Association Between rs1859168/HOTTIP Expression Level and Colorectal Cancer and Adenomatous Polyposis Risk in Egyptians

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LncRNA HOTTIP is a new lncRNA that is strictly linked to the susceptibility, growth, propagation, and prognosis of several human cancers together with colorectal cancer. lncRNA HOTTIP rs1859168 may confer colorectal cancer susceptibility through regulating its gene expression level. To elucidate its role in colorectal cancer risk, we genotyped rs1859168 A>C and measured serum HOTTIP expression level in colorectal cancer, adenomatous polyposis patients and controls by real-time polymerase chain reaction. The results displayed that rs1859168 A>C single-nucleotide polymorphism is a risk factor for colorectal cancer among adenomatous polyposis patients and controls, AC versus CC genotypes [adjusted odds ratio (OR)=2.256, 95% confidence interval (CI)=1.316–3.868, $P=0.003$] when compared with controls and (adjusted OR=9.521, 95% CI=3.330–27.217, $P<0.0001$) when compared with adenomatous polyposis. Serum HOTTIP was upregulated in the colorectal cancer group when compared with adenomatous polyposis or controls [median (interquartile

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JICR

Journal of Interferon & Cytokine Research

Conclusion: rs1859168A>C single-nucleotide polymorphism and high serum HOTTIP are risk factors for colorectal cancer among adenomatous polyposis patients and controls; rs1859168 CC homozygous mutant genotype was significantly higher in colorectal cancer (53.6%) than in healthy controls (26.7%, $P=0.003$) and adenomatous polyposis patients (11.1%, $P<0.0001$). Also, there was a significantly higher prevalence of mutant C allele in colorectal cancer patients (76.8%) than healthy controls (53.3%, $P<0.0001$) and adenomatous polyposis patients (55.6%, $P=0.001$). Serum HOTTIP was upregulated in the colorectal cancer group [median (interquartile range) = 3.64 (2.46–5.02) ($P<0.0001$)] when compared with adenomatous polyposis or controls. A significant difference in serum HOTTIP was found to be associated with different rs1859168 genotypes. rs1859168A>C and higher serum HOTTIP were significantly associated with distant metastasis, lymph nodes metastasis, and grade III of colorectal cancer. Both rs1859168 and high HOTTIP confer increased risk for colorectal cancer development.

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DOI: 10.1089/jir.2019.0105

Visual Abstract was created by Marwa A. Ali

Color images are available online.

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range)=3.64 (2.46–5.02) ($P<0.0001$)). A significant difference in serum HOTTIP was found to be associated with different rs1859168 genotypes. rs1859168 A>C and higher serum HOTTIP were significantly associated with distant metastasis, lymph nodes metastasis, and grade III of colorectal cancer. Both rs1859168 and high HOTTIP confer increased risk for colorectal cancer development.

Keywords: HOTTIP, rs1859168, colorectal cancer

Background

AMONG THE MOST PREVALENT and fatal cancers that were distributed all over the world and with increasing incidence every day is colorectal cancer (Bray and others 2018). The term “sporadic colorectal cancer” is defined as cancer that affects subjects older than 50 years with no apparent risk factors. However, hereditary causes of colorectal cancer refer to familial inherited disorders called adenomatous polyposis syndromes that manifest at a younger age of life by hundreds to thousands of adenomas polyps affecting any part of the large intestine (Souglakos 2007). They are life-threatening conditions; so if they are not removed, one or more of these adenomatous polyps ultimately become malignant with high invasion performance (Talseth-Palmer 2017).

lncRNAs are reported as a type of RNA transcripts exceeding 200 nucleotides in length and lacking protein-coding capacity. Recently, possible usage of lncRNAs as cancer diagnostic or prognostic biomarkers has been informed in numerous studies (Bhat and others 2016; Yin and others 2019). Most of these studies report variations chiefly in lncRNAs expression profiles in several cancers when compared with the normal tissues of similar origin. However, increasing evidence has confirmed that also genetic variations that occur within the lncRNA incriminate the tumor induction process through affecting the lncRNAs expression levels and subsequently, their biological functions (Luo and others 2018).

HOTTIP is a recently discovered lncRNA that is transcribed from 5' tip of the HOXA locus and promotes the activation of many HOXA genes that are found to be involved in the oncogenesis process (Wang and others 2011; Bhatlekar and others 2014). Also, it was recently reported that lncRNA HOTTIP enhances interleukin 6 (IL-6) expression, which potentiates the immune escape of cancer cells by upregulating the expression of PD-L1 in neutrophils (Shang and others 2019). rs1859168 was reported as a potential functional polymorphism on HOTTIP that alters its expression level (Hu and others 2017).

Therefore, the current research aims at demonstrating the effect of rs1859168 on HOTTIP expression level as well as its influence on colorectal cancer, adenomatous polyposis (precancerous lesion) susceptibility, and their clinical and pathological parameters.

Subjects and Methods

Sample selection

The objectives, steps, and procedures of this study were permitted by the institutional Ethics Committee (Fayoum University Ethics Committee). The research has been performed in compliance with Helsinki's medical guidelines, and we obtained the signature of all participants on written informed consent after a clear clarification of the study's objectives. This study was composed of a case-control population; all

the cases were recruited from colorectal cancer, adenomatous polyposis patients attending at Fayoum University Hospital (Internal Medicine and Tropical departments), Fayoum University. After an initial diagnosis of all patients, a definite diagnosis was established by histopathological analysis. Patients were newly diagnosed and did not previously receive chemotherapy and/or radiotherapy for colorectal cancer.

There were 3 groups in this study: Group I, including 140 colorectal cancer patients aged (53.4 ± 11.9), with males representing 82.1% and females representing 17.9% of them; Group II, including 45 adenomatous polyposis patients, where 55.6% were males, and 44.4% were females aged (41 ± 18.5). A full history was taken from all patients (demographic characteristics, symptoms including abdominal pain, chronic constipation, rectal bleeding, and unplanned weight loss) followed by clinical examination and laboratory inspection (complete blood picture, liver, and kidney function tests), imaging studies [chest X-ray, pelviabdominal ultrasound, and pelviabdominal computed tomography (CT) scan]. The diagnosis was confirmed by colonoscopy and pathology examination.

Group III, control group, consisted of 150 healthy subjects without colorectal cancer or history of other cancers and without familial or past history of adenomatous polyposis coli. Controls were recruited from healthy blood donors in the blood bank.

