ORIGINAL ARTICLE

https://doi.org/10.1590/1806-9282.20220777

Evaluation of the Rho-kinase gene expression and polymorphisms in adult patients with acute appendicitis: a differential impact of gender

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SUMMARY

OBJECTIVE: Acute appendicitis represents one of the most common causes of acute intra-abdominal emergencies worldwide. In this case-control study, we aimed to investigate associations of Rho-kinase gene expression and polymorphisms with acute appendicitis in a Turkish population. We also aimed to study the effects of gender on these parameters.

METHODS: A total of 93 unrelated patients with acute appendicitis and 93 healthy controls in the Department of Emergency Medicine, Erciyes University, between June 2019 and June 2021 were included in this study. Genomic DNA was isolated from peripheral leukocytes, and the LightCycler 480 II real-time polymerase chain reaction was utilized to detect Rho-kinase1 gene rs35996865 and Rho-kinase2 gene rs2230774 (Thr431Asn) polymorphisms. Quantitative real-time polymerase chain reaction was applied to determine Rho-kinase1 and Rho-kinase2 gene expressions.

RESULTS: There was a marked increase in Rho-kinase1, but not in Rho-kinase2, mRNA expression, and this increase was evident only in male patients (p=0.0008). No significant differences were found in allele and genotype frequencies for Rho-kinase1 gene rs35996865 and Rho-kinase2 gene rs2230774 polymorphisms between the patients with acute appendicitis and the control group.

CONCLUSIONS: Our data imply that Rho-kinase1 (rs35996865) and Rho-kinase2 (rs2230774) gene variants are not risk factors for the development of acute appendicitis in the Turkish population. However, increased mRNA expression of the Rho-kinase1 gene in males indicated that Rho-kinase1 is involved in the pathogenesis of acute appendicitis in a gender-specific way.

KEYWORDS: Appendicitis. Pharmacogenomic variants. Gene expression. Inflammation. Rho kinase.

INTRODUCTION

Acute appendicitis (AA) resulting from inflammation of the appendix is a leading cause of abdominal surgical emergency. Despite its classic signs and symptoms being well known, it is still difficult to diagnose. Any delay in diagnosis or untreated appendicitis is linked with perforation and increased complications, including abscess, ileus, and peritonitis[1]. Therefore, timely diagnosis is necessary to reduce morbidity and mortality. However, since symptoms of AA overlap with many gynecologic, abdominal, and urologic conditions, reaching a definitive diagnosis is a clinical challenge. In fact, after clinical diagnosis, a negative appendectomy rate of 5.8% and missed perforated appendicitis rate of 3.4% have been described in the previous studies[2]. An estimated 17.7 million cases (incidence 228/100,000) with over 33,400 deaths (0.43/100,000) have been reported in 2019[3].

Rho-kinase (ROCK) is a serine/threonine protein kinase with a molecular mass of ~160 kDa, which has been identified as the first downstream effector of the Rho family of small GTPases. Ubiquitously expressed and highly homologous ROCK1 and ROCK2 isoforms have been identified[4]. The Rho/ROCK signaling pathway regulates cellular migration, adherence, and proliferation through control of the cell contraction and actin-cytoskeletal assembly[4]. Experimental

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Conflicts of interest: the authors declare there is no conflicts of interest. Funding: This study was supported by a project (TSA-2020-9776) from the Scientific Research Projects Department of Erciyes University, Kayseri, Turkey.
Received on July 19, 2022. Accepted on July 19, 2022.

data suggest that ROCK activity regulates sepsis-induced systemic inflammation and organ injury[5]. There are no studies investigating the role of *ROCK* gene expression or polymorphisms in AA. We hypothesized that *ROCK* gene expressions and single-nucleotide polymorphisms (SNPs) contribute to the risk of AA development. Thus, the goal of this study was to assess an association between *ROCK* gene expressions/SNPs and AA in a Turkish population. Another objective of this study was to identify the effects of gender on *ROCK* gene expression or polymorphisms.

METHODS

Study design and patients

This prospective case-control study investigated 93 patients admitted to the Erciyes University Department of Emergency Medicine with suspected cases of AA. Of all admitted patients, only those with the intraoperatory diagnosis of AA, aged 18 years and older, were included. Clinical and surgical diagnoses were confirmed postoperatively by histopathological examination. Approval of this research was granted by the Erciyes University Clinical Research Ethics Committee (decision no.: 2019/374) and was performed in accordance with the principles of the Declaration of Helsinki. All participants submitted written informed consent to blood sampling, genotyping, and inclusion in the study. All genetic studies were carried out in the Erciyes University Genome and Stem Cell Center (GENKOK).

