin level was measured by ELISA. Apelin gene expression was undetectable in the studied samples. The SNP analysis revealed a greater frequency of the C (mutant) allele among patients compared with control subjects ($P = 0.012$; odds ratio, 3.67). The serum apelin level was significantly greater in patients with LC/varices (median, 31.6 ng/L) compared with patients without LC/varices (median, 2.9 ng/L; $P < 0.001$). A serum apelin level cutoff value of 16.55 ng/L predicted the presence of varices, with an area under the receiver operating characteristic curve value of 0.786. A positive correlation was found between serum apelin level and grade of liver fibrosis ($r = 0.346$, $P < 0.001$) and portal hypertension ($r = 0.438$, $P < 0.001$). In conclusion, the apelin rs3761581-C allele may be associated with the progression of HCV-related chronic liver disease and varices formation, and can be considered a potential therapeutic target to control fibrosis progression. The serum apelin level provided an accurate prediction of the presence of esophageal varices.

INTRODUCTION

Hepatitis C virus (HCV) is a globally prevalent pathogen and a leading cause of morbidity and mortality.[1] Liver cirrhosis develops in 5% to 20% of patients with chronic HCV and is complicated by portal hypertension, a serious consequence of chronic liver diseases[2,3] characterized by the formation of an extensive network of portosystemic collateral vessels that include the gastroesophageal varices. Hemorrhage from ruptured esophageal varices is one of the most notable causes of death in patients with cirrhosis.[4] Early diagnosis of varices before the first bleed is crucial to reduce the risk of variceal hemorrhage.[5] It is recommended that all patients with cirrhosis should be screened for the presence of esophageal varices by upper gastrointestinal (GI) endoscopy, and again at 2- to 3-year intervals in patients without varices and at 1- to 2-year intervals in patients with small varices.[6] However, this implies a number of unnecessary endoscopies, which increases the workload of endoscopy units,[7] in addition to being an unpleasant invasive procedure that may reduce patient compliance.[8] Therefore, there is a need for cost effective noninvasive tests to select people who are in need of undergoing upper GI endoscopy screening.[9] Multiple parameters can help predict the presence of esophageal varices, including splenomegaly, ascites, spider nevi, Child's grade, platelet count, prothrombin time/activity, portal vein diameter, platelet count/spleen diameter ratio, serum albumin, serum bilirubin, and transient elastography,[10,11] but none have been approved to replace regular upper GI endoscopic examination at scheduled time intervals.[12] Angiogenesis is a pathological hallmark of portal hypertension and varices formation.[13,14] The identification of novel molecules involved in angiogenesis is relevant from the clinical point of view because they may represent potential new targets to suppress pathological neovascularization in portal hypertension.[15]

Apelin is an endogenous ligand of the angiotensin II receptor-like 1 (APJ) that belongs to the G protein-coupled receptor family. Both apelin and APJ are widely distributed in different body tissues, and thus are thought to be involved in many physiological processes.[16] Apelin/APJ binding stimulates endothelial cell proliferation and can trigger vascular sprouting in vivo even in the absence of vascular endothelial growth factor.[17] Apelin is also found to be upregulated in hepatic stellate cells of patients with cirrhosis.[18] Furthermore, apelin behaves as a paracrine mediator of fibrogenesis-related gene induction in human hepatic stellate cells,[18] and APJ blockade was proved effective in reducing liver fibrosis and angiogenesis in rats with cirrhosis.[19] Twelve common polymorphisms in the apelin/APJ pathway were identified by direct sequencing.[20] Of these 12 common polymorphisms, four (rs3761581, rs56204867, T-1860C, rs7119375, and rs10501367) were considered the top ones.[21] The apelin rs3761581-A/C polymorphism is located in the promoter region and it is expected to be responsible for the transcriptional regulation of the apelin gene, either by itself or through linkage with other functional loci by modifying the coordinated action of multiple regulatory proteins through complex protein–DNA and protein–protein interaction.[22] The effect of apelin rs3761581-T/G on transcriptional activity has also been documented.[23]

*Address correspondence to Rania A. Zayed, Cairo University, El Saraya St., Infront of El Manial Palace, Cairo 11451, Egypt. E-mail: rania.zayed@medicine.cu.edu.eg

We studied apelin gene expression, apelin rs3761581 single-nucleotide polymorphism (SNP), and serum apelin levels in patients with chronic HCV to assess associations with hepatic fibrosis, esophageal varices formation, and grade of esophageal varices.