Collection of blood samples

Venous blood samples were withdrawn from all individuals and divided into 2 tubes. One was an ethylenediaminetetraacetic acid (EDTA)-coated tube and was utilized for the extraction of DNA and genotyping of HOTTIP single-nucleotide polymorphism (SNP). The other tube was used for serum assessment of HOTTIP. The samples were kept in the freezer at -80°C till they were handled.

DNA extraction and genotyping of rs1859168

EDTA-coated blood samples were used for genomic DNA extraction by utilizing the QIA-amplification extraction kit (Qiagen, Valenica, CA) as stated by the manufacturer's instructions. DNA sample quantity and pureness were measured by NanoDrop® (ND)-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington). SNP of lncRNA HOTTIP rs1859168 was genotyped via real-time polymerase chain reaction by using predesigned Taqman primer/probe sets (with the TaqMan allelic discrimination assay; Applied Biosystems). HOTTIP rs1859168 (PN-4351379, Lot. P180726-004A01).

Polymerase chain reaction (PCR) protocol includes time of denaturation of 10 min at 95°C followed by 45 cycles at 92°C for 15 s, then 60°C for 90 s for annealing and extension. Fluorescence was detected at the achievement of every cycle and at the endpoint. Rotor-gene Q Real-Time PCR System (Qiagen) was used to check PCR products.

Measurement of serum lncRNA HOTTIP expression level

It has been shown that lncRNAs were present in human serum samples, where they remain remarkably stable (Miao and others 2016; Shaker and others 2019a, 2019b). MiRNeasy extraction kit (Qiagen) was used for total RNA extraction (with conserved lncRNAs) from all participants' serum by following the manufacturer's guidelines. Quantitation and purity of RNA samples were estimated by NanoDrop (ND)-1000 spectrophotometer (NanoDrop Technologies, Inc.). Reverse transcription (RT) was done through using RT2 first-strand kit (Qiagen) as documented in the manufacturer's instructions.

The lncRNA HOTTIP serum level was determined by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a normalizing control (Stephens and others 2011; Shaker and others 2019a) and customized primers and Maxima SYBR Green PCR kit (Thermo) as specified by the manufacturer's guidelines. The primer sequences for GAPDH were: 5'-CCCTTCATTGACCTCAACTA-3', reverse, 5'-TGGAAGATGGTGGTGGGATT-3'. HOTTIP (C-11173652-30). Real-time PCR was done by using Rotor-gene Q System (Qiagen) with the subsequent conditions: 95°C for 10 min, 45 cycles at 95°C for 15 s, and 60°C for 60 s.

Delta-delta CT ($2^{-\Delta\Delta Ct}$) equation was used for the estimation of the fold change of serum HOTTIP. The cycle threshold (Ct) is the number of quantitative PCR cycles that are essential for the fluorescent signal to cross a certain threshold. ΔCt is the difference between Ct values of housekeeping (GAPDH) and those of HOTTIP. $\Delta\Delta Ct$ was obtained by deducting the ΔCt values of the healthy control samples from the ΔCt of the patients' samples. As for the control samples, $\Delta\Delta Ct$ equals 0, and 2^0 equals 1 (Livak and Schmittgen 2001; Shaker and others 2019b).

Sample size

Sample size calculation using (G power version 3) was achieved. The minimum sample size of patients was 124 in the group needed to get power level 0.90, alpha level 0.05, and 20% as an expected difference in the proportion of

subjects with CC in polymorphism rs1859168 between colorectal cancer cases and control (50% versus 30%). The problem of missing data was overcome by increasing the calculated sample size by 20% to reach 136 in the 2 groups.

Statistical analysis of data

Chi-square test by a specific calculator, available online, was utilized to determine whether the studied SNP was in accordance with Hardy-Weinberg equilibrium or not. The information was organized, formulated, and statistically evaluated by the statistical package SPSS version 22 (SPSS, Inc.). As for numerical variables, we obtained the mean, median, standard deviation, and interquartile range (IQR). Kolmogorov-Smirnov test was performed as a test of normality. If the parameter was not distributed normally, Mann-Whitney *U* test (comparing between any 2 groups) was conducted. Alternatively, Independent *t*-test or one-way analysis of variance (ANOVA) was executed.

Qualitative information was shown as numbers and proportions. Chi-square (χ^2) was performed as a test of significance. Crude and adjusted (for age and sex) Odds ratios (ORs) with 95% confidence intervals (CIs) of genotypes of rs1859168 associated with different clinicopathological features in the colorectal cancer group were estimated by using univariate and multivariate logistic regression analysis. Also, Crude and adjusted ORs for the association of the study groups with genotype and allelic frequency of rs1859168 were calculated. To estimate the cut-off point at which the highest sensitivity and specificity of HOTTIP was reached in differentiating between different conditions, the receiver operating characteristic (ROC) curve analysis was done. Significance was approved at $P < 0.05$.

Results

Basic characteristics and laboratory variables in patients (colorectal cancer and adenomatous polyposis), and controls

Basic characteristics, laboratory parameters in studied groups are shown in (Table 1).

TABLE 1. DEMOGRAPHIC AND LABORATORY DATA OF STUDIED GROUPS

Parameter	CRC (n=140)	AP (n=45)	Control (n=150)	P
Age, mean $\pm$ SD	53.4 $\pm$ 11.9	41 $\pm$ 18.5	47 $\pm$ 9.5	<0.0001^a
Sex, n (%)				
Male	115 (82.1)	25 (55.6)	80 (53.3)	<0.0001^b
Female	25 (17.9)	20 (44.4)	70 (46.7)	
Hg (g/dL)	10.2 $\pm$ 3	12 $\pm$ 1.4		<0.0001^c
TLC ($\times 10^3/\text{mm}^3$)	7.6 $\pm$ 2.7	5.6 $\pm$ 0.9		<0.0001^c
Platelets ($\times 10^3/\text{mm}^3$)	304.79 $\pm$ 97.71	261.11 $\pm$ 39.44		<0.0001^c
ALT (0–42 IU/L)	22.5 $\pm$ 8.6	27.3 $\pm$ 4.8		<0.0001^c
AST (0–42 IU/L)	25.9 $\pm$ 8.9	32.9 $\pm$ 4.5		<0.0001^c
BIL (0.1–1.2 mg/dL)	0.69 $\pm$ 0.28	0.76 $\pm$ 0.23		0.149 ^c
ALB (3.5–5.5 g/dL)	4 $\pm$ 0.8	3.9 $\pm$ 0.1		0.190 ^c
ALP (0–290 IU/L)	87.6 $\pm$ 29.1	87.9 $\pm$ 12		0.926 ^c
Creatinine (0.6–1.2 mg/dL)	0.9 $\pm$ 0.2	0.9 $\pm$ 0.3		0.138 ^c

Age is shown as mean $\pm$ SD, and gender is presented by n (%). *P* values in bold are statistically significant ($P < 0.05$).

