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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
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INVESTIGATION OF EGF AND EGFR GENE EXPRESSION LEVELS IN IRRITABLE BOWEL SYNDROME PATIENTS

Bahra Radhaa HAMARASHID

Master's Thesis

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This thesis, which was prepared according to the thesis writing rules of the Graduate School of Natural and Applied Sciences, Fırat University, was evaluated by the committee members who have signed the following signatures and was unanimously approved after the defense exam made open to the academic audience.

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DECLARATION

I hereby declare that I wrote this Master's Thesis titled " Investigation of EGF and EGFR Gene Expression Levels in Irritable Bowel Syndrome Patients " in consistent with the thesis writing guide of the Graduate School of Natural and Applied Sciences, Firat University. I also declare that all information in it is correct, that I acted according to scientific ethics in producing and presenting the findings, cited all the references I used, express all institutions or organizations or persons who supported the thesis financially. I have never used the data and information I provide here in order to get a degree in any way.

26 July 2021

Bahra Radhaa HAMARASHID

PREFACE

Irritable Bowel Syndrome (IBS) Is a gastrointestinal (GI) disorder characterized by altered bowel habits in association with abdominal discomfort or pain in the absence of detectable structural and biochemical abnormalities.

IBS is an obvious health problem Worldwide. IBS affects 7% to 21% of the general population, and especially in Mediterranean countries, like Turkey, and in people from Asia, Africa, and the Middle East. Because IBS pathogenes relate to many causes such as genetic, diet, environmental, and psychological factors.

This study is designed to determine the specific EGF gene expression, which has a great role in the regulation of bowel activity by contact with EGFR. This defect causes patients to have a disruption in bowel function. Therefore, need to establish an effective diagnosis and preventative technic for this disease. As part of this effort, we initiated this study to determine the new molecular line of study in this field to estimate the roles of genetic factors in a patient with IBS to help the medical staff for proper diagnosis and management with signs and symptoms of IBS.

First and foremost, praises and thanks to God, the Almighty, for His showers of blessings throughout my research work to complete the research successfully.

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ABSTRACT

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Irritable bowel syndrome (IBS) is one of the most frequent gastrointestinal disorders. It is characterized by altered bowel habits associated with abdominal pain or discomfort without the presence of structural and biochemical abnormalities. Because IBS is a multifaceted disorder that afflicts millions of individuals worldwide. This study was designed to determine the EGF and EGFR gene expression level in IBS, which have a great role in the regulation of bowel activity by contact with each other. This defect causes patients to have a disruption in bowel function. Therefore, need to establish an effective diagnosis and preventative technic for this disease. As part of this effort, we initiated this study to determine the new molecular line of study in this field to estimate the roles of genetic factors in patients with IBS to help the medical staff for proper diagnosis and management with signs and symptoms of IBS.

Ethics committee approval required for the study was obtained from the Firat University Medical Faculty Local Ethics Committee. The levels of expression of EGF and EGFR genes were investigated in IBS patients. In order to examine that, quantitative real-time PCR (qRT-PCR) was performed to analyze gene expression levels of two target genes, including; EGF, and EGFR, and one reference gene GAPDH. Gene expression level data from qRT-PCR confirmed that both genes (EGF and EGFR) were downregulated in 40 IBS blood, approximately 5-fold to 7-fold changes, respectively, as compared to the 40 healthy control group. Finally, the significant downregulation of EGF and EGFR genes in IBS patients can be considered as important biomarkers for IBS disease.

Keywords: Irritable Bowel Syndrome, EGF, EGFR, Gene expression, QRT-PCR

ÖZET

Huzursuz Bağirsak Sendromu Hastalarında EGF ve EGFR Gen İfade Düzeylerinin Araştırılması

Bahra Radhaa HAMARASHID

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Irritabl bağırsak sendromu (IBS), en sık görülen gastrointestinal bozukluklardan biridir. Yapısal ve biyokimyasal anormallikler olmadan karın ağrısı veya rahatsızlık ile ilişkili değişen bağırsak alışkanlıkları ile karakterizedir. Çünkü IBS, dünya çapında milyonlarca kişiyi etkileyen çok yönlü bir hastalıktır. Bu çalışma, bağırsak aktivitesinin birbirleriyle temas yoluyla düzenlenmesinde büyük rolü olan IBS'de EGF ve EGFR gen ekspresyon düzeyini belirlemek için tasarlanmıştır. Bu kusur, hastaların bağırsak fonksiyonlarında bozulmaya neden olur. Bu nedenle, bu hastalık için etkili bir teşhis ve önleyici teknik kurmaya ihtiyaç vardır.

Bu çabanın bir parçası olarak, IBS'li hastalarda genetik faktörlerin rollerini tahmin etmek için bu alandaki yeni moleküler çalışma hattını belirlemek için bu çalışmayı, IBS'nin belirti ve semptomları ile tıbbi personele doğru teşhis ve yönetim için yardımcı olmak üzere başlattık. Çalışma için gerekli etik kurul onayı Fırat Üniversitesi Tıp Fakültesi Yerel Etik Kurul'dan alındı. IBS hastalarında araştırılan EGF ve EGFR genlerinin ekspresyon seviyeleri. Bunu incelemek için, iki hedef genin gen ekspresyon seviyelerini analiz etmek için qkantitatif real-time PCR (qRT-PCR) yapıldı; EGF ve EGFR ve bir referans gen GAPDH. qRT-PCR'den elde edilen gen ekspresyon seviyesi verileri, 40 sağlıklı kontrol grubuna kıyasla, her iki genin (EGF ve EGFR) 40 IBS kanında aşağı regüle edildiğini, sırasıyla yaklaşık 5 kat ila 7 kat değişiklik olduğunu doğruladı. Son olarak, IBS hastalarında EGF ve EGFR genlerinin önemli ölçüde aşağı regülasyonu, IBS hastalığı için önemli bir biyobelirteç olarak kabul edilebilir.

Anahtar Kelimeler: Huzursuz bağırsak sendromu, EGF, EGFR, gen ifadesi, QRT-PCR

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SYMBOLS AND ABBREVIATIONS

Symbols

2-ΔΔCT : 2ˆ (–delta delta cycle threshold)
% : Percent
ºC : Centigrade
ml : Milliliter
µl : Microliter
µg : Microgram
g : Relative centrifugal force

Abbreviations

DNA : Deoxyribonucleic Acid
RNA : Ribonucleic Acid
IBS : Irritable Bowel Syndrome
EGF : Epidermal Growth Factor
EGFR : Epidermal Growth Factor Receptor
C-IBS : Constipation Irritable bowel syndrome
D-IBS : Diarrhea Irritable Bowel syndrome
A-IBS : Alternate Irritable Bowel Syndrome
U-IBS : Unsubtyped Irritable Bowel Syndrome
cDNA : Complementary Deoxyribonucleic Acid
PCR : Polymer Chain Reaction
RT-PCR : Real Time-Polymer Chain Reaction
GWAS : Genome-Wide Association Analysis
ENS : Enteric Nervous System
CNS : Central Nervous System
GBA : Gut-Brain Axis
GI : Gastrointestine
QoL : Quality of life
bp : Base pair
5-HT : 5-hydroxytryptamine Receptor
SLC6A4 : Solute Carrier Family 6 Member 4
SERT : Serotonin Transporter
KDa : Kilodalton
TGF α : Transforming growth factor α
BTC : Betacellulin
AR : Amphiregulin Tumor Necrosis Factor Superfamily-15
EPR : Epiregulin
KGF : Keratinocyte Growth Factor
HB-GFR : Heparin-Binding Epidermal Growth Factor
miRNA : Micro RNA
TNFSF15 : Tumor Necrosis Factor Superfamily-15
NCGS : Non-Celiac Gluten Sensitivity
GFD : Gluten-Free Diet
FODMAP : Fermentable Oligosaccharides, Di-saccharides, Momo-saccharides, And Polyps
RTK : Protein Tyrosine- Kinase
ERBB1 : Epidermal Growth Factor Receptor
HER1 : Human Epidermal Growth Factor Receptor 1
TATA : Thymine Adenine Thymine Adenine

SH1	: Src Homology 1 domain (kinase domain)
PLC3	: Phospholipase3
Grb2	: Growth Factor bound two
EDTA	: Ethylenediaminetetraacetic acid

1. INTRODUCTION

Irritable bowel syndrome (IBS) is defined as irregular dysfunction in gastrointestinal organs, which have complex signs in patients such as changes in intestine habits and abdominal pain with discomfort [1]. Besides of main symptoms, it makes minor symptoms such as abdominal distension, straining, and urgency. IBS has a major health burden that has an impact on quality of life significantly [2]. The prevalence estimation in the world is between 10% and 15% [3]. According to the common habit of the bowel, it was dividing into many subtypes; constipation-irritable bowel syndrome (C-IBS), diarrhea-irritable bowel syndrome (D-IBS), alternate-irritable bowel syndrome (A-IBS) with unsub typed constipation, and diarrhea-irritable bowel syndrome (U-IBS) [4]. IBS pathophysiology is heterogeneous include abnormal gastrointestinal motility [5], visceral hypersensitivity [6], brain-intestinal connections [7], environmental factors [8], changes of microflora in fecal [9], food sensitivity, carbohydrate malabsorption, and intestinal inflammation [10].

The molecular mechanism of IBS pathogenesis remains incompletely understood [11], some studies demonstrate genetic factors as a strong point of pathophysiology in it [12, 13]. As in twin research that is done in various area in the global world; have clarified the higher relationship among monozygotic twin rather than dizygotic twins, it means IBS have multilinked with the genetic factor [14]. The epidermal growth factor (EGF) is one of the natural ligands of the trans membranous tyrosine epidermal growth factor receptor (EGFR), essential for normal proliferation and differentiation of cells [15]. The binding of EGF on the surface of the enterocytes causes dimerization of EGFR, leading to the activation of EGFR tyrosine kinase and RTK autophosphorylation and the eventual activity of different intracellular signaling routes which lead to cellular proliferation and differentiation that contribute towards intestinal development and reparation of the intestinal mucosa.

It is essential to discovering biomarker genes in the IBS to fight the danger and combativeness with IBS symptoms in the gastrointestinal. This study interpreted the gene expression level of two specific genes; EGF and EGFR in the IBS. It is helpful to determine biomarker genes, which demonstrate a new line in the molecular study of IBS. As the result, it provides a significant improvement for diagnosis.

2. FEATURES OF IRRITABLE BOWEL SYNDROME

IBS is a persistent condition of the gastrointestinal tract that causes abdominal pain and bowel dysfunction [1]. IBS is fairly prevalent that up to 11% of the general population documented IBS-compatible symptoms in a meta-analysis of society-based cross-sectional surveys, but the prevalence in individual studies ranged from 1.1% to 45% [16]. Epidemiologic studies have shown that the prevalence of IBS is increasing in Asian communities, perhaps due to rapid socio-economic development and changes in dietary patterns. The characteristics of IBS in Asia may differ from those of Western societies based on diets, socio-cultural backgrounds, and genetic factors [17].

Clinical symptoms: abdominal pain which is poorly localized, but is frequently reported in the mid to low abdomen. The character of the pain is variable but is most commonly described as being cramp-like or aching. Pain typically occurs in bouts and classically is relieved by the passage of flatus and gases. Diarrhea and constipation are the main clear symptoms in IBS situations [18]. Diarrhea is commonly defined as loose stool before lower abdominal influences. Upon defecation, people with diarrhea may get an acute and inadequate relief or mucus in their stools. However, IBS was not characterized by huge quantities, blood streams, and overnight diarrhea. When there is constipation, it can continue for days or longer and can overlap with regular bowels or diarrhea [19]. Constipation is distinct as hard, pellet-shaped stools that make an incomplete sense of relief after defecation in IBS patients. The Bristol Stool Scale can be used to classify the intensity of the stools to differentiate diarrhea with constipation to assess therapies. The collection is divided into seven different types: separate hard lumps, snuffy sausage, smooth, clear-cut blobs with fuzzy borders, mushy pieces with furrows, a sticky stool, and watery pieces with no firm. Several additional pathways associated with the constipation pathology can often include certain disorders, such as poor movement constipation, pseudo-obstructive bowel movement, and unclear colonic large. The IBS symptoms occurrence is higher in individuals with chronic inflammatory diseases such as (inflammatory bowel, coeliac, small bowel syndrome), or acute gastroenteritis (post-infection IBS) incidents [20].