METHODS

This case–control study had 192 participants—112 patients with HCV-related chronic liver disease (40 patients with liver cirrhosis and low-grade varices, 33 patients with liver cirrhosis and high-grade varices, and 39 patients with fibrotic non-cirrhotic liver and no varices) and 80 age- and gender-matched healthy control subjects. Liver cirrhosis was diagnosed based on physical findings, laboratory investigations, ultrasound findings, and a transient elastography (TE)-FibroScan value of $\geq$ 14.6 kPa (cutoff value for the diagnosis of cirrhosis).[24] Patients were recruited from the Hepatogastroenterology Department, Kasr Alainy Hospital, Cairo University, Cairo, Egypt, over a 2-year period. Informed consent was obtained from each subject after explanation of the study objectives. The study was approved by Faculty of Medicine, Cairo University Ethics Committee, and was performed in accordance with the ethics guidelines of the Declaration of Helsinki. Patients with HBV co-infection, alcoholic liver disease, autoimmune liver disease, other inflammatory conditions, malignancy, other chronic diseases, diabetes, hypertension, morbid obesity and smokers were excluded from the study. Patients were subjected to detailed history taking, laboratory investigations (alanine aminotransferase, aspartate aminotransferase, albumin, prothrombin time and concentration, total and direct bilirubin, complete blood count, creatinine, and alpha fetoprotein), abdominal ultrasound for cirrhosis assessment and exclusion of focal hepatic lesions, TE-FibroScan to assess the degree of liver fibrosis, and upper endoscopy to assess the presence and grade of esophageal varices.

The apelin rs37661581 SNP was analyzed using a TaqMan allelic discrimination assay (SNP genotyping) by real-time polymerase chain reaction (PCR), apelin gene expression was evaluated using TaqMan real-time PCR, and serum apelin level was measured by ELISA.

Allelic discrimination of the apelin rs37661581 SNP using a TaqMan-based SNP genotyping assay. Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA blood Mini kit (Qiagen) according to the manufacturer's instructions. Isolated DNA was stored at –80°C until used for polymerase chain reaction (PCR) amplification. Real-Time PCR using sequence-specific primers was performed on the StepOne™ Real-Time PCR (Applied Biosystems) in a reaction volume of 25 μL containing 2 μL DNA, 1 μL forward primer (5′- CCGATGACCACATGACCAAG-3'), 1 μL reverse primer (3′-GAATAGGGCGGAGGGAAAG), 0.5 μL VIC labeled probe, 0.5 μL FAM labeled probe, 12.5 μL TaqMan PCR master mix, and 7.5 μL RNase free water. Cycle conditions were initial incubation at 50°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Allelic discrimination was determined using sequence detection software (version 2.1), where the fluorescence signals obtained were plotted to indicate alleles present in each sample.

Apelin gene expression analysis using real-time PCR. RNA extraction was done using the Pure link RNA mini kit (Ambion-Life Technologies) according to the manufacturer's instructions, following the protocol of RNA extraction from a leukocyte pellet to achieve better RNA yield. RNA concentration and purity were evaluated; 1.5 μL of the RNA sample was measured directly at an absorbance of 260 nm using the NanoDrop 1000A Spectrophotometer (NanoDrop Technologies, Waltham, MA), and RNA concentration was displayed in nanograms per microliter. The purity of RNA was determined by measuring the absorbance at 260 and 280 nm. The ratio of absorbance of 260 nm to 280 nm is a measure of RNA purity; pure RNA has a 260-nm:280-nm ratio between1.8 and 2.1. Reverse transcription of extracted RNA to complementary DNA (cDNA) was performed in a total volume of 20 μL reaction using a high-capacity cDNA reverse transcription kit (Qiagen). cDNA was then stored at –80°C until amplification. cDNA amplification was done using StepOne Real-Time PCR using a TaqMan gene expression assay in a total reaction volume of 20 μL containing 3 μL cDNA, 2 μL primers (sense primer, 5'-GGCCATCACCAGCCA TTCCTTG-3'; antisense primer, 5'-GGGCATCAGGCTCTTGT CTTCTCT-3'), 10 μL TaqMan universal master mix, 5 μL RNase free water, and housekeeping gene GADPH (forward primer, 5'-GAAGGTGAAGGTCGGAGTCA-3'; reverse primer, 5'-GAA-GATGGTGATGGGATTTC-3'), under the following conditions: 45 cycles of annealing and extension at 95°C for 15 seconds, and at 60°C for 1 minute. The relative quantification of APLN gene expression was set using the comparative C_T method ($2^{-\Delta\Delta CT}$ method).[25]

The serum apelin level was measured using a specific ELISA kit (Bioassay Technology Laboratory), with an assay range of 0.5 to 200 ng/L, according to the manufacturer's recommended protocol.