^aOne-way ANOVA.

^bChi-squared test.

^cIndependent *t*-test.

ALB, albumin; ALP, alkaline phosphatase; ALT, alanine transaminase; ANOVA, analysis of variance; AP, adenomatous polyposis; AST, aspartate transaminase; BIL, bilirubin; CRC, colorectal cancer; Hg, hemoglobin; TLC, total leucocytic count; SD, standard deviation.

Regarding demographic data of studied subjects, adenomatous polyposis patients were the youngest among studied subjects ($P < 0.0001$). Male gender was more prevalent in colorectal cancer (82.1%) and adenomatous polyposis (55.6%) groups than healthy controls (53.3%) ($P < 0.0001$).

Regarding laboratory data of the involved patients, anemia was found in all patients with a significantly lower hemoglobin (Hg) level in the colorectal cancer (10.2 ± 3) than the adenomatous polyposis group (12 ± 1.4) ($P < 0.0001$). However, total leukocytic counts for both groups were within normal reported ranges, a significant relative leukocytosis was detected in the colorectal cancer group (7.6 ± 2.7) when compared with the adenomatous polyposis group (5.6 ± 0.9) ($P < 0.0001$).

Normal mean platelet counts were reported in case groups with relative thrombocytosis detected in the colorectal cancer group ($P < 0.0001$). Alanine transaminase (ALT) and Aspartate transaminase (AST) values were within normal values, with higher levels presented in the adenomatous polyposis group (27.3 ± 4.8 , 32.9 ± 4.5 , respectively) than the colorectal cancer group (22.5 ± 8.6 , 25.9 ± 8.9 , respectively) ($P < 0.0001$). There were no significant differences between colorectal cancer and adenomatous polyposis patients as for bilirubin, albumin, alkaline phosphatase, and creatinine levels.

Clinical and pathological parameters of the patients

Regarding the colorectal cancer site, 60% of colorectal tumors were in the recto-sigmoid portion of the large bowel followed by transverse colon+flexures and cecum+ascending colon (21.4%, 17.9%, respectively).

Regarding reported symptoms, patients, whether they had colorectal cancer or adenomatous polyposis syndrome, were suffering from abdominal pain, rectal bleeding, and constipation. However, severe unexplained weight loss was detected in colorectal cancer patients but not in adenomatous polyposis patients.

Regarding pathological features of cancer, 89.3% of colorectal cancer tumors were adenocarcinoma (10.7% were mucinous adenocarcinoma), of whom more than half of them (67.1%) were with lower grades [Tubulovillous (carcinoma *in situ*)+grade I+grade II] whereas 22.2% were with higher grades (grade III+undifferentiated adenocarcinoma), and 10.7% of patients were with undetermined pathological grade. Distal liver metastasis was detected in 7.1%, and lymph node (LN) metastasis was determined in 32.1% of colorectal cancer patients.

Regarding colonoscopy, 57.1% of colorectal cancers were presented as circumferential malignant ulcers, 46.4% were fungating mass, whereas other presentations included were circumferential stricturing and irregular polypoidal (17.9% each).

In the adenomatous polyposis group, 33.3% of adenomatous polyps were present in the recto-sigmoid portion of the large intestine or distributed all over the colon, whereas 22.2% of adenomatous polyps affected the ascending and 11.1% affected the transverse colon. According to the size of polyps, there were 2 groups; polyps ≥ 3 cm (11.1%) and polyps < 3 cm (88.9%). According to the shape of polyps, 55.6% of polyps were pedunculated; meanwhile, 44.4% were sessile polyps. According to the numbers of polyps, there may be < 5 polyps (66.7%) or ≥ 5 polyps (33.3%). The pathological type of polyps includes non-adenomatous polyps (55.6%) and adenomatous polyps (44.4%) (Table 2).

TABLE 2. CLINICAL AND PATHOLOGICAL FEATURES OF STUDIED GROUPS

Parameters	CRC (n = 140)		AP (n = 45)	
	n	%	n	%
Site				
Cecum+ascending	25	17.9	10	22.2
Transverse+flexures	30	21.4	5	11.1
Rectosigmoid	85	60.7	15	33.3
All over the colon			15	33.3
Symptoms				
Abdominal pain				
Yes	100	71.4	25	55.6
No	40	28.6	20	44.4
Bleeding per rectum				
Yes	35	25.0	20	44.4
No	105	75.0	25	55.6
Constipation				
Yes	90	64.3	10	22.2
No	50	35.7	35	77.8
Loss of weight				
Yes	50	35.7	0	0.0
No	90	64.3	45	100.0
Tumor- related data				
CT_liver metastasis				
Yes	10	7.1		
No	130	92.9		
CT_LN_metastasis				
Yes	45	32.1		
No	95	67.9		
Pathological types and grades				
Adenocarcinoma	125	89.3		
Mucinous adenocarcinoma	15	10.7		
Tubulovillous (carcinoma <i>in situ</i>)	10	7.1		
Grade I	30	21.4		
Grade II	54	38.6		
Grade III	26	18.6		
Undifferentiated	5	3.6		
Colonoscopy				
Fungating mass				
Yes	65	46.4		
No	75	53.6		
Circumferential stricturing				
Yes	25	17.9		
No	115	82.1		
Irregular polypoidal				
Yes	25	17.9		
No	115	82.1		
Circumferential ulcerating				
Yes	80	57.1		
No	60	42.9		
Size of polyps				
≥ 3 cm			5	11.1
< 3 cm			40	88.9
Shape of polyps				
Pedunculated			25	55.6
Sessile			20	44.4
Number of polyps				
≥ 5			15	33.3
< 5			30	66.7
Pathological type of polyps				
Nonadenomatous			25	55.6
Adenomatous			20	44.4
Low-grade dysplasia			10	22.2
Moderate-grade dysplasia			10	22.2

Fifteen patients (10.7%) were with undetermined pathological grade. CT, computed tomography; LN, lymph node.