It is confirmed that the symptoms of gastrointestinal happen in combination with anxiety, and scare. In the emotional situation and stress, gut motility will become affected and have been a change. There are many types of research done on the effects and relation of psychiatric disorders with IBS [21]. All IBS subtypes correlate significantly with depression and stress. The highest score effects were in the IBS-M group, but until now the reason is not clear why this group has the highest ratio. Many ideas returning the factor to simultaneous that present in both constipation and diarrhea digestive problems [22].

In IBS, interventions have been mainly spread in two directions with a view to the manipulation of genetic and epigenetic mechanisms that include pathogenesis. Firstly, attempts to classify genes related to the development of IBS and the definition of it. Medical characteristics and predominance of sex-specific correlations, either through gene candidate methods or, more recently through the genome-wide association analysis (GWAS). GWAS studies are based primarily on DNA use in chip-microarray technology that uses hybridization, to analyze more than one million genetic markers [23].

2.1. Pathophysiology of IBS

Understanding the pathophysiology of IBS till now it does not clear. There is no consensus on the etiology underlying the disorders, and a combination of factors that include multiple mechanisms can describe the disorder [24]. Over the years, various criteria have been established in IBS, this makes therapists and researchers dependent on them (e.g., Manning, Kruis, and Rome). Several factors have evolved in the last 40 years, which have led to IBS pathophysiology. IBS pathogenesis-related with several factors like altered gastrointestinal motility, posts of infectious reactivity, visceral hypersensitivity, flora abnormality, over bacterial growth, environmental factors, food-material malabsorption, and intestinal inflammations. Historically IBS pathogenesis has concentrated on symptoms. Although in the majority of the IBS cases, one or more of these abnormalities is apparent, none of them can account for the symptoms. In recent times, several IBS cases are described as altered intestinal immune function, intestinal permeability, and intestinal and colonic microbiota [25].

Sensation and function of the gastrointestinal are stimulated by the state and action of multiple domains, including a gut lumen, mucosa, enteric nervous system (ENS), and central nervous system (CNS), as well as contact between these domains. A unifying factor in IBS pathogenesis is that complex interaction between these regions, which form the "gut-brain axis," collectively, is dysregulated. There is a signal connection between the stomach and brain, which makes communication between them, this signaling way, was named Gut-Brain Axis (GBA). The GBA signal incorporates actions and relationships between the microbiota from the stomach with the autonomous nervous system, which has a significant role in the externalization of IBS symptoms [26]. The interconnected behavior and coordination between the microbiota and the autonomous GBA are key players in the continuation of IBS symptoms. Therefore, any changes in the brain or gastrointestinal system physiology can make changes in the composition of the microbiota environment [27].

In twin and family study a heritable component of IBS has been shown, its valuate heritability has a difference between 0 and 57 %. in recent times, another study (Swedish nationwide) that done investigated nearby 50,000 cases illustrates that genetic inheritance risk of

IBS increases in the family especially among the first, second, and third-degree relatives. A huge genetic variation and hereditary background are present for settle majority in-patient of IBS [28]. Nowadays most focused factor, which continuously studying in IBS is the evaluation of specific "candidate" genes. The nominee genes are genes designated a priori by scientists, based on the biological legitimacy that the protein product of the gene will play its part in disease pathophysiology [29].

2.2. Genetics of IBS

In recent years, major strides have been made in the field of genetics. Our knowledge of the DNA sequence, design, and function has significantly improved as genotyping's performance, ease and costs have fallen [30]. The IBS was shown to be linked with polymorphisms or mutations of a variety of chromosomes. Genes that encode proteins that are involved in the homeostasis of the process epithelium barrier, such as cadherin 1 (CDH1), cycle 42 cell divisions (CDC42, IL6, IL10, TNF, and TNF superfamily 15 (TNF SF15 encoding cytokines and neuronal signal transduction) and others [neurexophiline 1 (NXPH1) and a sodium-voltage-gated α-subunit 5 (SCN5A)] channels have been reproduced in many investigations [31]. In the examination of function in the heritability in IBS initial researchers found that one-third of patients with IBS had a relative, even in the non-competitive psychiatric diagnosis cases [32]. Two reports employed GWAS to assess the hereditary link of IBS pathophysiology. Firstly, in a group of 172 IBS patients and 1,398 healthy persons, a limited pilot GWAS was performed and the protocadherin-15 (PCDH15) SNP interaction on chromosome 10 was established with IBS, Nonetheless, in additional samples, this result could not be verified. Second, Ek et al. studied 11,326 Swedish twins, including 534 IBS patients and 4932 control, and confirmed their results in samples in different European, U.S., and Australian centers, utilizing a common methodology. Epigenetic alteration explanation in IBS and associated diseases is still in its early stages, but there has been a decrease in mRNA rates in the candidate genes in many animal experiments consistent with altered trends of histone acetylation and methylation of DNA in the specific promoter regions.[33]. Notably, it has been identified that hsa-miR-150 is correlated with IBD and pain, while hsa-miR-342-3p is expected to communicate with miRs that include pain signals, colonic motility, and smooth muscle activity [34].

In a previous study, the nature of IBS was clarified by designing a network of protein-protein interactions (PPI) from the extracted genes/proteins of the document. PPI networks have shown 68822 associations of 3595 proteins. IBS interactome was analyzed with an interactome of the colon tissue, resulting in a sub-network of 153 genes [35]. Zucchini and co-workers find an overexpression of mRNA TNFSF15 in-patient with (IBS-D) comparing with controls. Besides, there is an association with Crohn’s disease [36]. Another study clearly shows correlations between neuropeptide S receptor gene (NPSR1) polymorphisms and colonic transit time as well as gastrointestinal sensory scores. NPSR1 protein is a neuropeptide S receptor, a neuropeptide implicated in anxiety, fear response, and nociception. Neuropeptides act in the GBA with IBS [37]. Past analysis of a study has shown that adding EGF to HeLa cells prompts the EGFR for general phosphorylation of 2244 proteins at 6600 locations. Moreover, stimuli by EGF have been shown to induce major expression variations in human mammalian epithelial cells (HMEC) of 3172 genes and 596 proteins [38].

2.3. Factors associated with IBS

Prevalence and factors of the IBS are complicated because of many points: first, the most signs are mutual with other diseases and are overlapping during treatment suggestion between the two diseases like (lactose or fructose intolerance with IBS), second, there is no specific biomarker for IBS patients that report chronic or recurring symptoms, and at last, because of absenting specific diagnosis for IBS patients recovering. the test for a positive result of recurrent IBS till now does not clearly illustrate [39].

2.3.1. Age

Alterations relating to age, such as gastro-intestinal activity, visceral tolerance, hormone and progesterone levels, cognitive capacity, and disease response can contribute to variations in pain or discomfort in the abdomen, anxiety, and depression, and lower rates of quality of life (QoL) [40]. Further aging implications for IBS are still unidentified and it is unclear whether the condition develops in the elderly. Indications of epidemiology studies proposed that the incidence of IBS decreases with age (perhaps linked with improvement in pain sensitivity), while IBS is still a widespread gastrointestinal condition in the olden years. [41]. The ages should be viewed in two terms of the old and young. The young-old for many diseases tend to be much like the regular. for example in individuals younger and older with unclarified gastrointestinal signs, age was not correlated with impaired upper gut activity, although both participants were under 70 years of age [42]. Differences in abdominal pain severity with age condition given the problem's therapeutic relevance. GI roles are frequently significantly impaired with age. There are only a few initial findings on systemic differences in visceral afferents consistent with age. The findings of the prevalence of IBS suggest that the incidence of IBS has risen with a declining age [43].

2.3.2. Gender

IBS in females is more common than in males, and women show more severe symptoms in IBS cases [44]. However, there have been only a few reports of gender differences in FGIDs, but in the IBS, cases were well established. Sex hormones or gender variations can play significant functions in the IBS pathophysiology. Sex is a biological difference between men and women while gender is a representation of sex-connected social norms, which therefore reflect acquired femininity or masculinity. Women with IBS may show constipation symptoms while men report diarrhea more often. Several reports have confirmed that women suffer significantly more constipation than men [45]. At least one of the gender disparities in IBS may be attributed to the different biobehavioral responses to stress in women and men [46].

In a systematic study comparing males and females for stress, Taylor et al suggested the response of women to stress, based on attachment/care systems, through the implementation method known as "tend and befriend" [47]. Depending on the female predominance in IBS and the relationship between IBS symptoms and hormonal status, the possible role of gonad hormones is debated. Many different models have been suggested to investigate the effect of sex hormones in gastrointestinal (GI) function, including variations in GI symptoms throughout the menstrual cycle and changes in symptom presentation in pre- and postmenopausal women, as well as shifts throughout pregnancy, hormonal therapy [48]. The gender-related variation in IBS prevalence occurs during puberty and peaks over the early adult years. In women, IBS most usually happens from late teens to mid-forties. When age subsequently continues to increase, the prevalence of IBS in women declines and exceeds the rate in men about 70 years of age and above. Conversely, the frequency of IBS between men stays stable between the ages of 20 and 70 [44, 49].

2.3.3. Diet

Diet plays a critical role in IBS pathophysiology as a concept is acknowledged [50, 51]. A large number of IBS patients report that different foods are susceptible to sensitivity [52]. In the present foods in the GI tract, GI motility, sensitivity, barrier activity, and intestinal microbiota will become changing. Hypersensitivity of certain materials may be inducing inflammatory, epithelial permeability, visceral hypersensitivity, and low-grade intestinal inflammation [53, 54]. Alcohol intake, Caffeine intake, spicy food intake, fat, and dietary fiber intake have potential triggers in the IBS symptoms [55, 56]. Restricting the channels of nitric oxide is believed to be the mechanism by which alcohol impacts gastrointestinal motility [29]. In spicy food, Capsaicin is the major component with hot peppers, which Improves gastrointestinal movement by receptors TRPV, which creates abdominal pain [57]. Food intolerance is a non-toxic response, non-immune, to bioactive chemicals in foods such as histamines, sulfites, or glutamate monosexuals, with symptoms caused typically beyond the gastrointestinal tract [58]. Bioactive chemical compounds in foods (e.g., salicylates) lead and cause GI symptoms in IBS, likely by causing prolonged contact gastrointestinal hypersensitivity [59].

FODMAPs (Fermentable Oligosaccharides, Disaccharides, Monosaccharides, And Polyols) are a technique to reduce the intake of short-chain carbohydrates that have been poorly processed and absorbed [60]. The low-FODMAP diet was developed by Dr. Peter Gibson and Dr. Susan Shepherd at Monash University in Australia, which is still widely seen as the foundation of treatment for IBS symptoms. depending on biological plausibility as well as evidence for more condition relief in about 75 % of patients [61]. The modes of action of FODMAPs are correlated with their osmotic activity, which pushes water through the gastrointestinal tract. Furthermore, they are a good food supply for the intestinal microbiota after reaching the colon, fermenting them and rising the development of hydrogen, methane, and carbon dioxide, in effect thus raising luminous distension [62]. It can contribute to abdominal discomfort with pain, flatulence, and bowel behaviors in the background of the gastrointestinal hypersensitivity commonly for IBS patients [63]. FODMAP fermentation primarily generates short-chain fatty acids (SCFAs) and products of some microbial SCFA that can be impacted with IBS. Low FODMAP diets have been linked with reduced bifidobacterial rates, which seem to be contradictory because of their possible health benefits [64].

Non-Celiac Gluten Sensitivity(NCGS) is clarified as presenting Gastrointestinal and extra gastrointestinal symptoms similar to the IBS without coeliac or wheat allergen, a recurrence to the gluten challenge [65]. Currently, foods containing wheat important because it is the source for more than 50% of human energy usage. [66]. (NCGS) gained recent media and general coverage, although it was puzzled by people's speculation about harmful health issues such as obesity-related to the high carbohydrate rates in wheat [67, 68].