Endoscopic examination. Esophagogastroduodenoscopies were performed on all patients using the Olympus GIF 160-Q165 (EXERA II). Esophageal varices were graded as follows: grade 0, no varices, grade I, varices disappearing with insufflation; grade II, larger and clearly visible, usually straight varices not disappearing with insufflation; grade III, more prominent varices locally coil shaped and partly occupying the lumen; grade IV, tortuous sometimes grape-like varices occupying the esophageal lumen.[26]

Assessment of the degree of liver fibrosis was based on a liver stiffness measurement using TE-FibroScan (Echosens™, Paris, France).[27]

Statistical analysis. Data were coded and entered using the statistical package SPSS (version 25; SPSS, Inc., Chicago, IL). Data were summarized using mean, SD, median, minimum, and maximum for quantitative data; and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests. For comparing categorical data, a χ^2 test was performed. An exact test was used instead when the expected frequency was less than 5%. Genotype and allelic frequencies were compared between the disease and control groups using χ^2 tests. Odds ratios (ORs) with 95% CIs were calculated. Correlations between quantitative variables were made using the Spearman correlation coefficient.

TABLE 1
Demographic data of patients and control subjects

Demographic data	Group I		Group II ($n = 39$)	Control subjects ($n = 80$)	P value
	Ia ($n = 40$)	Ib ($n = 33$)			
Age, y; mean ± SD	48.72 ± 7.38	57.64 ± 7.05	40 ± 4.43	48.3 ± 8.5	0.017
Gender, n (%)					
Male	37 (92.5)	22 (66.7)	34 (87.2)	62 (77.5)	0.338
Female	3 (7.5)	11 (33.3)	5 (12.8)	18 (22.5)	
Body mass index, mean ± SD	24.20 ± 1.14	24.6 ± 2.25	25.74 ± 2.27	23.38 ± 1.19	< 0.001

The P value is for a comparison of the control group (group III) and the patient groups (groups I and II).

RESULTS

Our study included 192 Egyptian adults. Group I included 73 patients with liver cirrhosis and esophageal varices, and this group was divided into two subgroups according to the grade of esophageal varices: 40 patients with small varices (group Ia) and 33 patients with large varices (group Ib). Group II included 39 patients with a fibrotic non-cirrhotic liver and no varices. Eighty age- and gender-matched healthy control subjects comprised group III. There was a male predominance in our study (83%, 93 of 112) in the patients groups (groups I and II) and 77.5% (62 of 80) in the control group (group III). Age and gender distribution in all groups are shown in Table 1.

Clinical and laboratory data for all groups. Laboratory and radiological data of the patients and control subjects are shown in Table 2. A statistically significant difference was found between patients and control subjects in hemoglobin, total leucocytic count, platelet count, and prothrombin concentration, the levels of which were significantly less in patients than control subjects ($P = 0.009$, < 0.001, < 0.001, and 0.001, respectively), whereas serum alanine transaminase (ALT), aspartate transaminase, total bilirubin, prothrombin time*, and international normalized ratio (INR) were significantly greater ($P < 0.001$, $^*P = 0.033$).

We further compared patient groups I and II regarding laboratory and radiological data. Hemoglobin, platelet count, and serum albumin were significantly less in group I than in group II ($P < 0.001$), and total and direct bilirubin, aspartate transaminase, ALT*, INR, HCV PCR, splenic size, and portal vein pressure showed statistically significant greater values in group I than in group II ($P < 0.001$, $^*P = 0.37$; Table 3).

Comparing group Ia to group Ib showed significantly lower values in hemoglobin, total leucocytic count, platelet count, serum albumin, prothrombin concentration, and ALT* in group Ib ($P < 0.001$, $^*P < 0.046$), whereas total and direct bilirubin, INR, splenic size, and portal vein pressure were significantly greater in group Ib patients ($P < 0.001$, Table 4).