The control group was composed of 93 healthy, gender-matched, and age-matched volunteers who had no recent surgery, history of medical illness, or diagnosis of genetic, neurologic, psychiatric, liver, infectious, or chronic inflammatory disease. The volunteers for the healthy control group were formed from resident doctors, employees of the hospital, and their families. Patients who had known or apparent systemic diseases such as heart failure, ischemic heart disease, malignancies, hypertension, autoimmune diseases, chronic pulmonary, renal, or liver diseases, pregnancy or breastfeeding, drug addiction, and receiving immunosuppressive therapy were excluded.

AA patients were classified before surgery using the Alvarado score, which is determined by the signs, symptoms, and diagnostic tests of suspected patients and is composed of a 10-point clinical scoring system for the AA diagnosis. Routine radiological imaging consisted of ultrasonography, computed tomography, or both. Appendectomies were performed via videolaparoscopy or laparotomy. Surgically removed all the appendix specimens were submitted to histopathological analysis according to routine protocols.

Blood samples and DNA isolation

Venous blood samples (6 mL) were drawn into EDTA-containing tubes from all individuals preoperatively in the emergency department and divided into two parts. One part of the samples was used for the measurements of complete blood count (CBC) and other biochemical parameters. Another part of the blood samples was quickly transferred to the GENKOK. Genomic DNA was extracted from peripheral leukocytes using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen, Germany) in accordance with the instructions of the manufacturer. The final DNA concentration was measured using a micro-volume UV-Vis spectrophotometer (BioSpec-nano, Shimadzu, Kyoto, Japan). An absorbance ratio of 1.8 at 260–280 nm was taken as an indicator of DNA purity. DNA samples were then stored at –20°C for further studies. Clinical parameters including pulse rate, mean arterial pressure, respiratory rate, and imaging data were recorded and analyzed as a routine evaluation at the emergency department.

Single-nucleotide polymorphisms selection and genotyping

The preliminary screening criteria for *ROCK1* and *ROCK2* gene SNPs were as follows:

1. minor allele frequency (MAF) and
2. on the basis of previously published studies.

This led to selection of one SNP in *ROCK1* (rs35996865 T>G MAF=0.26) and one SNP in *ROCK2* (rs2230774 G>T MAF=0.40) for inclusion in this study. A total of 15 µL mix was prepared using LightCycler Fast Start DNA Master Hyprobe, $MgCl_2$ stock solution, SNP Primer/Probe, and PCR-Grade water. To identify *ROCK1* gene rs35996865 and *ROCK2* gene rs2230774 (Thr431Asn) polymorphisms, genotyping was done using commercially synthesized primers and fluorescently labeled probes and the LightCycler 480 II real-time polymerase chain reaction (RT-PCR) system (Roche Diagnostics GmbH, Mannheim, Germany). Gene variants were detected by analyzing the detailed melting curve of the PCR product obtained.

RNA isolation and gene expression analysis

PureZol was applied to extract total RNA from patient whole blood samples (Bio-Rad, CA, USA) according to the manufacturer's recommendations. The quantity (absorbance at 260 nm) and quality (ratio of absorbance at 260 and 280 nm) of the RNA were evaluated using a NanoDrop spectrophotometer. RNA was stored at -80°C until use. An iScript cDNA Synthesis kit (CA, USA) was used to reverse transcribe 1g of RNA as stated by the manufacturer's instructions. In a 20-µl reaction volume, quantitative RT-PCR (qRT-PCR) test reactions were

performed. Initial denaturation at 95°C for 10 min was followed by 45 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 60 s. The LightCycler 480 II instrument was used to perform qRT-PCR on duplicate reactions for *ROCK1* and *ROCK2* gene expressions (Roche, Germany). β-Actin (*ACTB*), as a housekeeping gene, was used. The $2^{-\Delta\Delta Ct}$ method of relative quantification was used to evaluate changes in gene expression.