2.3.4. Stress and Emotional Trouble

Human emotional status can affect the safety and well-being of somatic status in humans. Stress has a direct role in the functions of the stomach due to the connection between the stomach and the brain. The severe condition for gastroenterologists is irritable bowel syndrome which is affected by negative emotions [69]. Exposure to stressors in the initial periods in life will raise the vulnerability of a person to irritable bowel syndrome [70]. The mechanism of stress-activated begins in a signaling system in which the key material is the corticotrophin-releasing factor (CRF) in the central part of the physiological process by which the brain converts a stimulus to an interpreted behavioral reaction. during body skilling stress, the neuroendocrine signaling network of CRF activates the HPA axis and serves as a neurotransmitter/neuromodulator in order to regulate the immune and visceral efferent limbs, and induces the locus coeruleus and noradrenergic projections . Stress stimulates the HPA axis, stimulates the release, directly or indirectly, of HPA, CRF, ACTH, and cortisol that enhances the role of the gut, affects microbiota development and composition, in addition, encourages the sympathetic nervous system [32].

2.4. Diagnosis

The lack of a reliable IBS diagnostic test led to the study for biomarkers, which are detectable biological features, such as physiologic reactions, proteins, metabolites, or genes, for clearer diagnosis [71]. The diagnosis may be established via a symptom-based strategy using a combination of patient history, exclusion of alarm symptoms/warning signs [72]. IBS may be classified as mild, moderate, or severe, based on several approaches, including symptoms (such as abdominal pain), comorbidities of health (such as fibromyalgia, anxiety/depression), medical QOL, and medical use [73].

Manning was the first and most widely researched global IBS diagnosis standards to be adopted. Manning guidelines were set in 1978 depending on the increasingly severe effects of IBS patients in contrast to those with organic disorders. The major signs were mentioned in the manning criteria were painful stomachs, decreased frequency of bowel motions after induction of pain, bowel movement relaxation, and abdominal discomfort. in addition (sensation of delayed removal and fecal mucus), two other signs have been identified [74]. For a clinician to be confident whether the disease under consideration is either present or incomplete, a diagnostic test performance will move the pre-test risk measurement of the disease up or down. The intestinal tissue is a plateau where novel biomarkers in IBS can be searched. The first experiments would be least intrusive. The appropriate clinical analysis will be the complete blood samples, urea, and electrolytes, the checks on C-reactive protein, and liver function [75]. For the precise diagnosis of IBS, the Rome IV guidelines are clinically beneficial. The requirements suggest that the patient must have abdominal pain ≥1 per day a week on average in conjunction with the ≥2 of these symptoms: defecation-related pain, change in stool frequency, or pain related to stool form changing [76].

Meta-analysis demonstrates that the pooled prevalence of a positive serological test for CD in individuals with suspected IBS is between 2.6 and 5.7%, and the OR for a positive test was up to threefold higher among those meeting criteria for IBS [77]. The diagnosis of IBS is often made by PCPs, particularly by internists and family practitioners, and usually without the need for specialized tests (e.g., endoscopic or radiologic tests. The nature of the signs or problems of an individual, however, often causes a physician to carry out unnecessary and/or onerous medical testing and turn IBS into an exclusive prescription. The occurrence of troubling signs in patients aged over 50 years such as (fever, loss of weight, rectal bleeding, severe variations of blood chemical behavior), the existence of visible abdominal masses; the occurrence of signs at night; and a family history of celiac disease; the prevalence of colorectal cancer and/or inflammatory bowel disorders are warranting study during IBS [23].

2.5. Treatment

The range of comorbidity in IBS treatment-seeking patients was from 54%-94% [78]**.** In 2018, revised guidelines for IBS therapies, based on a systemic study were released by the American College of Gastroenterology (ACG). 13 Non-pharmacological therapy such as fibers, non-prescription laxatives, and stool softeners normally require initial care, but therapy performance is weak [79]. A low gluten diet and a low FODMAP diet are two diets that most frequently manage IBS. Because the proof substantiates of a gluten-free diet uses is low therefore it is not suggested in IBS treatment [80]. In the United States, three prosecretory medicinal products for IBS-C are approved: linaclotide and leconotide, which are guanylate cyclase C agonists, and lubiprostone, a chloride channel stimulator.

Some medicines have been found (e.g., Neomycin) to be used off-label to better IBS symptoms [73]. The antibiotic most extensively investigated in the treatment for both IBS-D and IBS-M is rifaximin, which is a non-absorbable antibiotic [81]. The signs of irritable bowel syndrome may be substantially diminished by antidepressants. They are intended to function by inducing acute hypersensitivity and an excessive sensitization of central pain. The Tricyclic anti-depressants and active serotonin reuptake inhibitors (SSRIs) show the advantages. In addition, Tricyclics are known that have adverse effects on constipation therefore used for the subtype of diarrhea. Alosetron, Ramosetron are 5-HT3 receptor antagonists, mitigate IBS-D symptoms, minimize gastrointestinal activity, increase liquid absorption, and reduce pain. The 5-HTTLPR L / L genotype has been shown to interfere with alosetron medical responses in IBS-D to modify the risk-benefit ratio of this compound class [82]. Specific reaction to clonidine, and α2-adrenergic agonist, is amplified by genetic variations in ADRA2A, including such rs1800544, the SNP reportedly correlated with gastric discomfort and rectal gas bloating sensations and defecatory urgency [83].

It is an appealing target for the development of anti-cancer therapies due to the role of EGFR in tumorigenesis and tumor growth. A range of targeting strategies has been undertaken to manipulate the role of EGFR in tumors. Monoclonal antibodies and tyrosine kinase (TKIs) are the most evolved of these anti-EGFR approaches [84].

3. MATERIAL AND METHOD

In the following sections, we introduce the materials and techniques that we used for our study. Also, the information is summarized in tables and charts.

3.1. Subjects

Blood samples of IBS were collected from December 2019 to January 2021 in the Firat University Hospital – Elazig –Turkey. All blood samples of patients were collected in EDTA tubes (Greiner bio-one, Frickenhausen, Germany). Fresh blood samples were composed of different ages and genders. The total population of the study was 80 samples were divided into two groups; 40 patients and 40 controls. This study was approved by Firat University Local Ethic Committee (Ethical Approved). Total RNA extractions from fresh blood samples were performed by commercial RNA isolation kit (NucleoSpin® RNA Blood Kit, and RNA Mini Kit (Geneaid)), Total RNA isolation was performed according to the manufacturer's instructions. The quantities and qualities of total RNA were evaluated by a Qubit fluorometer and agarose gel electrophoresis.

3.2. Material

The equipment and tools that were used in this study are listed in the table below with their models, company, and origin. As shown in Table 3.1.

Table 3.1. Equipment and tools

NO	Equipment	Company/Brand	Origin
1.	Centrifuge	NUVE NF 800R	Turkey
2.	Gel image iBright 750	MedSan Tek	Turkey
3.	Vortex	MVS-1	USA
4.	PCR/Thermal cycle	SimpliAmp/Applied Biosystem	Singapore
5.	Real-time PCR	StepOnePlus/AppliedBiosystem	Singapore
6.	Qubit Fluorometer	Invitrogen	USA
7.	Mini Centrifuge Spin	ISOLAB-Laborgeräte GmbH	Turkey
8.	Refrigerator	Regal	UK
9.	Micropipettes	AXYPET, A.B.T.	USA,Turkey
10.	Water bath	MedSan Tek	Turkey
11.	Electron balance	AND HM-200	Japan
12.	Hot plate	VELP ARE Labex	UK
13.	96-Well Reaction Plate	Applied Biosystem	China

3.3. Chemicals and Kits

The chemicals and kits that were used in this study are listed in the table below with their company and origin. As shown in Table 3.2.

Table 3.2. Chemical and kits

No	Chemicals	Company	Origin
1.	Agarose	SIGMA-ALDRICH	USA
2.	RedSafe™ Nucleic Acid Staining	iNtRON	China
3.	DNA Gel Loading Dye (6X)	Thermofisher scientific	UK
4.	Ladder 1kb	Thermofisher scientific	UK
5.	High-Capacity cDNA Reverse	Applied biosystems	UK
6.	NucleoSpin® RNA Blood Kit	MACHEREY-NAGEL	Germany
7.	Qubit™ RNA HS Assay kit,	Invitrogen	UK
8.	TBE (Tris/Boric/EDTA) Buffer	SIGMA Life Science	Germany

3.4. Method

This study was included the investigation of expression levels of EGF and EGFR genes in IBS patients. A three-milliliter sample of venous blood was taken from each IBS patient to the scheduled blood transfusion by using a disposable syringe. 2 milliliters of the blood was delivered into a sterile Ethylenediaminetetraacetic acid (EDTA) tube. The blood was used for RNA extraction freshly and directly.

3.4.1. Design of the study

Design of the study started by collecting blood samples then isolating RNA by special kit. The quality control was done by running gel electrophoresis with Qubit measurement. cDNA was done from the collected RNA samples after that gene expression was done by quantitative RT-PCR. As shown in Figure 3.1.

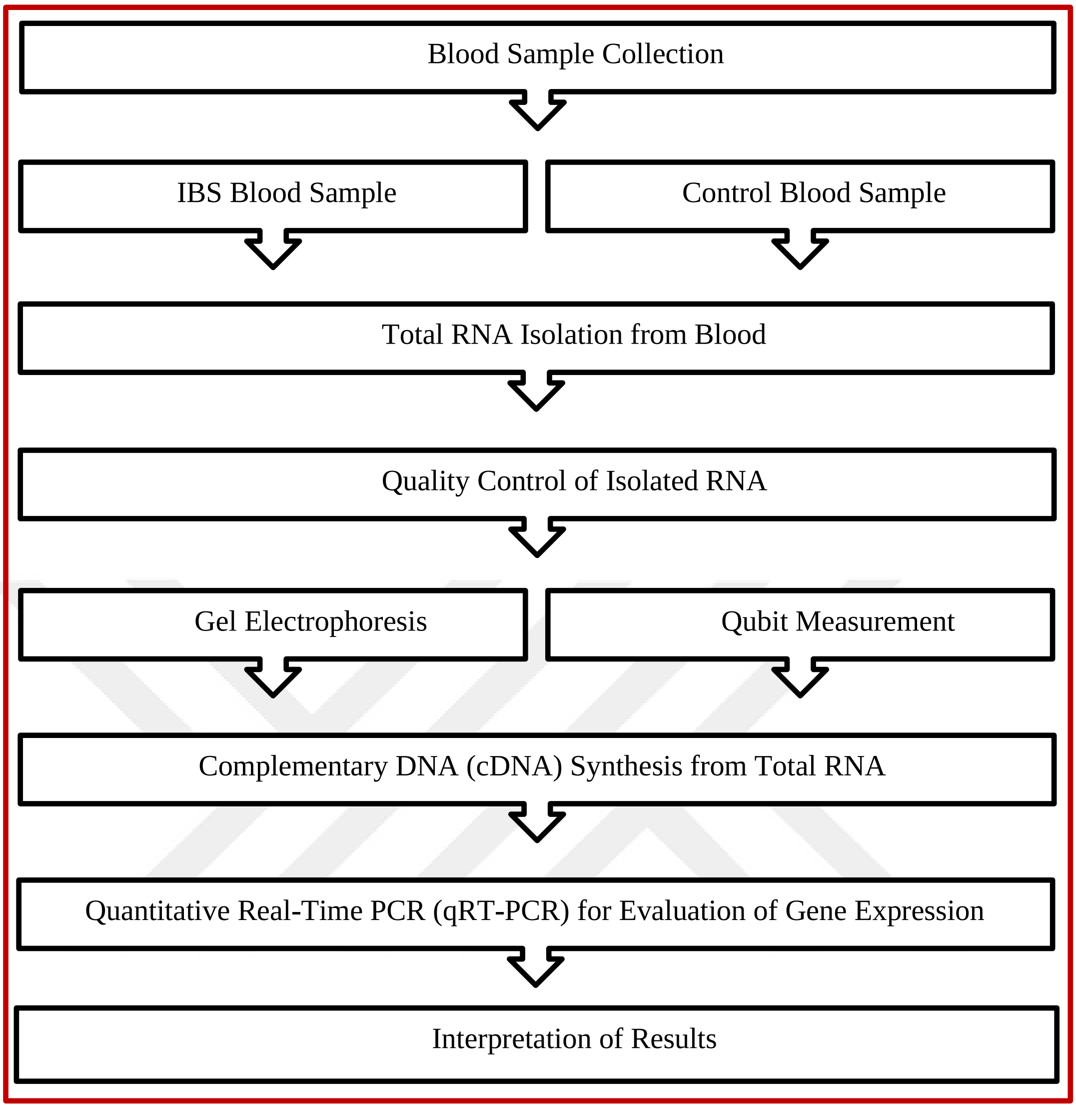

Figure 3.1. Experimental flow chart of the research

3.5. Isolation of Genomic RNA

The genomic RNA was isolated from the completely human fresh blood collected in EDTA anticoagulant tubes for molecular studies. Isolation was applied by using Total RNA Mini Kit (Geneaid) and NucleoSpin® RNA Blood.