Apelin rs37661581 SNP genotyping assay. Apelin genotyping results were categorized according to gender because the apelin gene is located on the X chromosome. In females, no statistically significant difference was found in apelin genotype or allelic frequency between patients and control subjects, nor in interpatient group comparisons (data not shown). In males, C allelic frequency was significantly greater in patients compared with control subjects ($P = 0.012$; OR, 3.67), and when comparing individual groups I and II to control subjects ($P < 0.001$ and OR, 3.8; and $P = 0.001$ and OR, 2.78, respectively). However, different patient group comparisons showed no statistically significant differences in C allelic frequency, as shown in Table 5. Therefore, the C allele may be associated with a greater risk of liver fibrosis and esophageal varices formation, but not with grade of varices or liver fibrosis stage.

Apelin gene expression. Apelin gene expression analysis was performed on 30 samples (20 patients and 10 control subjects). CT values of the housekeeping gene ranged from 19 to 27, with a mean of 23.8 ± 2.5, whereas apelin gene expression was not detected in all samples.

Serum apelin level. The serum apelin level was significantly greater in patients with varices (group I; median, 31.6 ng/L) compared with patients without varices (group II; median, 2.9 ng/L; $P < 0.001$). No statistically significant

TABLE 2
Laboratory and ultrasound data of patients and control subjects

Laboratory and ultrasound data	Patients, mean ± SD ($n = 112$)	Control subjects, mean ± SD ($n = 80$)	P value
Hemoglobin, g/dL	11.70 ± 1.69	12.39 ± 1.69	0.009
TLC, $\times 10^3$ leukocytes/μL	5.4 ± 1.22	7.01 ± 1.94	**< 0.001**
Platelet count, $\times 10^3$ platelets/μL	163.02 ± 84.13	264.1 ± 79.6	**< 0.001**
Alanine transaminase, U/L	38.54 ± 6.08	21.1 ± 6.2	**< 0.001**
Aspartate transaminase, U/L	49.4 ± 9.8	26.6 ± 4.7	**< 0.001**
Albumin, g/dL	3.9 ± 0.75	–	–
Total bilirubin, mg/dL	1.1 ± 0.54	0.8 ± 0.4	**< 0.001**
Direct bilirubin, mg/dL	0.5 ± 0.38	–	–
Creatinine, mg/dL	0.96 ± 0.19	0.75 ± 0.12	**< 0.001**
Prothrombin time, s	12.6 ± 0.19	12.4 ± 0.48	0.033
Prothrombin concentration, %	85.4 ± 16.6	95.1 ± 4.6	**0.001**
International normalized ratio	1.17 ± 0.29	1.01 ± 0.04	**< 0.001**
Triglycerides, mg/dL	132.3 ± 15.3	90.3 ± 20.9	**< 0.001**
Total cholesterol, mg/dL	223.19 ± 18.07	172.1 ± 27.8	**< 0.001**
Alpha fetoprotein, ng/mL	4.52 ± 2.5	–	–
HCV PCR, $\times 10^6$ IU/L	1.97 ± 5.4	–	–
MELD score	8.8 ± 3.2	–	–

HCV = hepatitis C virus; MELD = Model for End-Stage Liver Disease; PCR = polymerase chain reaction; TLC = total leucocytic count. Values in bold type are significant.

TABLE 3
Laboratory and ultrasound data for different patient groups

Laboratory and ultrasound data	Patients with varices, group I; mean ± SD (n = 73)	Patients without varices, group II; mean ± SD (n = 390)	P value
Hemoglobin, g/dL	10.97 ± 1.59	13.8 ± 0.7	**< 0.001**
TLC, $\times 10^3$ leukocytes/μL	5.4 ± 1.46	5.49 ± 0.56	0.250
Platelet count, $\times 10^3$ platelets/μL	114.6 ± 42.5	253.5 ± 65.8	**< 0.001**
Alanine transaminase, U/L	39.3 ± 7.2	37.05 ± 2.5	0.37
Aspartate transaminase, U/L	54.33 ± 8.5	40.3 ± 2.6	**< 0.001**
Albumin, g/dL	3.6 ± 0.73	4.4 ± 0.3	**< 0.001**
Total bilirubin, g/dL	1.26 ± 0.62	0.84 ± 0.05	**< 0.001**
Direct bilirubin, g/dL	0.61 ± 0.42	0.29 ± 0.08	**< 0.001**
Creatinine, mg/dL	0.97 ± 0.2	0.94 ± 0.16	0.655
Prothrombin time, s	12.9 ± 9 2.99	11.87 ± 0.62	**0.001**
Prothrombin concentration, %	79.4 ± 17.77	96.5 ± 3.6	**< 0.001**
International normalized ratio	1.2 ± 5 0.34	1.03 ± 0.06	**< 0.001**
Triglycerides, mg/dL	133.7 ± 15.2	129.9 ± 15.2	0.159
Total cholesterol, mg/dL	222.2 ± 17.8	224.9 ± 18.6	0.316
Alpha fetoprotein, ng/mL	4.5 ± 2.4	4.5 ± 2.7	0.833
HCV PCR, $\times 10^6$ IU/L	2.7 ± 6.5	0.49 ± 0.87	**0.001**
Spleen, cm	14.1 ± 2	10.5 ± 1.2	**< 0.001**
Portal vein diameter, cm	15.12 ± 0.69	10.23 ± 0.99	**< 0.001**
MELD score	9.9 ± 3.5	6.8 ± 1.1	**< 0.001**