TABLE 3. GENOTYPES AND ALLELIC DISTRIBUTION OF HOTTIP rs1859168 A/C IN COLORECTAL CANCER, ADENOMATOUS POLYPOSIS CASES, AND CONTROL GROUP

HOTTIP Rs8159168 (A/C)	CRC (n= 140)		CRC versus control		CRC versus AP		AP versus control		Control (n= 150)
	n (%)	n (%)	OR (95% CI), P ^a		OR (95% CI), P ^b		OR (95% CI), P ^a		
			OR (95% CI), P ^b	OR (95% CI), P ^b	OR (95% CI), P ^b	OR (95% CI), P ^b	OR (95% CI), P ^a	OR (95% CI), P ^b	
Genotypic model									
AA	0	0	—	—	—	—	—	—	30 (20.0)
AC	65 (46.4)	40 (88.9)	r	r	r	r	r	r	80 (53.3)
CC	75 (53.6)	5 (11.1)	2.308 (1.394–3.821), 0.001	9.231 (3.439–24.774), <0.0001	0.250 (0.092–0.682), 0.007	0.250 (0.092–0.682), 0.007	0.250 (0.092–0.682), 0.007	0.250 (0.092–0.682), 0.007	40 (26.7)
			2.256 (1.316–3.868), 0.003	9.521 (3.330–27.217), <0.0001	0.206 (0.072–0.587), 0.003	0.206 (0.072–0.587), 0.003	0.206 (0.072–0.587), 0.003	0.206 (0.072–0.587), 0.003	
Allele									
A	65 (23.2)	40 (44.4)	r	r	r	r	r	r	140 (46.7)
C	215 (76.8)	50 (55.6)	2.894 (2.023–4.142), <0.0001	2.646 (1.605–4.362), <0.0001	1.094 (0.681–1.756), 0.711	1.094 (0.681–1.756), 0.711	1.094 (0.681–1.756), 0.711	1.094 (0.681–1.756), 0.711	160 (53.3)
			2.757 (1.886–4.030), <0.0001	2.630 (1.519–4.553), 0.001	0.829 (0.578–1.549), 0.827	0.829 (0.578–1.549), 0.827	0.829 (0.578–1.549), 0.827	0.829 (0.578–1.549), 0.827	

Hardy–Weinberg equilibrium; $\chi^2 = 0.77$, $P = 0.380$. P values in bold are statistically significant (<0.05).

^aCrude P value done by univariate logistic regression.

^bAdjusted P value done by multivariate logistic regression (adjusted for age and sex).
CI, confidence interval; OR, odds ratio.

TABLE 4. ASSOCIATION BETWEEN rs1859168 GENOTYPES AND COLORECTAL CANCER STRATIFIED BY AGE AND SEX

Rs1859168	CC		AC		OR (95% CI); P ^a
	n	%	n	%	
Age					
>50					
CRC	50	71.4	45	52.9	2.222 (1.136–4.347);
Control	20	28.6	40	47.1	0.019
≤50					
CRC	25	55.6	20	33.3	2.500 (1.128–5.542);
Control	20	44.4	40	66.7	0.038
Sex					
Male					
CRC	65	76.5	50	47.6	3.575 (1.903–6.717);
Control	20	23.5	55	52.4	<0.0001
Female					
CRC	10	33.3	15	37.5	0.833 (0.309–2.249);
Control	20	66.7	25	62.5	0.917

P values in bold are statistically significant (<0.05).
^aUnivariate logistic regression.

Associations between HOTTIP rs1859168 genotypes and the risk of colorectal cancer and adenomatous polyposis

The genotype and allele distributions of HOTTIP SNP (rs1859168) in cases (colorectal cancer, adenomatous polyposis) and controls are shown in Table 3.

The rs1859168 genotype distributions in controls were in accordance with Hardy–Weinberg equilibrium ($P = 0.380$).

Our results revealed that wild genotype AA was not present in the colorectal cancer or adenomatous polyposis group but represented 20% of controls. So on comparing 2 mutant genotypes AC with CC, we determined that the occurrence of the rs1859168 CC homozygous mutant genotype was significantly higher in colorectal cancer than in healthy controls (53.6% versus 26.7%, respectively, crude $P = 0.001$) and this significance also was present after adjustment of age and sex (AC versus CC, adjusted OR = 2.256, 95% CI = 1.316–3.868, $P = 0.003$). Also, there was a significantly higher prevalence of mutant C allele in colorectal cancer patients than healthy controls in both adjusted and non-adjusted models ($P < 0.0001$).

On comparing the distribution of rs1859168 genotypes and alleles between colorectal cancer and adenomatous polyposis groups, we got a significant difference with higher mutant genotype (CC) and allele (C) prevalence in colorectal cancer than adenomatous polyposis patients (53.6% versus 11.1%, respectively, for genotypes) and (76.8% versus 55.6%, respectively, for alleles) (AC versus CC, adjusted OR = 9.521, 95% CI = 3.330–27.217, $P < 0.0001$) (A versus C, adjusted OR = 2.630, 95% CI = 1.519–4.553, $P = 0.001$).

As for the adenomatous polyposis group, there was a significant difference between adenomatous polyposis and controls as regards genotypes with a higher percentage of heterozygous mutant AC genotype in the adenomatous polyposis group (88.9%) when compared with the homozygous mutant CC genotype (11.1%) (AC versus CC, adjusted OR = 0.206, 95% CI = 0.072–0.587, $P = 0.003$). However, as regards allele distribution, there were no significant differences between the adenomatous polyposis group and healthy individuals ($P = 0.827$).

FIG. 1. Relative serum expression level of lncRNA HOTTIP in studied groups. Increased serum HOTTIP was observed in the colorectal cancer group when compared with adenomatous polyposis and/or controls [median (IQR)=3.64 (2.46–5.02) ($P<0.0001$)]. Also, increased serum HOTTIP was noted in the adenomatous polyposis group when compared with controls [median (IQR)=1.2 (0.92–1.21) ($P=0.016$)]. The horizontal line represents the expression level of the control group. Control was considered as 1. IQR, interquartile range.

Stratification analysis of rs1859168 by age and sex in the colorectal cancer group

We further stratified rs1859168 risk in the colorectal cancer group by age and sex (Table 4). The results documented that rs1859168 CC was the risky genotype in all age groups as well as in male patients ($P<0.0001$).