3.5.1. Total RNA isolation Mini Kit (Geneaid)

Fresh human blood was collected in anticoagulant-treated collection tubes. 1 ml of RBC Lysis Buffer was added to 300 µl of whole human blood in a 1.5 ml microcentrifuge tube. Then the mixture was incubated for 10 minutes on ice with Mixing by vortex twice during incubation. After incubation, centrifuged at 3,000 x g for 5 minutes then the supernatant was removed completely. Cell lysed by adding 400 µl of RB Buffer with 8 µl of freshly prepared 2M

Dithiothreitol. Then the cells were resuspended by pipetting then incubated at room temperature for 5 minutes. For RNA binding 400 µl of 70% ethanol was added to the mixture then vigorously Shacked. 500 µl of the mixture was transferred to the RB Column then centrifuged at 16,000 x g for 1 minute then the flow-through was discarded. The remaining mixture was transferred to the same RB Column then centrifuged at 16,000 x g for 1 minute then RB Column was placed in a new 2 ml Collection Tube. DNase digestion mixture was prepared according to the protocol of the kit then 50 µl of prepared DNase solution was added to the RB column matrix then incubated for 15 minutes at room temperature (25ºC). For the washing step, 400 µl of W1 Buffer was added into the RB Column then centrifuged at 16,000 x g for 30 seconds. The flow-through discarded then .600 µl of Wash Buffer added. Centrifuged at the same speed for 30 seconds then the flow-through was discarded. Washing repeated by adding 600 µl of Wash Buffer. Centrifuge at 14-16,000 x g for 30 seconds then discard the flow-through. To dry the column matrix RB Column centrifuged at 14-16,000 x g for 6 min. finally, RNA was Eluted by adding 50 µl of RNase-free Water to the center of the column matrix. Waited for 5 minutes to ensure the RNase-free Water is absorbed. then Centrifuged at 16,000 x g for 3 minutes.

3.5.2. Protocol of RNA Isolation (NucleoSpin® RNA Blood kit)

All venous blood samples of the patient and control groups were taken into the tubes with EDTA in a volume of 5 ml. Blood samples were stored in a refrigerator at + 4 ° C until RNA isolation. 400 µl of whole blood sample was taken put in a 2 ml microcentrifuge tube, 400 µl of Lysis Buffer DL was added. After mixing 10 µl Liquid Proteinase K was added then incubated at room temperature for 15 minutes on a shaker. To homogenized lysate, 400µl of Ethanol 70 % was added and well mixed by pipetting. 610 µl of lysate were transferred into a NucleoSpinR RNA Blood Column placed in a Collection Tube and centrifuged for 30 s at 11,000 x *g.* flow-through was discarded and the NucleoSpinR column was placed in a new collection tube. The remaining lysate was applied into the column then centrifuged for 30 s at 11,000 x *g*. Membrane Desalting Buffer 350 µl MDB added onto the column and centrifuged 30 s at 11,000 x *g*. to remove DNA from lysate 95µl DNase was added and incubated for 15 minutes at 20-25 °C. The Spin column was washed by adding 200 µl Buffer RB2 then centrifugated at 11,000 x *g*. for 30 *s*. The flow-through was discarded. the second washing was done by adding 600 µl Buffer RB3 and centrifuged the same at 30 s at 11,000 x g. the flow-through was discarded finally, the third washing was done by 250 µl Buffer RB3 to the NucleoSpinR. And centrifuged for 2 min at 11,000 x g. at the ending of the third washing the column was placed into a nuclease-free Collection Tube (1.5 mL) and the collection tube discarded RNA was collected by adding 60 µl RNase-free H2O onto the column and centrifuge 30 s at 11,000 x *g*.

3.6. Agarose Gel Electrophoresis

After genomic RNA extraction, Agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted RNA. As shown in Table 3.3.

Table 3.3. Materials for gel electrophoresis

Materials	Amounts
Agarose	1.0 gr
Tris/Boric Acid/EDTA (1xTBE) loading buffer	100 ml
Red Safe	8 μL
6× loading dye	2 μL
RNA Ladder Marker (1kb)	3 μL

3.6.1. Preparation of Agarose gel

Weight 1.0 g Agarose powder by electron balance and put in a conical flask, then 100ml 1 × TBE buffer was added. Swirled gently and put in microwave for 2-3 minutes until the Agarose has dissolved. Allowed the Agarose solution to cool under running the tap water, and then the gel apparatus was constructed. When the Agarose solution was hand hot, 2μL of the red safe was added and mixed slowly. After sealing both edges of the gel tray with cellophane tape and fixing the comb 1 cm away from one edge, the agarose solution was poured into the gel tray. The Agarose was allowed to solidify at room temperature for 30 minutes. The fixed comb was carefully removed and the gel tray was placed in the gel tank. The tank was filled with 1X TBE buffer until it reached 1-2 mm over the surface of the gel.

3.6.2. RNA Loading and Electrophoresis Running

On the paraffin paper, 2μl 6× loading dye was put and 5μl of RNA samples were added with mixing well by pipette. The mixture was loaded into the gel with a 3 μl control ladder 1kb. The voltage machine was set at 90 V and run for 30 min. After running ended, the gel electrophoresis machine using a UV transilluminator given the photo of the gel. Then the result of RNA running was analyzed.

3.7. Qubit RNA Concentration Measurement

For standards and samples, 0.5 ml tubes were set with labeling all-tube lids according to the samples The Qubit® RNA HS Assay requires 2 standards. For each standard tube 190 µL of Qubit working solution was added and 10 µL of each Qubit standard to the appropriate tube, then mixed by vortexing 2–3 seconds. For samples tubes, 198 µL of the working solution was added with 2 c of the RNA sample. the correct volume of Qubit® working solution with samples RNA became 200 µL. After vortexing for few seconds allowed all tubes to incubate for two min at room temperature. Finally, standards and samples were read

3.8. cDNA Reverse Transcription

cDNA synthesis from total RNA of 80 samples, which divided into two groups (40 IBS patients and 40 control groups). It was performed with a cDNA Reverse Transcription Synthesis Kit (Cat. No.4368814). The volumes were determined according to the lowest RNA Molar concentration amount between the samples (10 ng/ µl). The calculations were done and the RNAs were adjusted to (10 ng/ µl) final volume. At first 2X RT master mix was prepared according to sample number. For each sample the cDNA was done like this: Nuclease-free H2O (4.2 µl), 10X RT Random Primers (2.0 µL) was added then 10X RT Buffer (2.0 µL), 25X dNTP Mix (100 mM) (0.8 µL) and MultiScribe™ Reverse Transcriptase (1.0 µL) was added to the RNA sample. The total volume 20 µL per every 0.5 tubes. As shown in Table 3.4 and Table 3.5.

Table 3.4. Component of the cDNA Reverse Transcription

Component	Reaction Volume Without RNase inhibitor
10X RT Buffer	2.0 µL
25X dNTP Mix (100 mM)	0.8µL
10X RT Random Primers0	2.0 µL
MultiScribe™ Reverse Transcriptase	1.0 µL
Nuclease-free H2O	4.2 µL
Total per reaction	10µL

Table 3.5. Program of the thermal cycling conditions

Setting	Step1	Step 2	Step 3	Step.4
Temperature	25°C	37 °C	85°C	25°C
Time	10 minutes	120 minutes	5 minutes	∞

3.9. Gene expression qRT-PCR

QRT-PCR was performed using the SYBR Green master mix (AM02-020) to determine the expression levels of two target genes: EGF, and EGFR. The StepOnePlus® Real-Time PCR

System (Applied Biosystems, Singapore) was carried out in 10 µl, including 5 µl of 2x Amplifyme SG Universal Mix), 0.3µl forward primer, 0.3µl reverse primer, 0.2µl 50X High ROX Solution, 2.2µl PCR grade water, and 2 µl cDNA sample, as shown in Table 3.6. The program was set to run for one cycle at 95°C for 3 min, followed by 40 cycles of the following protocol, as shown in Table 3.8, and Table 3.9: 95°C for 5s, 54°C for 10s, and 72°C for 20s. For normalization of gene expression, the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control, and 40 control samples were used as external control. Agarose gel electrophoresis and melting curve analysis were used to confirm the accuracy of PCR amplification. The primer sequences are shown in Table 3.7. Results of gene expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method, and data were expressed as the mean fold changes ± SD for three independent amplifications.

Table 3.6. Quantitative RT- PCR reaction mixture content

Reagent	**Suggested amount per reaction**
2x AMPLIFYME SG Mix	10µ
Forward primer (10µM)	0.6 µM (0.3 µM)
Reverse primer (10µM)	0.6 µM (0.3 µM)
50x ROX solution	0.4µl
DNA or cDNA template	1 – 100 ng
PCR-grade water	fill up to 20 µl

Table 3.7. The list of primer sequences

Gene names	**Primer sequence**	
	Forward (5'-3')	Reverse (5'-3')
EGF	AGCAATTGGTGGTGGATG	ACTCTTTGCAAAAGTTGTC
EGFR	AAATCCTGCATGGCGCCGTG	GGTGGTTCTGGAAGTCCATC
GAPDH	GAA GGT GAA GGT CGG AGT C	GAA GAT GGT GAT GGG ATT TC

Table 3.8. Three-step thermal cycling profile

Step	Temperature	Time	Cycle
Activation and denaturation	95°C	180 s	
Denaturation	95°C	5 s	35 – 45 cycles
Annealing	60°C	10 s	
Extension／Fluorescence Detection	72°C	5 – s	
Melt curve analysis	according to the qPCR instrument manual		

Table 3.9. Two-step thermal cycling profile

Step	Temperature	Time	Cycle
Activation and denaturation	95°C	180 s	
Denaturation	95°C	5 s	35 – 45 cycles
Annealing／Extension／ Fluorescence Detection	60°C	15 – 30 s	
Melt curve analysis	according to the qPCR instrument manual		

3.10. Statistics analysis

SPSS (version 22), GraphPad Prism (version 8), and Inkscape (version 0.92.4) software were used to design the experiment and perform statistical analysis of data. Independent sample t-test was performed, and values of $p < 0.01$ were considered significant.

4. RESULTS AND DISCUSSION

From December 2019 to January 2021, forty-five IBS and fifty control blood samples were collected daily at Firat University's hospital in Elazig City, Turkey. All blood samples (95) had total RNA extracted. The quality and amount of RNA were assessed, and 15 RNA samples were excluded, 5 from the IBS group and 10 from the control group. Data on the patients' and controls' ages and genders were also obtained and there were no significant differences between them. The characteristics of patients and controls are shown in Table 4.1.

Table 4.1. Population characteristics

Patients			**Controls**		
Age					
Mean	Median	Range	Range	Median	Mean
40	37	50 (19-69)	51 (20-71)	44	43
Gender					
Male		Female	Male	Female	
18		22	18	22	

There are numerous methods for isolating RNA. The phenol/chloroform-based approach and the column-based rotating approach were the most often used methods for extracting RNA from blood. The phenol/chloroform method employs a monophasic phenol and guanidinium isothiocyanate solution that solubilizes organic materials while also lysing protein. Following the addition of chloroform, the phase separates (similar to phenol extraction: chloroform: isoamyl alcohol), with the protein being replaced in the organic phase, the DNA being dissolved at the interface, and the RNA existing in the aqueous phase [85]. In our research, the quality control for total RNA extraction from 40 IBS and 40 control blood samples by two different kits were evaluated by agarose gel electrophoresis as shown in Figure 4.1, and Figure 4.2 the product was shown clear RNA bands (28s and 18s) on an agarose gel, without any gDNA contamination. The quantity evaluations of total RNA samples by Qubit measurement, the normal Qubit values (ng/µL) for each sample as shown in Figure 4.3 and Figure 4.4. NucleoSpin RNA Blood Mini kit and Total RNA Mini Kit (Geneaid) were performed to found an optimized and standard amount of total RNA from IBS and control blood group samples.