HCV = hepatitis C virus; MELD = Model for End-Stage Liver Disease; PCR = polymerase chain reaction; TLC = total leucocytic count. Values in bold type are significant.

difference was found between patients with small varices (group Ia) and those with large varices (group Ib, $P = 0.833$). Therefore, the serum apelin level at a cutoff value of 16.55 ng/L can predict the presence of varices with an area under the receiver operating characteristic curve of 0.786, a sensitivity of 61.6%, a specificity of 97.4%, a P value of < 0.001, and 95% CI of 0.702 to 0.870, but it cannot discriminate varices grade (Figure 1).

A positive correlation was found between serum apelin level and grade of liver fibrosis ($r = 0.346$, $P < 0.001$) as well as portal hypertension ($r = 0.438$, $P < 0.001$). No correlation was found between serum apelin level and the apelin rs37661581 genotype.

DISCUSSION

HCV infection is associated with changes in both the liver and blood cytokine microenvironments.[28] We studied apelin gene expression in peripheral blood samples as a noninvasive alternative to liver tissue, assuming that changes in the cytokine microenvironment could affect apelin expression in blood in the same way it affects liver tissue expression. Also, the apelin rs3761581 SNP and serum apelin level in patients with chronic HCV were studied.

Although apelin gene expression is low in many tissues, including the liver and blood,[29,30] apelin gene expression in liver tissues was found to be high in liver fibrosis, and messenger RNA level somehow reflected the severity of liver fibrosis and cirrhosis.[25] Previous studies have investigated apelin gene expression in liver tissue and its role in liver fibrosis and cirrhosis by studying apelin expression in animal models, which showed that apelin expression was greater in hepatic stellate cell line (LX2) cells than hepatocyte cell lines (HEPG2).[18] In addition, APJ blockade was found to be effective in reducing liver fibrosis and angiogenesis in rats with cirrhosis[19] and in cultured cell lines, as in the experimental model conducted by Melgar-Lesmes et al.,[18] in which LX2 and HEPG2 were studied. This emphasizes the role of apelin

TABLE 4
Comparison between group Ia and group Ib patients with regard to laboratory and clinical data

Laboratory and clinical data	Patients with small varices, group Ia; mean ± SD (n = 40)	Patients with large varices, group Ib; mean ± SD (n = 33)	P value
Hemoglobin, g/dL	11.9 ± 1.1	9.7 ± 1.1	**< 0.001**
TLC, $\times 10^3$ leukocytes/μL	5.9 ± 1.1	4.7 ± 1.6	**< 0.001**
Platelet count, $\times 10^3$ platelets/μL	132.9 ± 30.5	92.4 ± 44	**< 0.001**
Alanine transaminase, U/L	40.7 ± 5.2	37.6 ± 8.7	**0.046**
Aspartate transaminase, U/L	55.3 ± 9.3	53.06 ± 7.8	0.235
Albumin, g/dL	4.06 ± 0.36	3.05 ± 0.69	**< 0.001**
Total bilirubin, g/dL	0.96 ± 0.19	1.63 ± 0.74	**< 0.001**
Direct bilirubin, g/dL	0.43 ± 0.21	0.82 ± 0.51	**< 0.001**
Creatinine, mg/dL	0.99 ± 0.19	0.93 ± 0.21	0.137
Prothrombin time, s	11.88 ± 0.76	14.3 ± 4.01	**< 0.001**
Prothrombin concentration, %	91.6 ± 9.9	64.6 ± 13.4	**< 0.001**
International normalized ratio	1.07 ± 0.11	1.5 ± 1.4	**< 0.001**
Triglycerides, mg/dL	136.4 ± 9.5	130.3 ± 19.8	0.408
Total cholesterol, mg/dL	223.4 ± 16.3	220.3 ± 19.4	0.393
Alpha fetoprotein, ng/mL	4.23 ± 1.9	4.8 ± 2.8	0.31
HCV PCR, $\times 10^6$ IU/L	2.87 ± 6.1	2.6 ± 7	0.282
US, spleen	13.6 ± 1	14.6 ± 2.7	0.114
US, portal vein diameter	15.02 ± 0.6	15.24 ± 0.75	0.180