Statistical analysis

The results are presented as mean (SD) for parameters with parametric distribution and median (IQR) for nonparametric data. The normal distribution of numerical variables was analyzed using the Kolmogorov-Smirnov normality test. For the normally distributed data, an unpaired Student's t-test was applied. Mann-Whitney U test was used for data with nonparametric distribution, or for comparing gene expression data. Categorical data were analyzed using the chi-square test. Hardy-Weinberg distribution was tested using the chi-square test by comparing the observed and expected genotype frequencies. Differences in allele and genotype frequencies among the controls and cases were compared using chi-square or Fisher's exact test. Analysis of data was carried out using GraphPad Instat version 3.05 (GraphPad Software Inc., San Diego, CA, USA). All tests were two-sided, and significance was considered at $p<0.05$.

RESULTS

After matching the exclusion and inclusion criteria, all cases of surgically and clinically diagnosed AA were taken for this study. Histopathological analysis was used to confirm the preoperative diagnosis.

A total of 93 patients with AA and 93 healthy volunteers were enrolled in this study. The demographic, laboratory, and clinical characteristics of the study population are given in Table 1. Compared with the controls, the average age, gender, systolic and diastolic blood pressure, respiratory and pulse rates, platelet and lymphocyte counts, glucose, blood urea nitrogen, creatinine, aspartate aminotransferase, and alanine aminotransferase in AA group were similar. Neutrophil and white blood cell (WBC) counts, total bilirubin, lactate dehydrogenase, and C-reactive protein (CRP) levels were found to be increased in the AA group when compared with the controls (Table 1). Our data showed a slight male predominance, and the male/female ratio was 2.6:1.

All patients underwent a videolaparoscopic or laparotomic appendectomy, and no incidences of complications were reported during their hospitalization with an average stay of 1.5 days. A total of 54 patients were evaluated with ultrasonography, 28 patients with computed tomography, and 11 patients with both methods. Using the Alvarado system, 2 (2.2%) patients had a score of 5 or 6, 84 (90.3%) had a score of 7 or 8, and 7 (7.5%) had a score of 9 or 10.

Both the control (*ROCK1,* p=0.9998; *ROCK2,* p=0.4621) and patients (*ROCK1,* p=0.3013; *ROCK2,* p=0.9271) groups were found to be in Hardy-Weinberg equilibrium. For the *ROCK1* gene rs35996865 polymorphism, no marked differences in both genotype (T/T, 60.2; T/G, 37.6; G/G, 2.2%) and allele (T, 79.0; G, 21.0%) frequencies in the AA group were detected when compared with controls (T/T, 59.1; T/G, 35.5; G/G, 5.4; T, 76.9; G, 23.1%, p>0.05) (Table 2). For the *ROCK2* gene rs2230774 (Thr431Asn) polymorphism, genotype (AA:

Table 1. Demographic, clinical, and laboratory characteristics of the study cases.

	Patients with AA (n=93)	Controls (n=93)	p
Age (years)	33.7 (9.4)	34.0 (8.9)	0.8228
Gender, n (%)			
Male	67 (72.0)	67 (72.0)	
Female	26 (28.0)	26 (28.0)	1.0000
Systolic BP (mmHg)	120.6 (13.3)	119.1 (10.8)	0.3775
Diastolic BP (mmHg)	74.2 (8.1)	75.4 (10.0)	0.4010
Pulse rate (beats/min)	87.3 (9.1)	90.1 (11.0)	0.0627
Respiratory rate (beats/min)	18.2 (2.4)	18.4 (7.3)	0.8218
Platelet (10^3/μL)	269.3 (87.8)	273.6 (45.1)	0.6741
Neutrophils (10^3/μL)	11.0 (4.2)	7.0 (0.9)	<0.0001
Lymphocytes (10^3/μL)	2.1 (1.3)	2.0 (0.9)	0.2662
WBC (10^3/μL)	14.1 (4.3)	9.9 (2.0)	<0.0001
Glucose (mg/dL)	109.1 (26.3)	106.9 (18.7)	0.5138
BUN (mg/dL)	14.2 (5.6)	13.9 (9.6)	0.8088
Creatinine (mg/dL)	0.9 (0.2)	0.9 (0.6)	0.6384
Na^+ (mmol/L)	139.8 (2.8)	–	
K^+ (mmol/L)	4.4 (0.4)	–	
AST (U/L)	23.2 (13.2)	20.8 (11.5)	0.1827
ALT (U/L)	19.2 (10.2)	21.5 (12.7)	0.1863
Total bilirubin (mg/dL)	0.7 (0.5)	0.5 (0.2)	0.0013
LDH (U/L)	277.5 (84.0)	254.9 (62.3)	0.0386
C-reactive protein (mg/L)	43.6 (9.2)	0.7 (0.3)	<0.0001

AA: acute appendicitis; BP: blood pressure; WBC: white blood cells; BUN: blood urea nitrogen; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; MAP: mean arterial pressure. All results were parametric and are presented as mean (SD), except gender which is shown as n (%).