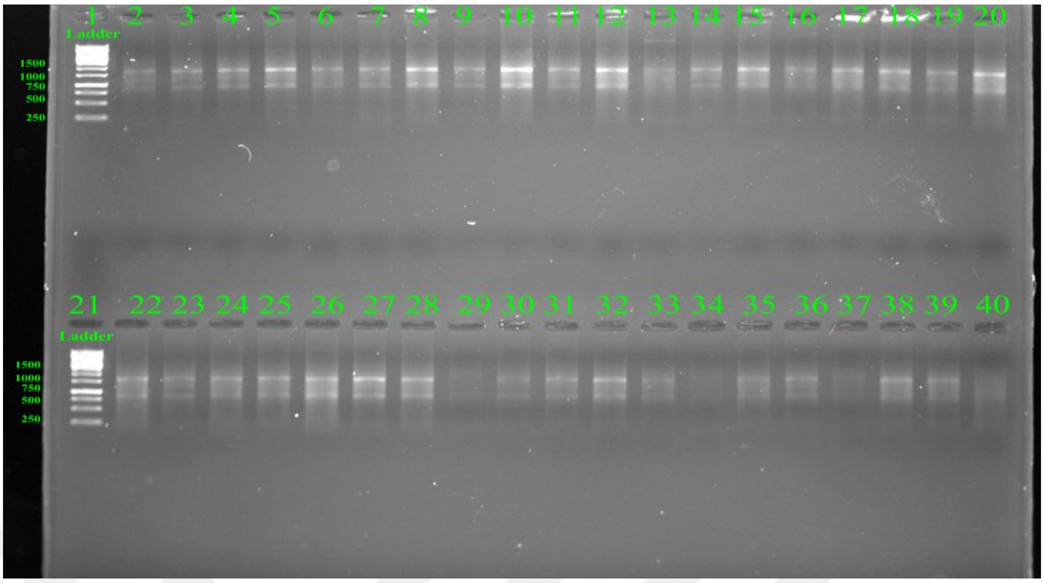

Figure 4.1. Total RNA samples of 40 control volunteers were run on a 1% agarose gel in 110V for 30 minutes, ladder (1 kb) used in line (1, 21), and samples arranged from (2 - 40), analysis image system iBrightTM750 performed for evaluation of total RNA quality from fresh blood.

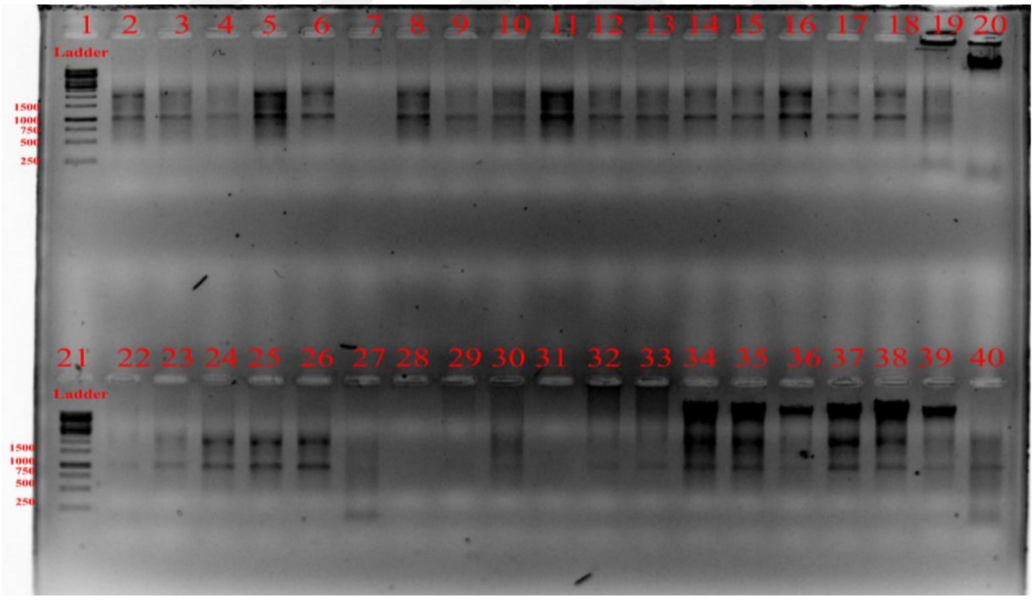

Figure 4.2. Total RNA samples of 40 IBS patients were run on a 1% agarose gel in 110V for 30 minutes, ladder (1 kb) used in line (1, 21), and samples arranged from (2 - 40), analysis image system iBrightTM750 performed for evaluation of total RNA quality from fresh blood.

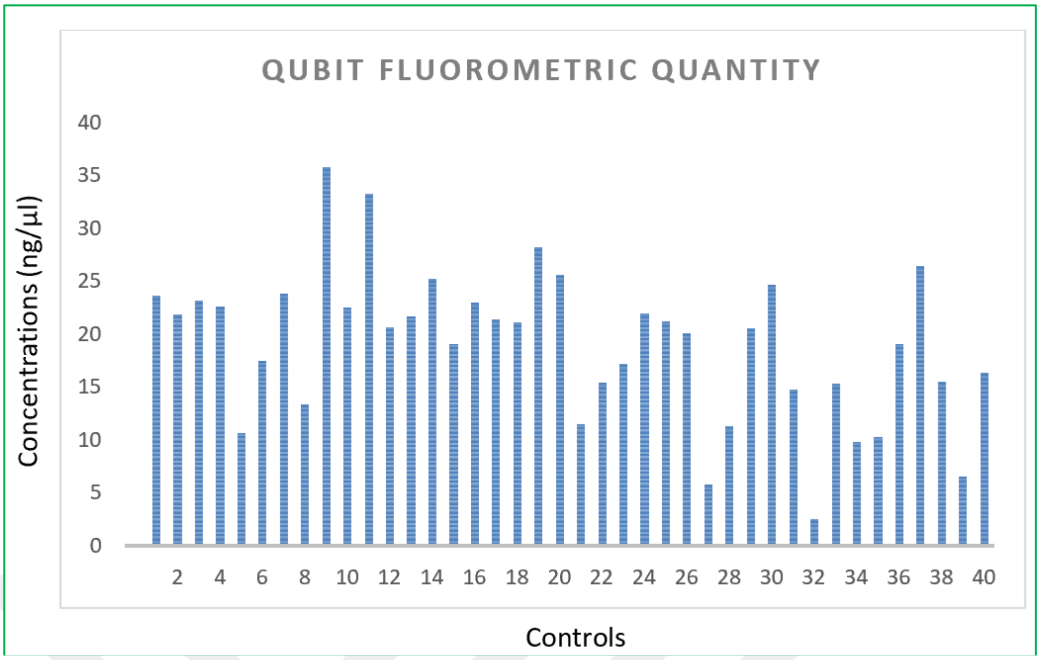

Figure 4.3. Evaluation of quantity of total RNA extraction from fresh blood samples of 40 control by Qubit® 2.0 Fluorometer, QubitTM RNA HS Assay kit (REF: Q32852, LOT 1913928), Qubit values of IBS samples arranged from (1,2,3 – 38, 39, 40).

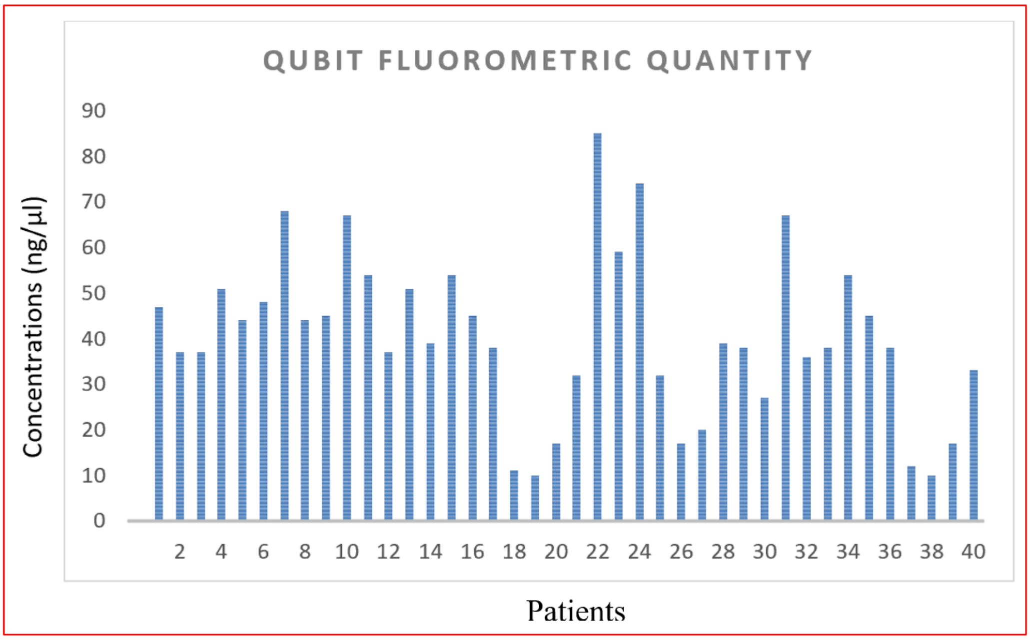

Figure 4.4. Evaluation of quantity of total RNA extraction from fresh blood samples of 40 IBS patients byQubit® 2.0 Fluorometer, QubitTM RNA HS Assay kit (REF: Q32852, LOT 1913928), Qubit values of IBS samples arranged from (1,2,3 – 38, 39, 40).

4.1. Genetics and IBS

One of the most common gastrointestinal illnesses is IBS. It is distinguished by changed bowel habits and abdominal pain without structural abnormalities. Therefore, IBS is a complicated condition that affects millions of people throughout the world. The purpose of this study was to investigate the amount of EGF and EGFR gene expression in IBS patients, which have a great role in the regulation of bowel activity by contact with each other. It is becoming a new molecular line of study in this field to estimate the roles of genetics. IBS is a disease that results in persistent changes such as diarrhea, constipation, and alternating patterns in bowel function. IBS is a complex multifactorial condition in most patients, in which the manifestations may differ with the period. in long-term follow-up of clinician-based IBS patients, a consistent analysis found that 2% to 18% of patients deteriorated, 30 % to 50% remained stable and 12% to 38% increased [86]. EGF is a natural component of saliva generation, is divided into the intestinal lumen, present in colostrum, and milk while feeding. In several experiments and some clinical applications, the use of EGF was investigated in a dietary technique to provide enhancements to the intestinal mucosa in impaired patients [87]. There is a lot of biological control in many cell types affected by EGF as a study has shown that it affects both the growth and the function of gastrointestinal tissue. In addition, EGF was applied to enhance intestinal transition in the animal model with substantial bowels resection. The multiple intestinal hyperplasias in response to EGF have increased [88].

Polymorphisms or mutations have been discovered to be correlated with IBS in many genes. Genes encoding proteins involved in homeostasis and epithelial barrier regulation, such as cadherin 1 (*CDH1*) and cell division cycle 42 (*CDC42*), the immune system, such as *IL6*, *IL10*, *TNF*, and TNF superfamily member 15 (*TNFSF15*; encoding cytokines and neuronal signal transduction) and others (such as neurexophilin 1 (*NXPH1*) and sodium voltage-gated channel α-subunit 5 (*SCN5A*)) In numerous studies, it has been repeated [89]. Wu et al in a research study investigated the relation between EGF A61G polymorphism and the CRC colorectal cancer risk in the community of Germany. Their findings suggest that EGF A61 G polymorphism raises the risk of CRC. The genotype EGF 61 G/G and allele G correlated with CRC sensitivity. Yet the this SNP has not associated tumor levels or tumor grades in the CRC [90].