HCV = hepatitis C virus; PCR = polymerase chain reaction; TLC = total leucocytic count; US = ultrasound. Values in bold type are significant.

TABLE 5
Apelin rs3761581 A/C allele frequency in males

	Allele A (reference allele)	Allele C	*P* value	Odds ratio	95% CI
Patients	68 (36.6%)	118 (63.4%)	**0.012**	3.675	1.327–10.180
Control subjects	82 (66.1%)	42 (33.9%)			
Group I	40 (33.9%)	78 (66.1%)	**< 0.001**	3.807	2.235–6.485
Control subjects	82 (66.1%)	42 (33.9%)			
Group II	28 (41.2%)	40 (58.8%)	**0.001**	2.789	1.516–5.131
Control subjects	82 (66.1%)	42 (33.9%)			
Group I	40 (33.9%)	78 (66.1%)	0.322	1.365	0.738–2.525
Group II	28 (41.2%)	40 (58.8%)			
Group Ia	24 (32.4%)	50 (67.6%)	0.663	0.840	0.384–1.839
Group Ib	16 (36.4%)	28 (63.6%)			

Values in bold type are significant at $P < 0.05$.

in fibrosis because LX2 cells are the principal fibrogenic cell type of liver tissue.[31]

An apelin gene expression assay was performed on 20 patients with HCV and 10 control subjects. None of the studied samples showed apelin gene expression in peripheral blood. Lack of apelin gene expression was concluded, as no signal was detected during the assay. Validity and reliability of the assay results were confirmed by detection of amplification signals of the GADPH internal control in the studied samples. We conclude that the apelin gene has very low expression in peripheral blood and that liver tissue is required for studying gene expression, as proved in previous studies.[18,19,25] This difference in expression of apelin between liver tissue and blood may be related to the cytokine effect of the liver microenvironment in patients with HCV. Tumor necrosis factor (TNF)-α was found to have the greatest hepatic expression in liver cirrhosis specimens compared with non-cirrhotic chronic HCV and hepatocellular carcinoma.[32] This hypothesis can be ascertained further by studies that investigated the putative regulation of apelin expression in adipocytes by TNF-α, in which a close correlation between apelin and TNF-α expression in adipose tissue of lean and obese humans was found. Apelin regulation by TNF-α in cultured explants of human adipose tissue showed that the endogenous expression of TNF-α in adipocytes isolated from the explants was accompanied by a 6- to 9-hour subsequent increase of apelin expression in adipocytes.[33] Although HCV infection causes changes in the liver cytokine microenvironment as well as in serum, orchestrated by TNF-α and IL-10,[28] our results suggest there are other factors that might influence apelin expression in liver tissue without a similar effect on apelin expression in the blood.