Table 2. Genotype and allele frequencies of Rho-kinase1 gene rs35996865 and Rho-kinase2 gene rs2230774 (Thr431Asn) polymorphisms among cases and controls.

Genotypes/ alleles	Patients with AA (n=93) n (%)	Controls (n=93) n (%)	p
ROCK1 rs35996865			
T/T	56 (60.2)	55 (59.1)	
T/G	35 (37.6)	33 (35.5)	0.8946
G/G	2 (2.2)	5 (5.4)	0.4392
T	147 (79.0)	143 (76.9)	
G	39 (21.0)	43 (23.1)	0.7075
ROCK2 rs2230774 (Thr431Asn)			
G/G	30 (32.3)	25 (26.9)	
G/T	44 (47.3)	52 (55.9)	0.3890
T/T	19 (20.4)	16 (17.2)	0.9808
G	104 (55.9)	102 (54.8)	
T	82 (44.1)	84 (45.2)	0.9169

AA: acute appendicitis; ROCK: Rho-kinase.

G/G, 32.3; G/T, 47.3; T/T, 20.4%, and controls: G/G, 26.9; G/T, 55.9; T/T, 17.2%) and allele (AA: G, 55.9; T, 44.1%, and controls: G, 54.8; T, 45.2%) frequencies were not significantly associated with AA (Table 2).

We found significant differences in leukocytes' *ROCK1* and *ROCK2* gene mRNA expressions in healthy controls and in patients with AA (Table 3). *ROCK1*, but not *ROCK2*, gene expression was markedly elevated in the AA group (p=0.0027). This marked increase in *ROCK1* gene expression was observed in males (p=0.0008), but not in females (p=0.5252).

DISCUSSION

In our study, we showed insignificant associations between AA and *ROCK1* gene rs35996865 and *ROCK2* gene rs2230774 (Thr431Asn) polymorphisms in the Turkish population. However, we demonstrated a marked increase in *ROCK1* gene mRNA expression in male AA patients. To the best of our knowledge, this is the first work to assess the link of the

Table 3. Comparison of mRNA content in leukocytes for the control and patient with acute appendicitis.

		Control Median (min–max)	Patients with AA Median (min–max)	Fold	p*
ROCK1					
Total (n=93)	$\Delta C_T = C_t(\text{target}) - C_t(\text{housekeeping})$	1.22 (0.02–7.01)	2.11 (0.01–42.08)	$\Delta\Delta C_T$: 1.22–2.11= -0.89	0.0027
	Content=$2^{-\Delta CT}$	0.43 (0.01–0.98)	0.23 (0.00–0.99)	Fold: 0.43/0.23=1.87	
Male (n=67)	$\Delta C_T = C_t(\text{target}) - C_t(\text{housekeeping})$	1.19 (0.02–7.01)	2.01 (0.01–42.08)	$\Delta\Delta C_T$: 1.19–2.01=-0.82	0.0008
	Content=$2^{-\Delta CT}$	0.44 (0.01–0.98)	0.25 (0.00–0.99)	Fold: 0.44/0.25=1.76	
Female (n=26)	$\Delta C_T = C_t(\text{target}) - C_t(\text{housekeeping})$	1.79 (0.02–4.66)	2.18 (0.01–6.75)	$\Delta\Delta C_T$: 1.79–2.18= -0.39	0.5252
	Content=$2^{-\Delta CT}$	0.29 (0.04–0.98)	0.22 (0.01–0.99)	Fold: 0.29/0.22=1.32	
ROCK2					
Total (n=93)	$\Delta C_T = C_t(\text{target}) - C_t(\text{housekeeping})$	1.40 (0.23–21.93)	1.20 (0.15–40.09)	$\Delta\Delta C_T$:1.40–1.20=0.20	0.0570
	Content=$2^{-\Delta CT}$	0.38 (0.00–0.85)	0.43 (0.00–0.90)	Fold: 0.38/0.43=0.88	
Male (n=67)	$\Delta C_T = C_t(\text{target}) - C_t(\text{housekeeping})$	1.49 (0.24–21.93)	1.12 (0.15–40.09)	$\Delta\Delta C_T$:1.49–1.12=0.37	0.0719
	Content=$2^{-\Delta CT}$	0.36 (0.00–0.85)	0.46 (0.00–0.90)	Fold: 0.36/0.46=0.78	
Female (n=26)	$\Delta C_T = C_t(\text{target}) - C_t(\text{housekeeping})$	1.20 (0.23–4.61)	1.29 (0.15–3.85)	$\Delta\Delta C_T$:1.20–1.29= -0.09	0.4671
	Content=$2^{-\Delta CT}$	0.43 (0.04–0.85)	0.41 (0.07–0.90)	Fold: 0.43/0.41=1.05	