Intestinal barrier dysfunction is correlated with increased intestinal permeability and the progression of many disorders, such as celiac disease, inflammatory bowel disease (IBD), irritable bowel syndrome, food allergy, and infectious enterocolitis. EGF through different underlying mechanisms can protect the epithelial of gastrointestinal, additional many compounds have been documented to have a role in protection like glutamine, sodium butyrate, bile acid, lactic acid bacteria [91].

4.1.1. EGF

EGF is a well-known sequence of polypeptides consists of 53-amino acid contains six cysteine residues that make three interchain disulfide bonds. It is a transmembrane glycoprotein that contains four members of family erbB (erbB2/HER-2, erbB3/HER3, and erbB4/HER's-4) which is tyrosine kinase receptors, as shown in Figure 4.5. It is a polypeptide of 6000 kDa that is mostly formed in the gastrointestinal tract [92]. It is located at 4q25 in the human chromosome with 26 exons.

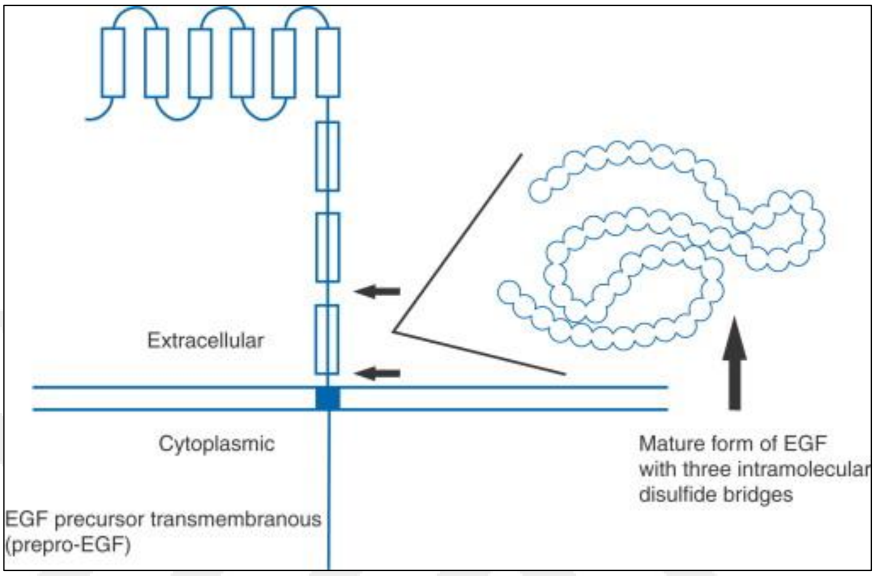

Figure 4.5. The EGF precursor molecule

EGF has a major role in proliferation, regulating cell growth, survival, migration, and apoptosis. In addition to enhancing cellular proliferation and differentiation, also EGF has a direct role in protecting homeostasis of the intestinal epithelial by different mechanisms [93]. The discovering of EGF is more than half a century ago by Stanley Cohen. He has found the induced precocial opening of the eyelid and incisor eruption due to direct stimulation of epidermal growth and keratinization by injection of a submaxillary extract into newborn Mice then EGF was isolated, purified, and differentiated [94]. It is the most widely studied growth factor in animals that found in different fluids in the body such as milk, saliva, plasma, amniotic fluid, and intestinal fluid, but the kidney is the main origin of EGF manufacturing and urine has high concentrations of EGF, although the largest level of EGF has been identified in prostate liquids [94]. Different EGF family ligands show different rates of expression in the cell kinds and tissue contexts and the topic is being examined extensively whether each may play a different physiological function [95]. EGF has been used in skin infection treatments, such as diabetic foot ulcers [96]. In addition, since 1989, EGF topical administration has been used for the therapy of various peripheral tissue injuries (16 clinical studies) as well as for the treatment of gastrointestinal track [97]. Therefore in the past, the name of EGF was (epidermal healing factor) which was more correct. Besides all advantages and affected role of EGF and its receptor in the body have significant performance in the developing IBS symptoms through changing the habits of the gastrointestinal and colon [36].

In our study, the expression levels of the EGF gene in the 40 IBS blood sample were significantly (p<0.01) decreased by 5.2-folds when compared to the 40-control group, as shown in Table 4.2, and Figure 4.6. EGF seems to have little impact on the development of the non-damaged intestinal epithelium [98]. EGF needs a mediate to bind through it to start biological actions in the body; this mediate is (EGFR), which is receptor tyrosine kinase transmembrane of the family ErbB. EGFR is a prototypical member of the family tyrosine kinase receptor, which is first discovered in this family. Many ligands can activate this receptor to vary cellular activities such as proliferation, cell growth, apoptosis, and cell division [15].

Table 4.2. Top 10 downregulated IBS patient's comparison to 10 control samples

The housekeeping gene (GAPDH)		**Target gene (EGF)**				
Sample	Ct Mean	**Sample**	Ct Mean	ΔCt	ΔΔCt	2^-(ΔΔCt)
Control 1	25.63407898	**Control 1**	32.50759125	6.873512268	-4.48349954	22.4
Control 2	19.82203293	**Control 2**	34.39074707	14.56871414	3.21170232	0.1
Control 3	20.7931366	**Control 3**	36.23467636	15.44153976	4.084527943	0.1
Control 4	25.20994568	**Control 4**	31.78126335	6.571317673	-4.78569449	27.6
Control 5	26.06271935	**Control 5**	31.84964371	5.786924362	-5.57008759	47.5
Control 6	19.42203293	**Control 6**	33.44234467	14.02031174	2.663299915	0.2
Control 7	26.62818146	**Control 7**	35.06289291	8.434711456	-2.92230065	7.6
Control 8	18.2771366	**Control 8**	36.95114136	18.67400476	7.316992939	0.0
Control 9	26.59532166	**Control 9**	34.49676514	7.901443481	-3.45556834	11.0
Control10	26.81953812	**Control10**	32.81341553	5.993877411	-5.36313411	41.2
IBS 1	18.40416336	**IBS 1**	28.32345009	11.8698082	0.512796375	0.7
IBS 2	17.1313591	**IBS 2**	29.0011673	13.23363876	1.876626942	0.3
IBS 3	16.8715477	**IBS 3**	30.10518646	13.06394196	1.706930134	0.3
IBS 4	18.61240387	**IBS 4**	31.67634583	11.87590408	0.518892262	0.7
IBS 5	17.31906128	**IBS 5**	29.19496536	15.07843971	3.721427891	0.1
IBS 6	20.7760601	**IBS 6**	35.85449982	13.00201035	1.644998524	0.3
IBS 7	18.84941483	**IBS 7**	31.85142517	9.930170059	-1.42684762	2.7
IBS 8	17.66415215	**IBS 8**	27.5943222	12.86949158	1.512479756	0.4
IBS 9	19.5029335	**IBS 9**	32.37242508	11.20523643	-0.15177587	1.1
IBS10	16.6470356	**IBS10**	27.85227203	9.483919144	-1.87309278	3.7

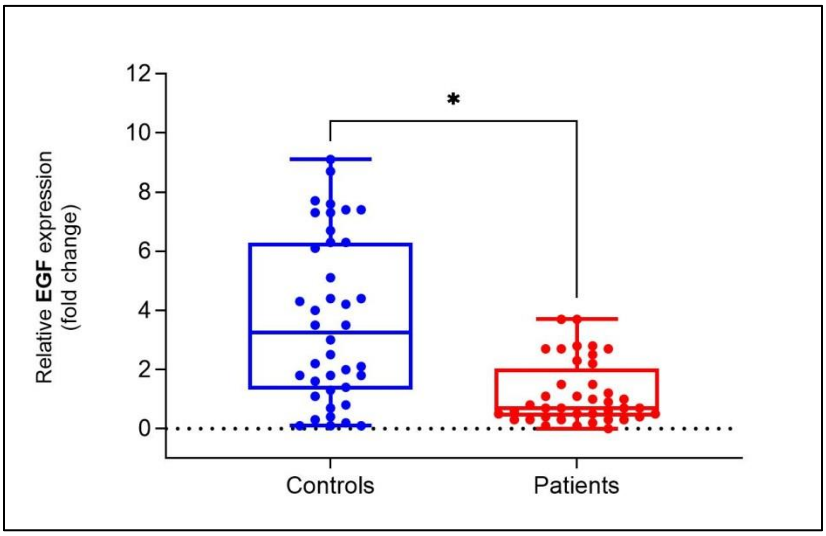

Figure 4.6. Quantitative real-time PCR was used to evaluate the impacts of IBS on the expression level of the EGF gene. Relative EGF gene expression in 40 IBS patients was compared to EGF gene expression in 40 control samples. Expression was normalized to the endogenous control; the GAPDH gene. Significant differences were analyzed by student t-test, where: *p<0.01 compared to control.

4.1.2. EGFR

The EGFR is a transmembrane glycoprotein that consists of 1,186 amino acids. It is located at 7p11-13 in the human chromosome and has 31 exon counts; TATA-less promoter gene-encoded EGFR. It composes of three parts, extracellular domain makes up of 621 amino acids which connect the ligand-binding site, a single transmembrane alpha-helical domain is 23 amino acid, and the cytoplasmic domain contains 542 amino acids which also includes three domains, juxtamembrane, Src homology (SH1) tyrosine kinase and carboxyl terminus tail, as shown in Figure 4.7. In addition, EGFR has many autophosphorylation locations for protein SH2 linking domains or PTB (phosphotyrosine binding) domains [99]. Expression of epidermal growth factor receptor in the colon tissue is mainly on the basolateral side of all animal epithelial cells and has decreased in the subepithelial myofibroblasts and monocytic cells, but the significant functions of the latter cells are not clearly defined in either the infected or the healthy colon [100]. In the gastrointestinal organ both submandibular glands and Brunner's glands are producing EGF and prototypical EGFR ligands in a limited range, however, during the formation of a wound on epithelial cell production of EGF decreased as a response to injury in all parts of the gastrointestinal organ [101].

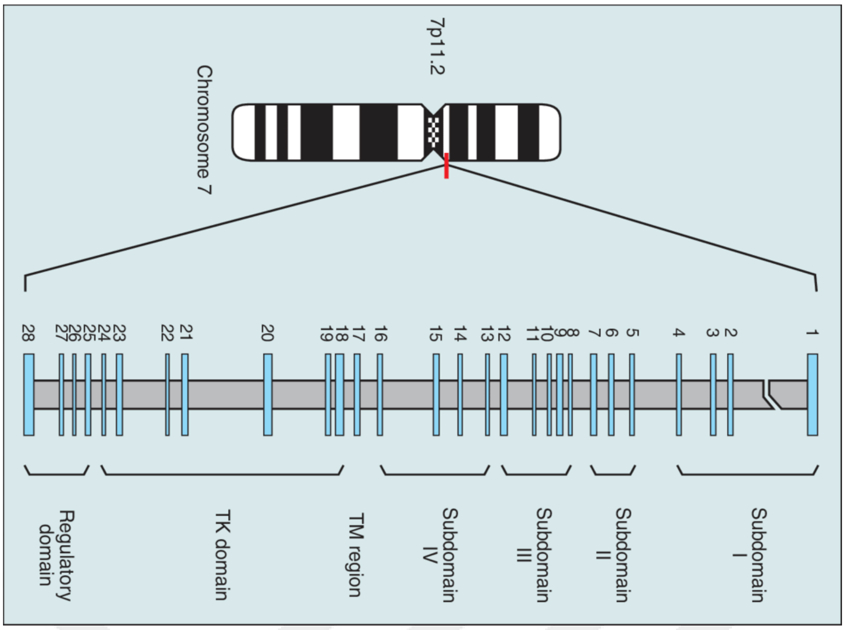

Figure 4.7. Location of the EGFR gene on the Chromosome, with its exon and protein domains [102]