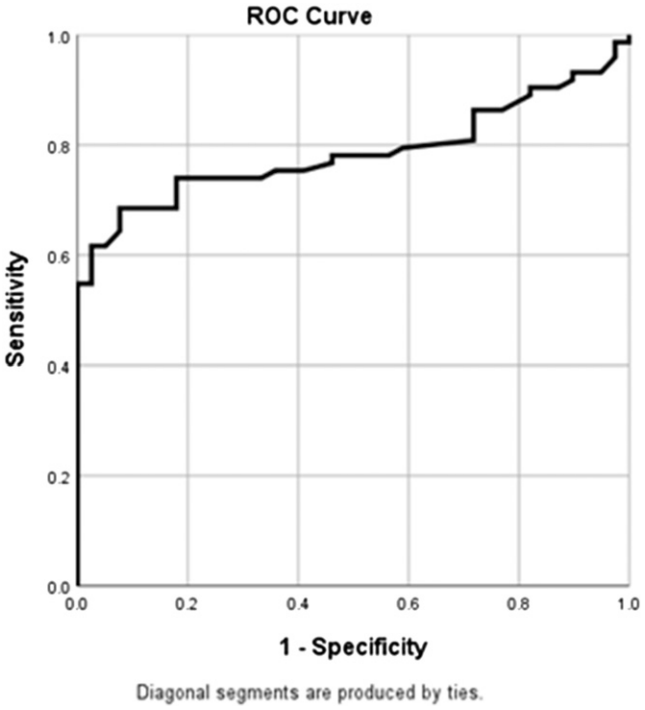

FIGURE 1. Receiver operating characteristic (ROC) curve using the serum apelin level cutoff to predict the presence of esophageal varices. A serum apelin level at cutoff value of 16.55 ng/L predicts the presence of esophageal varices (area under the ROC curve, 0.786; sensitivity, 61.6%; specificity, 97.4%; $P < 0.001$; 95% CI, 0.702–0.870). Diagonal segments are produced by ties.

Because apelin is an X-linked gene, the results of the apelin rs3761581 SNP were classified according to gender; however, the female group was less represented in our study population, which may reflect the prevalence and natural history of HCV in the Egyptian population. Previous studies have shown that HCV is more prevalent in males than females in the Egyptian population.[34,35]

Studying the prevalence of apelin rs3761581 SNP in the control subjects showed that the frequency of the A allele was greater than the C allele; thus, the A allele represents the major allele in our studied group of control subjects. Our results are in concordance with several ethnic groups of Asian, African, and European populations[36] in which the T allele of the apelin polymorphic site rs371581 is the major allele in these studied populations, with allele expression ranging from 55% to 100% (median, 77%). Only two populations from East Asia, Uyghur and Xibe, revealed a greater frequency of the G allele in 65% and 67% of the population, respectively.[36] In the allelic frequency database, the apelin rs3761581-A/C SNP is reported as rs3761581-T/G because the description is based on a "genomic reference sequence," starting with "g," because of the availability of a reliable reference sequence of the complete human genome. When a coding DNA reference sequence is used, the description of the variant starts with "c."[37] To our knowledge we were the first to study this polymorphism in a control group of healthy Egyptians, and our results could be used as a reference for additional population studies.

The apelin rs3761581-A/C SNP in male patients and control subjects showed that the C allele was significantly greater in patients ($n = 118$, 63.4%) than in control subjects ($n = 42$, 33.9%), with a *P* value of 0.012. In females, no significant difference in A/C allele distribution was found, which

could be referred to the small sample size. The C allele is probably associated with the progression of HCV-related chronic liver disease, as patients with cirrhosis and varices (group I) showed a greater frequency of the C allele compared with control subjects ($P < 0.001$; OR, 3.8). Moreover, patients with no varices (group II) showed a greater frequency of the C allele compared with control subjects ($P = 0.001$; OR, 2.78). There was no statistically significant difference between A and C allelic frequency among the different groups of patients. However, although not statistically significant, the risk was greater in those patients who developed varices. Thus raising the probability that, although no statistically significant difference in C allelic frequency was observed among groups, the association may be found if larger patient sample is studied. It was stated by Tiani et al.[38] that apelin and the apelin signaling system contribute to portal collateralization and splanchnic neovascularization in rats with portal hypertension. Lv et al.[39] found that apelin/APJ activation was reported in patients with cirrhosis, and blockage of the apelin/APJ system was thought to improve liver cirrhosis, suggesting that the apelin/APJ system is a potential therapeutic target of liver cirrhosis. In these studies, apelin gene expression was studied in liver tissue, providing an accurate quantitative estimation of apelin, allowing a wide intergroup range of positivity for comparison. However, in our work, we studied allelic frequency, which is qualitative and provides less accurate information. Although we failed to establish an association between the apelin rs3761581-A/C SNP and varices, this failure of validation may be a result of the disregard of gene–gene and gene–environment interactions.[40] However, we provided information regarding gender difference in the apelin rs3761581 SNP. In females, no difference in allelic frequency was observed, in contrast to male patients, in whom the C allele was highly expressed in patients with chronic HCV compared with control subjects.