HOTTIP Serum level in colorectal cancer and adenomatous polyposis

Serum HOTTIP was higher in colorectal cancer and adenomatous polyposis patients when compared with healthy controls with median=3.64, IQR=(2.46–5.02) ($P<0.0001$) for the colorectal cancer group and median=1.2, IQR=0.92–1.21 ($P=0.016$) for the adenomatous polyposis group. Serum HOTTIP level also could distinguish between colorectal cancer patients and adenomatous polyposis patients ($P<0.0001$) (Fig. 1).

Association between rs1859168 and serum HOTTIP expression

Lower serum HOTTIP was significantly associated with the CC genotype or the C allele than the AC genotype or the A allele in colorectal cancer patients ($P<0.0001$). Conversely, in the adenomatous polyposis group, serum HOTTIP was not significantly different between either genotypes or alleles (Fig. 2A, B).

Correlation of rs1859168 genotypes and serum HOTTIP with clinical and pathological parameters in colorectal cancer patients

Associations between rs1859168, serum HOTTIP, and clinical and pathological parameters of colorectal cancer are shown in Tables 5 and 6.

FIG. 2. (A) Association between rs1859168 genotypes and HOTTIP serum fold change in studied groups. (B) Association between rs1859168 alleles and HOTTIP serum fold change in studied groups.

Regarding symptoms of colorectal cancer, a significantly larger percentage of patients who had heterozygous AC genotype had abdominal pain ($P=0.002$), unintended weight loss ($P=0.009$), but with less incidence to have constipation ($P<0.0001$). Higher HOTTIP was significantly associated with the presence of abdominal pain ($P<0.0001$) and the presence of bleeding per rectum ($P=0.019$).

As for tumor-related data, both the AC genotype and high HOTTIP expression level were associated with LN metastasis ($P<0.0001$, $P=0.012$ respectively), distal liver metastasis ($P<0.0001$, $P=0.043$ respectively), and stage III and/or undifferentiated colorectal cancer ($P=0.046$, $P<0.0001$ respectively). The AC genotype but not serum HOTTIP was associated with adenocarcinoma than mucinous adenocarcinoma ($P<0.0001$).

As regards colonoscopy findings, we found that higher serum HOTTIP fold changes were significantly associated with fungating mass or an irregular polypoidal pathological picture of colorectal cancer.

TABLE 5. RELATIONSHIP BETWEEN GENOTYPES OF HOTTIP rs8159168 AND DIFFERENT CHARACTERISTICS IN COLORECTAL CANCER GROUP

Parameter	CC		AC		Crude OR (95% CI), P ^a	Adjusted OR (95% CI), P ^b
	n	%	n	%		
Site						
Colon	25	33.3	30	46.2	1	1
Rectosegmoid	50	66.7	35	53.8	1.714 (0.865–3.398); 0.123	1.748 (0.878–3.479); 0.112
Symptoms						
Abdominal pain						
Yes	45	60.0	55	84.6	0.273 (0.120–0.617); 0.002	0.276 (0.122–0.625); 0.002
No	30	40.0	10	15.4	1	1
Bleeding per rectum						
Yes	15	20.0	20	30.8	0.563 (0.260–1.219); 0.145	0.585 (0.266–1.288); 0.183
No	60	80.0	45	69.2	1	1
Constipation						
Yes	60	80.0	30	46.2	4.667 (2.211–9.849); <0.0001	4.922 (2.301–10.529); <0.0001
No	15	20.0	35	53.8	1	1
Loss of weight						
Yes	20	26.7	30	46.2	0.424 (0.209–0.860); 0.017	0.376 (0.180–0.785); 0.009
No	55	73.3	35	53.8	1	1
Tumor-related data						
CT_liver metastasis						
Yes	0	0.0	10	15.4	<0.0001^a	
No	75	100.0	55	84.6		
CT_LN_metastasis						
Yes	15	20.0	30	46.2	0.292 (0.138–0.616); 0.001	0.168 (0.078–0.443); <0.0001
No	60	80.0	35	53.8	1	1
Pathological types and grades						
Adenocarcinoma	60	80.0	65	100.0	<0.0001^a	
Mucinous adenocarcinoma	15	20.0	0	0.0		
Tubulovillous+Grade I+	50	83.3	44	67.7	2.386 (1.015–5.612); 0.046	2.333 (0.988–5.507); 0.053
Grade II						
Grade III+	10	16.7	21	32.3	1	1
undifferentiated						
Colonoscopy						
Fungating mass						
Yes	30	40.0	35	53.8	0.571 (0.292–1.119); 0.103	0.590 (0.298–1.168); 0.130
No	45	60.0	30	46.2	1	1
Circumferential stricturing						
Yes	10	13.3	15	23.1	0.513 (0.212–1.238); 0.137	0.539 (0.216–1.345); 0.185
No	65	86.7	50	76.9	1	1
Irregular polypoidal						
Yes	10	13.3	15	23.1	0.513 (0.212–1.238); 0.137	0.539 (0.216–1.345); 0.185
No	65	86.7	50	76.9	1	1
Circumferential ulcerating						
Yes	60	80.0	45	69.2	1.778 (0.821–3.851); 0.145	1.704 (0.772–3.761); 0.187
No	15	20.0	20	30.8	1	1

P values in bold are statistically significant (<0.05).

^aCrude P value done by Univariate logistic regression.

^bAdjusted P value done by Multivariate logistic regression (adjusted for age and sex).

Correlation of rs1859168 genotypes and serum HOTTIP levels with clinical & pathological data in adenomatous polyposis patients

Regarding rs1859168, a significantly large proportion of patients who had AC genotype had rectal polyposis ($P < 0.0001$), abdominal pain ($P = 0.013$), but were less likely to have constipation ($P < 0.0001$) (Table 7). Results of the association between clinicopathological data of adenomatous polyposis and rs1859168 should be taken into consideration carefully due to the quite small number of adenomatous polyposis patients.

We got significant value with higher serum levels of HOTTIP in larger size of polyps ($P < 0.0001$), the larger number of polyps ($P < 0.0001$), pedunculated shape of polyps ($P = 0.022$), and with the existence of rectal bleeding ($P = 0.004$) (Table 8).

ROC curve analysis for serum HOTTIP

The ROC curve analysis of serum HOTTIP in colorectal cancer and adenomatous polyposis patients is determined in Table 9.