There is evidence that EGF downgrading the expression of its intestinal receptor (EGFR) after small bowel resection in rats.78 Similar mechanisms may operate in the stomach in response to injury, thus forming a positive feedback loop to augment the reparative and healing ability of the injured mucosa [98]. The binding of EGF with EGFR results in dimerized, activated, and auto phosphorylated on its tyrosine residues. Five fairly well-understood pathways are downstream of EGFR [103]. The first is phospholipase-y (PLC3), which binds to the activated EGFR, which induces PLCy's active tyrosine phosphol3A action, which makes the activation of it, and catalyzes membrane phospholipid phosphatidylinositol-4, 5-biphosphate hydrolysis [104]. The second is the Ras-mitogen-activated protein kinase pathway through the adaptor proteins such as Grb2 and Shc. The third pathway involves the phosphorylated activating protein of Ras-guanosine 5'-triphosphatase (GTPase)- activating EGFR protein, enhancing the activities of the Ras GTPase and increasing the rate for the transformation of Ras into the inactive state of 5'-diphosphate (GDP)-bonded guanosine [15]. The fourth is phosphatidylinositol-3-kinases (PI3-K) activation via the Erb-3, Cbl, and Gab1 mediators. EGF stimulates the Jak-Stat pathway is the last pathway that was initially known as interferon [105]. In mature EGFR mutation in G711S, G772S, E780) ligands make enhancing cell growth and invasion in prostate cancer by increasing phosphorylation of four tyrosine residues [106]. As shown in Figure 4.8

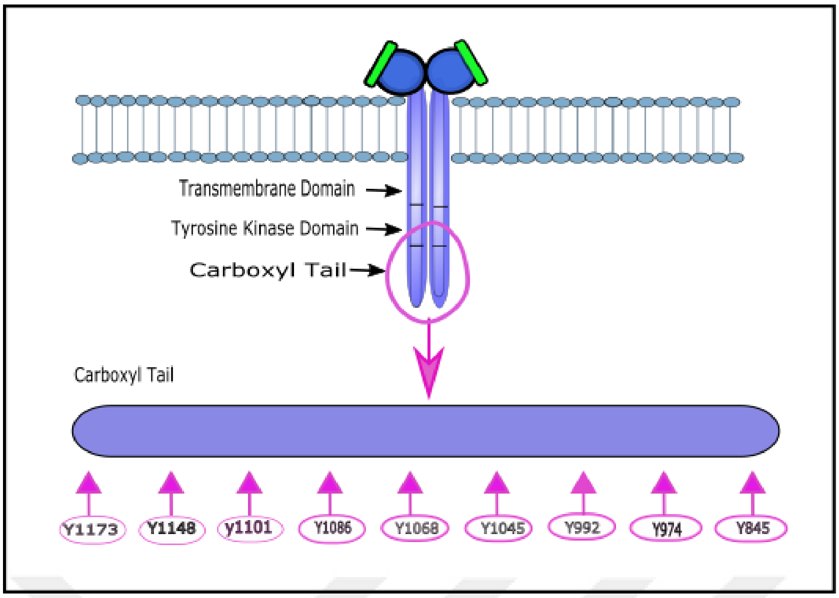

Figure 4.8. Phosphorylation of EGFR-ligand binding induces the EGFR to dimerize with another receptor. This leads to allosteric activation of the kinase domain and phosphorylation of several cytoplasmic residues. Phosphorylation of these residues can activate several signaling cascades.

In the present research, we evaluated the expression level of EGFR gene among 40 IBS patients and compared with 40 control group. We found that the expression levels of the EGFR gene significantly downregulated in IBS patients, as shown in Table 4.3, and Figure 4.9. While the EGFR Overexpression has been proven by promoting autocrine and paracrine loops and cell proliferation in EGF and TGF- production. Many processes combined in tumor progression (for example apoptosis, angiogenesis, and metastasis), rising of EGFR-mediated signaling is significant. HER-2 is observed in 10% to 30% of breast, esophageal, abdomen, and urinary bladder carcinoma cases to be overexpressed and seem to have a link with increased rates of growth, tumorigenicity, and a tumor cell metastatic potential [15, 107]. The relation of the EGFR to cancer was first identified when a mutant homolog of human EGFR was discovered in the transforming v-ErbB oncogene of the avian erythroblastosis virus because v-erbB oncogene contains recombination of the transmembrane and cytoplasmic domains of the EGFR, entangling EGFR aberrations to the tumor [108]. The level of Overexpression of HER2 in colorectal carcinoma more frequently appears to associate with the elevated stage of cancer and reduced patient survival in contrast with ordinary colonic mucosa. The differentiation of gene expression was not observed in all types of IBS. it can be seen in only a type of IBS patient. Moreover, may indicate the heterogeneity of the typical symptom-phenotype molecular mechanisms [109].

Table 4.3. Top 10 downregulated IBS patient's comparison to 10 control samples

The housekeeping gene (GAPDH)		Target gene (EGFR)				
Sample	Ct Mean	**Sample**	Ct Mean	ΔCt	ΔΔCt	2^-(ΔΔCt)
Control 1	25.63407898	**Control 1**	33.55948	7.92539978	-2.59276	6.0
Control 2	19.82203293	**Control 2**	33.20086	13.37882233	2.860659	0.1
Control 3	20.7931366	**Control 3**	31.55861	15.76547241	5.247309	0.0
Control 4	25.20994568	**Control 4**	36.24453	11.03458786	0.516424	0.7
Control 5	26.06271935	**Control 5**	34.01211	7.949388504	-2.56878	5.9
Control 6	19.42203293	**Control 6**	35.91319	16.49115982	5.972996	0.0
Control 7	26.62818146	**Control 7**	31.99405	5.3658638	-5.1523	35.6
Control 8	18.2771366	**Control 8**	30.68447	20.40733072	9.889167	0.0
Control 9	26.59532166	**Control 9**	32.57393	5.978610992	-4.53955	23.3
Control10	26.81953812	**Control10**	33.57728	6.757743835	-3.76042	13.6
IBS 1	18.40416336	**IBS 1**	29.90765	18.50348282	7.985319	0.0
IBS 2	17.1313591	**IBS 2**	32.31964	15.18828201	4.670118	0.0
IBS 3	16.8715477	**IBS 3**	30.51745	13.64590263	3.127739	0.1
IBS 4	18.61240387	**IBS 4**	34.1664	15.55399323	5.03583	0.0
IBS 5	17.31906128	**IBS 5**	28.52267	11.20361328	0.68545	0.6
IBS 6	20.7760601	**IBS 6**	34.74766	13.97160149	3.453438	0.1
IBS 7	18.84941483	**IBS 7**	31.39054	12.54112625	2.022963	0.2
IBS 8	17.66415215	**IBS 8**	34.06472	16.40056801	5.882404	0.0
IBS 9	19.5029335	**IBS 9**	35.39603	15.89310074	5.374937	0.0
IBS10	16.6470356	**IBS10**	38.16401	21.5169735	10.99881	0.0

The extent to which the expression of the multiple genes in the mucosa of IBS patients is increasing by folds varies from 1.2 to 1.52, which is compatible with the gradual degree of cytological or functionally assessed immune activation studies [110]. In some cases, the inherited genotype has been affected with the tissue mucosal gene expression, such as the gene TNFSF15 is associated with IBS and related to functional mucosal immune modifications [111]. In addition, in another research Different genes have been interpreted abnormally in rectal mucosa by patients coming back from IBS. Moreover, a rise in peripheral mononuclear blood cells (PBMC) and IL-10 cytokines have also been recorded in IBS [112]. A murine-producing gene (e.g. MUC20) is upregulated and can be associated with the commonly observed excessive mucus transient in IBS patients [113]. EGFR co-expression, together with EGF, has resulted in autocrine activation of EGF-R through its ligands as the way for tumor cells to evade normal growth controls in the human lines of cancerous colon cells and colon carcinoma tissue. Patients with IBS reported an increased frequency of CD4 and CD8 T peripheral blood cells expressing intestinal homing integrin 7, elevated lamina propria CD8 T cells in ascending colon biopsy samples, and raised integrin 7 receptor expression, Mucosal addressin cell adhesion molecule-1, as compared to control subjects on endothelial cells in colon biopsy samples [114]. Evaluation of total gene expression profile in the colon in the IBS is various by using multivariate analysis[115].

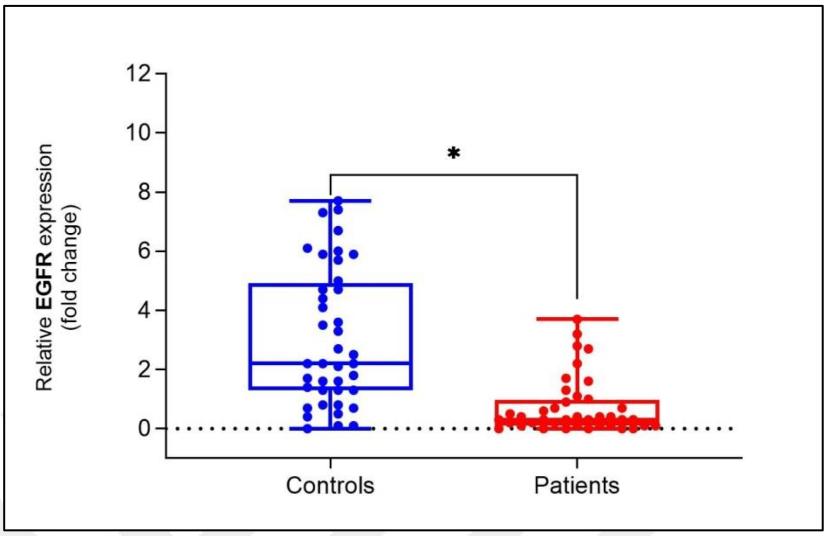

Figure 4.9. Quantitative real-time PCR was used to evaluate the impacts of IBS on the expression level of the EGFR gene. Relative EGFR gene expression in 40 IBS patients was compared to EGF gene expression in 40 control samples. Expression was normalized to the endogenous control; the GAPDH gene. Significant differences were analyzed by student t-test, where: *p<0.01 compared to control.

Finally, by carefully analyzed the expression data from quantitative real-time PCR, this study indicated that EGF and EGFR gene expression levels in IBS patients were significantly downregulated as compared to control groups. This finding confirmed that the IBS has effectively associated with downregulation of both EGF and EGFR expression. These results suggest that these two genes play a critical role in the gastrointestinal tract and especially in IBS patients. Our results have shown the expression level of the EGF gene was downregulated and approximately 5-fold changed compared to the control group. In addition, the expression level of the EGFR gene was more downregulated and approximately 7-fold changed compared to the control group. This study is possibly the first that reports the impact of IBS disease on gene expression levels of EGF and EGFR. There are no previous studies about the expression levels of EGFR and EGF genes in IBS patients. However, few studies illustrate that the EGF and EGFR play a major role in the growth, maturation, and differentiation of the intestines [116].

5. CONCLUSIONS

One of the most common gastrointestinal disorders is irritable bowel syndrome (IBS). It is distinguished by changed bowel habits and abdominal pain with the absence of structural abnormalities. Because IBS is a complicated condition that affects millions of people throughout the world. This study aimed to assess the level of EGF and EGFR gene expression in IBS patients, as these two genes play an important role in the control of bowel movement by interacting with one another. The results of this research revealed that the expression levels of both EGF and EGFR genes are strongly associated with IBS diseases. There is an insufficiency of data on the expression levels of EGF and EGFR genes in IBS patients. The current study is likely the first to show that downregulation of EGF and EGFR genes is associated with IBS disease. The findings of this study may provide a different angle on the gene biomarkers for IBS diagnosis. This study contributes to the scientific field of utilizing gene expression levels as a biomarker for IBS. More study is required to better understand the molecular mechanism underlying IBS diseases.

RECOMMENDATIONS

Our findings could be valuable to other researchers who want to look at additional genes associated with IBS to see if they can uncover more efficient genes for different biological pathways. For additional research, who would be interested in looking at the genetic role in the clinical condition in IBS patients. We recommend that the investigator increase the sample size and include additional groups, such as other types of IBS disease and colon cancer groups. We believe that including these two groups will help the researcher detect the genetic problems and determine the gene expression biomarker more precisely.