The serum apelin level was significantly greater in patients with varices (group I) compared with patients without varices (group II, $P < 0.001$). No statistically significant difference was found between patients with small and large varices ($P = 0.833$). The serum apelin level at a cutoff value of 16.55 ng/L can predict the presence of varices with an area under the receiver operating characteristic curve of 0.786, a sensitivity of 61.6%, a specificity of 97.4%, a P value of < 0.001, and a 95% CI of 0.702 to 0.870, but it cannot discriminate the grade of varices. A positive correlation was found between the serum apelin level and grade of liver fibrosis ($r = 0.346$, $P < 0.001$). Therefore, apelin contributes to the fibrogenic process occurring in liver disease and participates in disease progression.[41] El-Mesallamy et al.[42] reported that patients with fibrosis displayed greater median values of apelin when compared with asymptomatic patients with chronic HCV. In addition, a positive correlation was found between the serum apelin level and portal hypertension ($r = 0.438$, $P < 0.001$). It was previously stated that the apelin level was high in patients with chronic HCV with portal hypertension.[42]

Regarding the clinical and ultrasound parameters associated with varices, our study showed that the average portal vein diameter and spleen size in patients with varices (group I) were 15.12 ± 0.69 mm and 14.1 ± 2 cm, respectively, versus 10.23 ± 0.99 mm and 10.5 ± 1.2 cm, respectively, in patients without varices (group II, $P < 0.001$). This implies that an increase in portal vein diameter and splenic size is directly associated with varices formation.[10,43] Previously reported studies showed a correlation between portal vein diameter and the presence of varices; however, a definite cutoff was not estimated. It was reported that patients with a portal vein diameter > 11.5 mm and a spleen size of > 10.3 cm[44] are more likely to have varices. In other studies, Dib et al.[45] reported a portal vein diameter of > 13 mm as the cutoff for varices. Thomopoulos et al.[46] showed that the majority of patients with varices had a spleen size > 13.5 cm.

In conclusion, to our knowledge, this is the first study to investigate the apelin rs3761581-A/C SNP in Egyptian patients with chronic HCV. Our study added a reference value for the apelin rs3761581-A/C SNP in the healthy Egyptian population. We studied the association of the apelin rs3761581-A/C SNP with liver fibrosis and varices in a cohort of patients with chronic HCV. Patients with the C allele had a greater risk of liver fibrosis and varices.

The serum apelin level provided an accurate prediction of the presence of varices, which provides a cost-effective noninvasive alternative to upper GI endoscopy screening to detect the presence of esophageal varices. Accordingly, regular GI endoscopy at scheduled intervals can be limited to those suspected to have esophageal varices according to their serum apelin level.

The apelin gene has very low expression in peripheral blood, and liver tissue is required for studying gene expression, as proved by previous studies.[18,19,25]

Our work had several limitations: we studied only one SNP in the apelin promoter region, the sample size in each group was limited, and most of the patients included were male. Being unable to find an association between the apelin rs3761581 SNP and varices does not necessarily assume that the apelin gene polymorphism has no role in varices formation. Therefore, larger studies are needed, including a large number of patients, to study other SNPs in the apelin gene promotor region. The mechanism of varices formation is multifactorial, and angiogenesis plays a crucial part in its pathogenesis. Identifying candidate genes involved in angiogenesis and varices formation is fundamental to allow targeted gene blockage and spare patients with chronic HCV one of the most drastic and morbid complications of chronic HCV infection, especially in countries where HCV infection is endemic and its economic and social burden is unaffordable.

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Authors' addresses: Lamyaa Abdellatif Soliman, Rania A. Zayed, Fadwa Said, Doaa Mohamed Ghaith, and Iman Rifaat Elmahgoub, Clinical and Chemical Pathology Department, Faculty of Medicine, Cairo University, Cairo, Egypt, E-mails: lelbialy@gmail.com, rania.zayed@medicine.cu.edu.eg, f2said@yahoo.com, doaaghaith@gmail.com, and imanelmahgoub@gmail.com. Dalia Omran, Samar Kamal Darweesh, Rasha Eletreby, and Mahmoud Salama Barakat, Department of Endemic Medicine and Hepatogastroenterology, Faculty of Medicine, Cairo University, Cairo, Egypt, E-mails: daliaomran@kasralainy.edu.eg, samarkad@hotmail.com, r_m_tawfik@yahoo.com, and dr.mahmoudsala1987@gmail.com. Mahmoud M. Bendary, Department of Microbiology and

Immunology, Faculty of Pharmacy, Port Said University, Port Said, Egypt, E-mail: m.pendary@pharm.psu.edu.eg. Doaa Zakaria Zaky, Department of Tropical Medicine, Ain Shams University, Cairo, Egypt, E-mail: drdoaazakaria@gmail.com. Eman Amer, Department of Biochemistry, Faculty of Pharmacy, Ahram Canadian University, Cairo, Egypt, E-mail: dr_eman_amer@yahoo.com.

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