TABLE 6. DIFFERENCE IN SERUM HOTTIP IN RELATION TO CLINICOPATHOLOGICAL CHARACTERISTICS IN COLORECTAL CANCER

Parameter	Median	IQR	P ^a
Site			
Colon	3.92	3.1–5.02	0.631
Rectosegmoid	3.57	2.25–5.03	
Symptoms			
Abdominal pain			
Yes	4.19	2.9–5.59	<0.0001
No	3.08	1.67–3.54	
Bleeding per rectum			
Yes	3.72	3.06–5.02	0.019
No	3.1	1.09–5.01	
Constipation			
Yes	3.46	2.25–5.01	0.277
No	4	3.06–6.18	
Loss of weight			
Yes	3.42	3.06–4.09	0.446
No	3.92	2.25–5.18	
Tumor-related data			
CT_liver_metastasis			
Yes	4.56	4.09–5.02	0.043
No	3.46	2.25–5.02	
CT_LN_metastasis			
Yes	4.09	3.06–6.18	0.012
No	3.36	2.25–5.01	
Pathological types and grades			
Adenocarcinoma	3.72	2.25–5.02	0.273
Mucinous adenocarcinoma	3.57	3.06–7.08	
Tubulovillous+	3.13	1.68–5.01	<0.0001
Grade I+Grade II			
Grade III+undifferentiated	4.28	3.92–6.18	
Colonoscopy			
Fungating mass			
Yes	4.72	3.57–5.18	<0.0001
No	3.1	1.68–4.28	
Circumferential structuring			
Yes	3.92	3.36–4.28	0.946
No	3.57	2.25–5.02	
Irregular polypoidal			
Yes	6.18	4.28–7.06	<0.0001
No	3.36	2.11–5.01	
Circumferential ulcerating			
Yes	3.35	2.65–5.02	0.833
No	3.82	2.18–5.51	

P values in bold are statistically significant (<0.05).

^aMann–Whitney U test.

IQR, interquartile range.

As for the HOTTIP's diagnostic role in colorectal cancer, by ROC curve performance we determined that serum HOTTIP differentiated colorectal cancer from healthy individuals with 96.4% sensitivity and 100% specificity (Fig. 3). Serum HOTTIP also differentiated colorectal cancer patients from adenomatous polyposis patients with 89.3% sensitivity and 88.9% specificity (Fig. 4). These results verified that serum HOTTIP fold change could be used as a sensitive and specific diagnostic tumor marker for colorectal cancer. In addition, serum HOTTIP discriminated adenomatous polyposis patients from healthy subjects with cut-off equals 1.04, 64.4% sensitivity, and 100% specificity (Fig. 5).

As for HOTTIP's prognostic role in colorectal cancer, ROC analysis revealed that serum HOTTIP could predict distant liver metastasis in colorectal cancer; cut-off=4.01, AUC=0.692, 95% CI=0.606–0.779, $P=0.043$, with 100%

TABLE 7. RELATIONSHIP BETWEEN GENOTYPES OF HOTTIP rs8159168 AND DIFFERENT CHARACTERISTICS IN ADENOMATOUS POLYPOSIS GROUP

Parameter	CC		AC		P ^a
	n	%	n	%	
Site					
Colon	5	100.0	25	62.5	<0.0001
Rectal	0	0.0	15	37.5	
Symptoms					
Abdominal pain					
Yes	0	0.0	25	62.5	0.013
No	5	100.0	15	37.5	
Bleeding per rectum					
Yes	0	0.0	20	50.0	0.056
No	5	100.0	20	50.0	
Constipation					
Yes	5	100.0	5	12.5	<0.0001
No	0	0.0	35	87.5	
Size of polyps					
≥3 cm	0	0.0	5	12.5	1.000
<3 cm	5	100.0	35	87.5	
Shape of polyp					
Pedunculated	5	100.0	20	50.0	0.056
Sessile	0	0.0	20	50.0	
Numbers of polyps					
≥5	0	0.0	15	37.5	0.153
<5	5	100.0	25	62.5	
Pathological types of polyps					
Nonadenomatous	5	100.0	20	50.0	0.056
Adenomatous	0	0.0	20	50.0	
Low-grade dysplasia	0	0.0	10	25.0	
Moderate-grade dysplasia	0	0.0	10	25.0	

P values in bold are statistically significant (<0.05).

^aChi-squared test.

sensitivity, 61.5% specificity (Fig. 6). Serum HOTTIP also predicts LN metastasis in colorectal cancer with best cut-off=3.82, AUC=0.632, 95% CI=0.529–0.734, $P=0.012$, with 66.7% sensitivity, and 63.2% specificity (Fig. 7). In addition, serum HOTTIP differentiated colorectal cancer patients with grade III and undifferentiated adenocarcinoma from lower pathological grades; cut-off=3.81, AUC=0.744, 95% CI=0.649–0.838, $P<0.0001$, with 80.6% sensitivity, and 62.8% specificity (Fig. 8).

Discussion

This case-control study was conducted on 140 colorectal cancer, 45 adenomatous polyposis patients, and 150 healthy controls. Significant differences were detected between studied groups as regards age and gender, with older and male patients being prevalent in the colorectal cancer group. Increasing incidence of colorectal cancer in patients older than 50 years and male patients was stated in several previous research studies (Ansa and others 2018; White and others 2018).

Regarding laboratory data in our patients' groups, anemia was a hallmark that characterized all colorectal cancer and adenomatous polyposis patients; this result was consistent with preceding reports that documented that anemia is a clinical feature frequently detected in colorectal cancer

TABLE 8. DIFFERENCE IN HOTTIP IN RELATION TO CLINICOPATHOLOGICAL CHARACTERISTICS IN ADENOMATOUS POLYPOSIS GROUP

Parameter	Median	IQR	P ^a
Site			
Colon	1.2	0.92–1.21	0.070
Rectal	1.2	0.86–1.2	
Symptoms			
Abdominal pain			
Yes	1.2	0.9–1.21	0.566
No	1.2	0.96–1.6	
Bleeding per rectum			
Yes	1.21	1.06–1.61	0.004
No	1.09	0.9–1.2	
Constipation			
Yes	1.21	1.09–1.21	0.179
No	1.2	0.9–1.21	
Size of polyp			
≥3 cm	2.1	2.02–2.12	<0.0001
<3 cm	1.2	0.91–1.2	
Shape of polyp			
Pedunculated	1.2	1.08–1.21	0.022
Sessile	1.05	0.88–1.21	
Number of polyps			
≥5	1.21	1.2–2.02	<0.0001
<5	0.96	0.9–1.2	
Pathological types of polyps			
Nonadenomatous	1.2	1.08–1.21	1.000
Adenomatous	1.06	0.91–1.6	
Low-grade dysplasia	1.46	0.92–2.1	0.063
Moderate-grade dysplasia	1.05	0.9–1.2	

P values in bold are statistically significant ($P < 0.05$).