ROCK: Rho-kinase. The results are presented as median (IQR). *Mann-Whitney U test.

ROCK gene polymorphisms with AA susceptibility. This is also the first research reporting that there was a gender-dependent effect on AA in terms of gene expression. The findings of this study indicate that rs35996865 and rs2230774 polymorphisms are unlikely to play a role in AA development.

The rs35996865 polymorphism is located in the *ROCK1* promoter region, about 2 kb upstream of the transcription start site. However, it is not known whether this polymorphism is able to alter the expression level of the *ROCK1* gene[6]. The *ROCK1* gene rs35996865 variant mapping to the 5′-UTR has been reported to be markedly associated with obesity-related metabolic syndrome[7], respiratory distress syndrome[8], and nonsyndromic cleft palate[6], but not with sepsis[9] or primary open-angle glaucoma[10]. The result of our study showed that there was no association between the rs35996865 variant and AA.

The rs2230774 polymorphism is located in the exon 10 of the *ROCK2* gene and causes amino acid change (Thr431Asn). This polymorphism is markedly associated with breast cancer metastases[11] and obesity-related metabolic syndrome[7]. In contrast, there are several reports showing that this polymorphism is not associated with respiratory distress syndrome of the newborn[8], mantle cell lymphoma[12], and primary open-angle glaucoma[10]. The result of the present study demonstrated that there was no association between rs2230774 polymorphism and AA.

Reactive oxygen species (ROS) and imbalance in the oxidant/prooxidant defense system may play an important role in the pathology and progression of AA[13]. ROS have been indicated to have a relationship with the RhoA/ROCK pathway[14]. These reports may support our findings, showing that upregulation of *ROCK1* gene expression in male AA patients could be related to increased oxidative stress. Estrogens are capable of diminishing oxidative stress and increasing antioxidative cell potency[15]. This may explain the result of the present study, showing that there was no upregulation of *ROCK* gene expression in female AA patients.

We observed increased neutrophil and WBC counts and CRP levels in AA patients. CRP can activate RhoA/ROCK to elevate endothelial plasminogen activator inhibitor-1 expression, which may lead to atherothrombogenesis[16]. Serum levels of interleukins (IL-1, IL-6, and IL-8), INF-γ, and TNF-α were markedly elevated in patients with appendicitis[17]. TNF activates different Rho GTPases, enhances filamentous actin, remodels endothelial cell morphology, and induces actin stress fibers through RhoA and ROCK. A rise in Rho-ROCK activity and F-actin promotes morphological changes in the endothelium on TNF exposure[18]. Thus, increased CRP or TNF levels may contribute to the elevated *ROCK1* gene expression in the present study.

The main limitation of this study is related to the small sample size in polymorphism studies. This could be the source of potential bias or imprecision. Therefore, further large population studies are needed to demonstrate the contribution of ROCK gene polymorphisms.

CONCLUSIONS

This study identified that AA has a genetic background and is influenced by the *ROCK* gene. We suggest that AA can be influenced by gene expressions in a gender-specific manner. These findings can improve understanding of the genetic factors influencing AA, which may also result in more accurate diagnosis, more targeted therapy, and eventually personalized treatment of AA.

ACKNOWLEDGMENTS

This study was supported by a project (TSA-2020-9776) from the Scientific Research Projects Department of Erciyes University, Kayseri, Turkey.

AUTHORS' CONTRIBUTIONS

NEG: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – review & editing**. EB:** Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – review & editing. **SD:** Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – review & editing. **EFŞ:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **RT:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **NG:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **ATD:** Formal analysis, Writing – original draft, Writing – review & editing.

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