^aMann–Whitney *U* test.

patients (Knight and others 2004) and adenomatous polyposis patients (Half and others 2009).

Moreover, relatively significant leukocytosis and thrombocytosis was detected in colorectal cancer patients than adenomatous polyposis patients, consistent with Zhou and others (2017) and Zhu and others (2018). Further, AST and ALT were significantly elevated in the adenomatous polyposis group versus the colon cancer group ($P < 0.0001$). It was found that 42% of non-alcoholic steatohepatitis cases were associated with colorectal adenomatous polyps, with a subsequently higher level of AST and ALT, which serve as liver function tests (Bhatt and others 2015).

Regarding rs1859168, on comparing the mutant genotypes (homozygous CC against heterozygous AC as well as C against A allele) in all studied groups, homozygosity of

the mutant genotype (CC) or the C allele was associated with a significantly increased risk of colorectal cancer when compared with adenomatous polyposis or controls. Also, the CC colorectal cancer-associated risk was more obvious in male patients in all age groups.

Previous studies conveyed the probable effect of the rs1859168 genetic variant on HOTTIP and cancers. Our results were compatible with the results of a meta-analysis achieved by Huang and others (2018), who reported that the HOTTIP rs1859168 A/C polymorphism was associated with an increased overall incidence of cancer in all genetic models. Also, our findings were consistent with Gong and others (2016), who reported that the rs1859168 was significantly associated with lung cancer risk in the dominant model.

However, our results were not in agreement with Hu and others (2017) and Duan and others (2018), who determined that the rs1859168 was associated with a lower risk of pancreatic and gastric cancers in the Chinese population. Meanwhile, Richards and others (2015) found no relationship between rs1859168 and epithelial ovarian cancer risk. This incongruity and variation of the influence of HOTTIP rs1859168 on cancer susceptibility perhaps referred to ethnic differences in the studied populations and the type of the cancer tissue.

Abnormalities in lncRNAs expression levels in cancer tissue biopsies have arisen as potential novel markers that predict cancer susceptibility and outcomes; however, the invasive procedure of biopsies could restrict their usage (Saus and others 2016). Circulating lncRNAs are easily reachable and are sufficiently stable for reasonable periods to serve as clinical biomarkers (Yuan and others 2017). In this study, we verified, for the first time, that serum HOTTIP was upregulated in colorectal cancer patients, differentiating them from adenoma patients or controls, and ROC curve analysis discriminated colorectal cancer patients from adenomatous polyposis cases or controls with almost 100% sensitivity and specificity when compared with controls, which identified serum HOTTIP as an applicable non-invasive early biomarker for colorectal cancer diagnosis.

Previous studies determined that upregulated HOTTIP can influence cancer susceptibility through regulation of cancer-promoting genes (HOXA genes) (Wang and others 2011; Bhatlekar and others 2014). Recently, it was demonstrated that HOTTIP also can break immune tolerance to cancer cells via increasing IL-6 expression, which modifies a crucial checkpoint of immunity by upregulating PD-L1 expression that binds to the PD-1 receptor on neutrophils, resulting in suppression of host immune response and enhancement of cancer cells' immune escape (Shang and others 2019). Increased serum HOTTIP in the current

TABLE 9. DIAGNOSTIC AND PROGNOSTIC PERFORMANCE OF SERUM HOTTIP

Variable	AUC (95% CI)	P	Cut-off point	Sensitivity (%)	Specificity (%)
CRC versus control	0.964 (0.934–0.995)	<0.0001	1.05	96.4	100
CRC versus polyp	0.913 (0.871–0.954)	<0.0001	1.38	89.3	88.9
Polyp versus control	0.656 (0.518–0.793)	0.002	1.04	64.4	100
CT_liver_metastasis	0.692 (0.606–0.779)	0.043	4.01	100	61.5
CT_LN metastasis	0.632 (0.529–0.734)	0.012	3.82	66.7	63.2
Grade III +undifferentiated	0.744 (0.649–0.838)	<0.0001	3.81	80.6	62.8

P values in bold are statistically significant ($P < 0.05$).

AUC, area under curve.

FIG. 3. ROC curve for serum HOTTIP differentiated colorectal cancer patients from controls and proved the efficacy of HOTTIP as a tumor biomarker with 100% specificity. ROC, receiver operating characteristic.

research was consistent with preceding research in colorectal cancer tumor tissues and cell lines (Lian and others 2016; Liu and others 2018; Rui and others 2018), which could be reflected in the serum (Miao and others 2016).

The rs1859168 has been reported as a potential functional SNP on the HOTTIP that affects the transcription activity

Diagonal segments are produced by ties.

FIG. 5. ROC curve indicating that serum HOTTIP could differentiate adenomatous polyposis patients from controls; (cut-off = 1.04, sensitivity = 64.4%, specificity = 100%).

and alters its expression level (Hu and others 2017). So we examined the correlation between serum HOTTIP and different genotypes. Unexpectedly, our results determined that lower serum HOTTIP was significantly related to risky homozygous genotype (CC) than heterozygous (AC) genotype in colorectal cancer patients ($P < 0.0001$).

FIG. 4. ROC curve for serum HOTTIP differentiating colorectal cancer patients from adenomatous polyposis patients at cut-off value = 1.38 with almost 90% sensitivity and specificity.

FIG. 6. ROC curve revealed that serum HOTTIP could predict distant liver metastasis in colorectal cancer at cut-off = 4.01, sensitivity = 100%, and specificity = 61.5%.

FIG. 7. ROC curve revealed that serum HOTTIP could predict distant lymph node metastasis in colorectal cancer at cut-off=3.82 and with sensitivity=66.7%, specificity=63.2%.

The above-mentioned finding could be explained by the fact that several factors could regulate HOTTIP expression levels other than rs1859168 SNP, for instance, epigenetic modifications (Zhou and others 2018), transcription factors (Wu and others 2014), and post-transcriptional regulation by miRNA-lncRNA interactions such as miR125b, miR192,

FIG. 8. ROC curve of serum HOTTIP differentiating grade III tumor and undifferentiated tumors from other grades (Tubulovillous+I+II) in colorectal cancer.

and miR-204, which suppress HOTTIP expression (Tsang and others 2015; Ge and others 2016). Also, its expression could be affected by other functional HOTTIP SNPs (Lv and others 2019). The study results need to be confirmed by further studies on larger sample sizes with the involvement of other functional polymorphisms in the studied gene and by taking into consideration other factors affecting HOTTIP expression level.

We further investigated the correlation between clinical and pathological data of cancer, studied SNP and lncRNA with site, symptoms, tumor-related pathological data, and colonoscopy finding.

As regards tumor-related pathological data, we found that the AC genotype was significantly associated with the determinants of tumor aggressiveness, including LN metastasis, distant liver metastasis, and stage III of colorectal cancer. These findings could be explained by the previously reported results in the current study, which verified that AC was associated with significantly higher expression of HOTTIP. This explanation was confirmed by our detected correlation between higher serum HOTTIP and the same tumor-related data. Also, by ROC curve analysis, serum HOTTIP could predict distant metastasis, nodal metastasis, and higher grades of colorectal cancer.

Several studies reported that HOTTIP is an unfavorable factor in colorectal cancer patients, as the higher expression level of HOTTIP was closely related to higher TNM staging, distant metastasis, and advanced clinical grade of colorectal cancer (Ren and others 2015). These findings proved the utility of usage of both serum HOTTIP and its SNP as non-invasive clinical markers not only for early colorectal cancer diagnosis but also for prediction of colon cancer prognosis and aggressiveness. Also, the AC genotype was significantly associated with adenocarcinoma than mucinous adenocarcinoma. These results should be verified in a large-scale population due to the relatively small number of mucinous adenocarcinoma with the involvement of distant, nodal metastasis and clinical grade of colorectal cancer.

Regarding colonoscopy findings, in our study, we found that higher serum HOTTIP levels were significantly associated with fungating mass or irregular polypoidal but not with stricturing or ulcerating pathological pictures of colorectal cancer determined by colonoscopy. Prior reports proved that HOTTIP could promote colorectal cancer progress and inhibit cell apoptosis by multiple mechanisms (Lian and others 2016; Liu and others 2018; Rui and others 2018) and its higher expression level was firmly related to patients with larger tumor size (Rui and others 2018). These data may explain that higher HOTTIP is associated with bulky tumor pathological type.

Regarding symptoms, our results determined that the AC genotype and/or higher HOTTIP were associated with abdominal pain, bleeding per rectum in the colorectal cancer or adenomatous polyposis group, and weight loss in colorectal cancer. But colorectal cancer or adenomatous polyposis patients who had heterozygous CC genotype were more likely to have constipation.

It was found that bleeding per rectum and weight loss were associated with advanced cancer grade and/or with higher TNM staging of colorectal cancer, which explain their association with high HOTTIP expression and which also has been demonstrated to be linked to tumor aggressiveness data of colorectal cancer (Ren and others 2015; Rui

and others 2018). However, abdominal pain and constipation were the 2 most common colorectal cancer manifestations that presented early and long lasting throughout the course of the disease and were detected to be associated with the site of colorectal cancer rather than stages and the grades of the tumor (Bedir and others 2016).

As adenomatous polyposis is a precancerous lesion and was commonly converted to colorectal cancer through carcinogenesis sequence (Pitot 1993), we verified whether the rs1859168 SNP or HOTTIP is implicated in this tumorigenesis process. We detected that a significantly higher heterozygous AC genotype was detected in the adenomatous polyposis group than controls ($P=0.003$). Also, serum HOTTIP was significantly higher in adenomatous polyposis versus control group, differentiating the 2 groups from each other. Besides, higher serum HOTTIP fold changes were detected in the larger size of the polyp, the larger numbers of polyps, and the pedunculated shape of polyps.

As cancer progression is a multistep process that requires supplementary molecular modifications, particularly amplified cell proliferation (Pitot 1993) and, indeed, HOTTIP stimulates the survival, proliferation, and propagation of many cancer cells (Ren and others 2015; Rui and others 2018). So, these results elucidate that HOTTIP dysregulation is a crucial pro-tumorigenic factor in colorectal cancer induction on top of colorectal adenomas; specifically, the higher AC genotype in the adenomatous polyposis group than colorectal cancer in our study possibly proposes that adenomatous polyposis may require a high level of oncogenic factors such as HOTTIP (higher HOTTIP in AC than CC) to transform to carcinoma.

The current used colorectal cancer screening method is colonoscopy; however, it is technically an invasive method (Young and Womeldorph 2013). This study suggested new applicable genetic biomarkers depending on blood samples and PCR techniques, which are both non-invasive and economical. The strengths of this study include that it is the first study to implicate rs1859168 as a potential genetic marker of colorectal cancer susceptibility among controls and adenomatous polyposis patients. Also, it is the first research to introduce serum HOTTIP as a new possible early diagnostic and prognostic tumor marker for colorectal cancer and as a new probable immunotherapeutic approach for colorectal cancer treatment.

Limitations facing this study include a quite small sample size, as it is a case-control study based on hospital collected patients; so, selection bias is unavoidable. The communication of the serum HOTTIP and its SNP with other risk factors of colorectal cancer, other functional SNPs in HOTTIP should be assessed in larger studies to confirm our results.

Conclusions

In conclusion, both rs1859168 and high serum HOTTIP confer increased risk for colorectal cancer development; Also, a significant difference in serum HOTTIP expression level was found to be associated with different rs1859168 genotypes.

Authors' Contributions

O.G.S.: gathered blood samples and data from patients' reports, executed the practical tests. M.A.A.: supposed the protocol of the research, contributed to the clinical experi-

ments, interpreted the information, wrote the article, and corrected the last copy of the article. E.M.E., S.N.G., E.A.H., M.Y.A., M.N.A., M.A.F.K., and S.A.: shared in the collection of blood samples and data from patients, shared in the writing of the article.

Acknowledgment

All authors acknowledge help from all paramedical personnel to accomplish and finalize the clinical research work.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

No funding was received for this article.

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Received 2 July 2019/Accepted 11 March 2020