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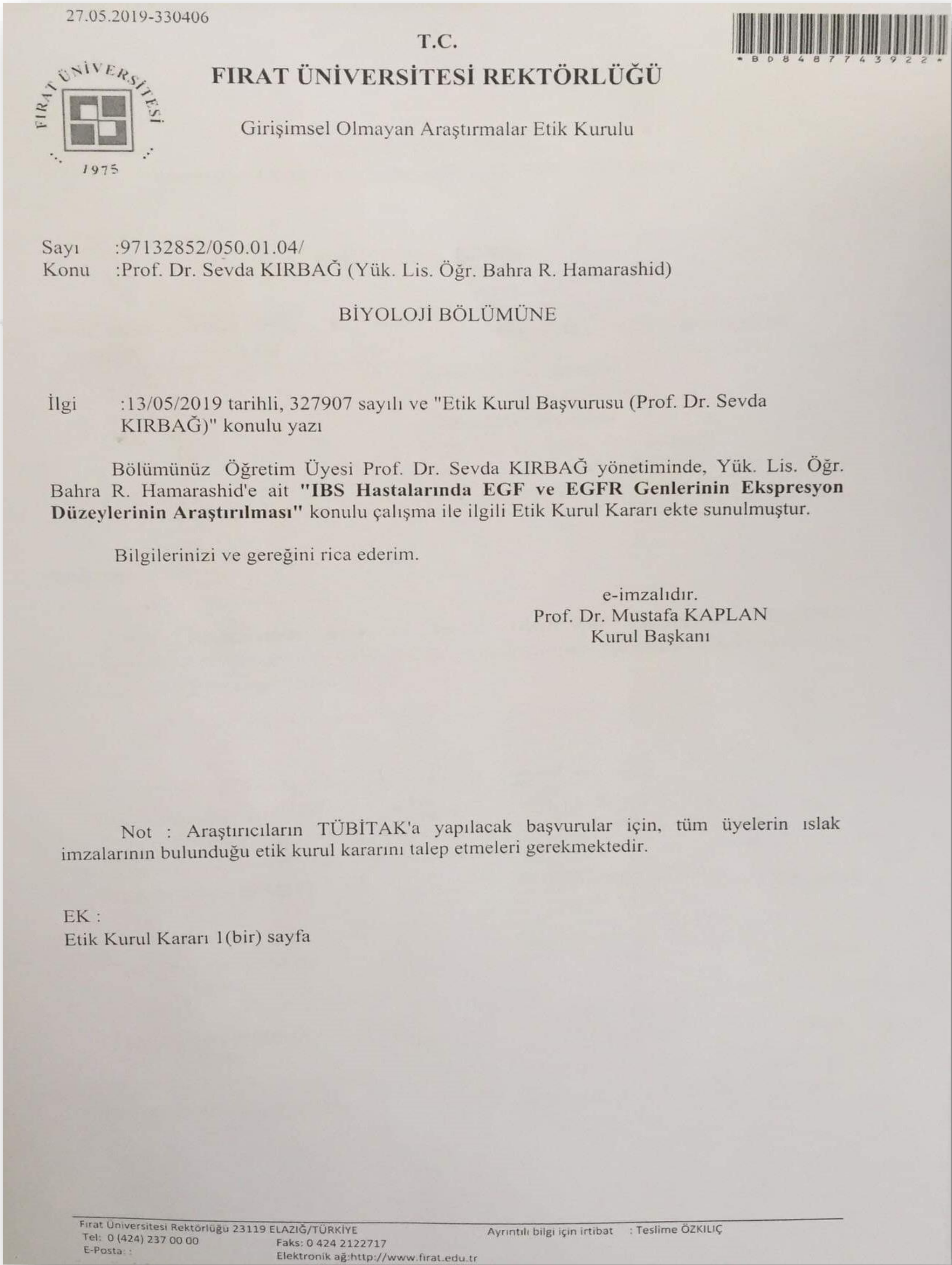
APPENDICES

APPENDIX- 1: ETHICAL COMMITTEE AND SOME EQUIPMENT



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T.C.

FIRAT ÜNİVERSİTESİ REKTÖRLÜĞÜ

Girişimsel Olmayan Araştırmalar Etik Kurulu

Sayı :97132852/050.01.04/
Konu :Prof. Dr. Sevda KIRBAĞ (Yük. Lis. Öğr. Bahra R. Hamarashid)

BİYOLOJİ BÖLÜMÜNE

İlgi :13/05/2019 tarihli, 327907 sayılı ve "Etik Kurul Başvurusu (Prof. Dr. Sevda KIRBAĞ)" konulu yazı

Bölümünüz Öğretim Üyesi Prof. Dr. Sevda KIRBAĞ yönetiminde, Yük. Lis. Öğr. Bahra R. Hamarashid'e ait **"IBS Hastalarında EGF ve EGFR Genlerinin Ekspresyon Düzeylerinin Araştırılması"** konulu çalışma ile ilgili Etik Kurul Kararı ekte sunulmuştur.

Bilgilerinizi ve gereğini rica ederim.

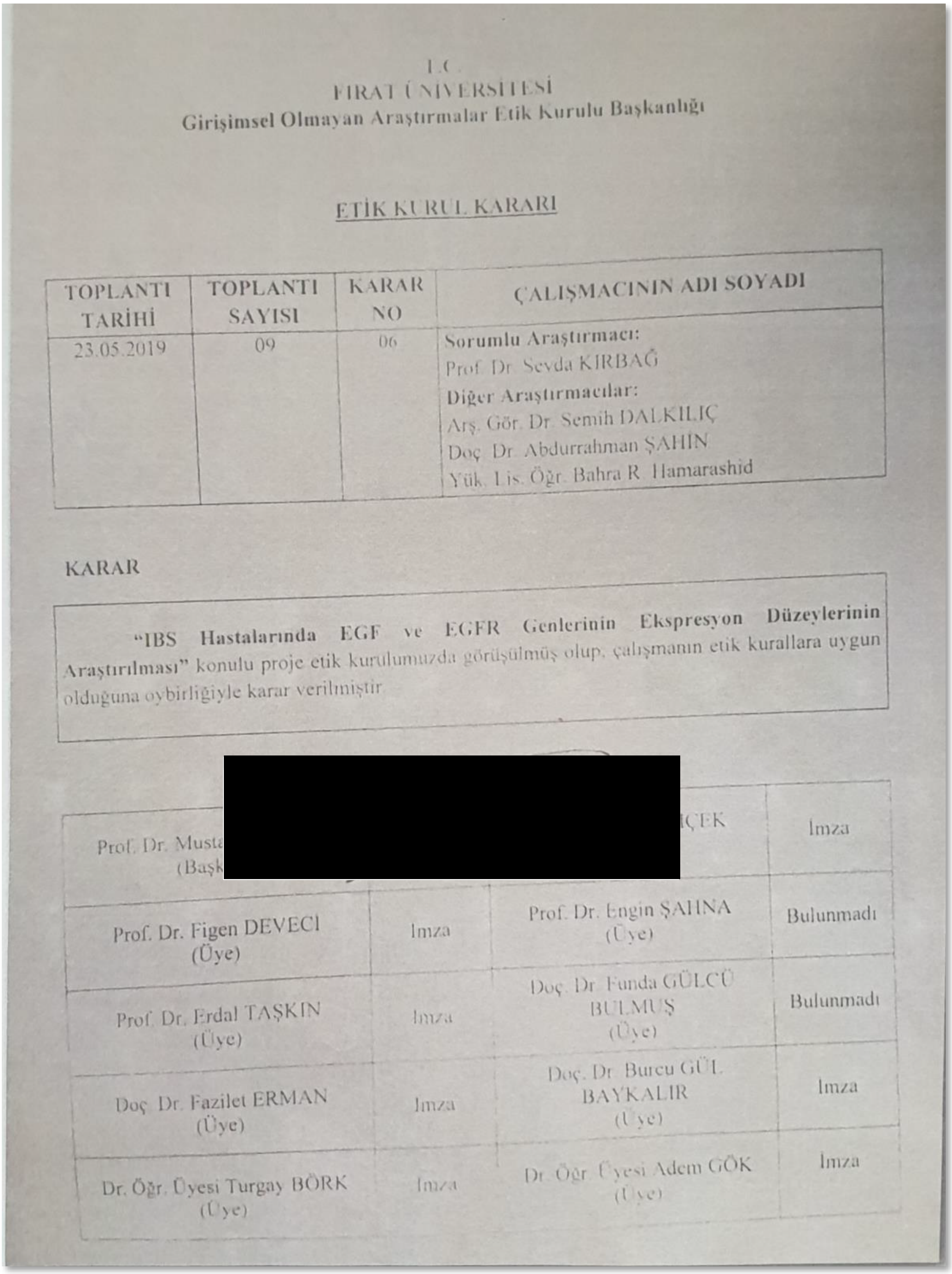
e-imzalıdır.
Prof. Dr. Mustafa KAPLAN
Kurul Başkanı

Not : Araştırıcıların TÜBİTAK'a yapılacak başvurular için, tüm üyelerin ıslak imzalarının bulunduğu etik kurul kararını talep etmeleri gerekmektedir.

EK :
Etik Kurul Kararı 1(bir) sayfa

Fırat Üniversitesi Rektörlüğü 23119 ELAZIĞ/TÜRKİYE Ayrıntılı bilgi için irtibat : Teslime ÖZKILIÇ
Tel: 0 (424) 237 00 00 Faks: 0 424 2122717
E-Posta: : Elektronik ağ:http://www.firat.edu.tr

Figure A1.1. Ethical Committee (approval of research ethics committee)



T.C.
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Girişimsel Olmayan Araştırmalar Etik Kurulu Başkanlığı

ETİK KURUL KARARI

TOPLANTI TARİHİ	TOPLANTI SAYISI	KARAR NO	ÇALIŞMACININ ADI SOYADI
23.05.2019	09	06	**Sorumlu Araştırmacı:** Prof. Dr. Sevda KIRBAĞ **Diğer Araştırmacılar:** Arş. Gör. Dr. Semih DALKILIÇ Doç. Dr. Abdurrahman ŞAHİN Yük. Lis. Öğr. Bahra R. Hamarashid

KARAR

"IBS Hastalarında EGF ve EGFR Genlerinin Ekspresyon Düzeylerinin Araştırılması" konulu proje etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oybirliğiyle karar verilmiştir.

Prof. Dr. Musta[redacted] (Başk[redacted]	[redacted]	[redacted]ÇEK	İmza
Prof. Dr. Figen DEVECİ (Üye)	İmza	Prof. Dr. Engin ŞAHNA (Üye)	Bulunmadı
Prof. Dr. Erdal TAŞKIN (Üye)	İmza	Doç. Dr. Funda GÜLCÜ BULMUŞ (Üye)	Bulunmadı
Doç. Dr. Fazilet ERMAN (Üye)	İmza	Doç. Dr. Burcu GÜL BAYKALIR (Üye)	İmza
Dr. Öğr. Üyesi Turgay BÖRK (Üye)	İmza	Dr. Öğr. Üyesi Adem GÖK (Üye)	İmza

Figure A1.2. Ethical Committee (approval of research ethics committee)

Evrak Tarih ve Sayısı: 28.02.2020-381433

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T.C.
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GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR
ETİK KURULU
GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR
ETİK KURULU KARARLARI

Oturum Tarihi	Oturum Saati	Oturum Sayısı
20/02/2020	13:30	2020/04-09

Girişimsel Olmayan Araştırmalar Etik Kurulu 20/02/2020 tarihinde saat 13:30'da Kurul Başkanı Prof. Dr. Mustafa KAPLAN başkanlığında, aşağıda imzaları bulunan kurul üyelerinin katılımlarıyla toplanarak gündemdeki konuları görüşmüş ve aşağıdaki kararları almıştır.

Sorumlu Araştırmacı : Prof. Dr. Sevda KIRBAĞ
Diğer Araştırmacılar : Arş. Gör. Dr. Semih DALKILIÇ, Yük. Lis. Öğr. Bahra R.Hamarashid

"IBS Hastalarında EGF ve EGFR Genlerinin Ekspresyon Düzeylerinin Araştırılması" başlıklı çalışma 23.05.2019 tarihinde 09/06 nolu kararla tarafımızca onaylanmıştır.

Sorumlu araştırmacının 19.02.2020 tarih ve 379420 sayılı dilekçesinde belirtildiği üzere; Doç. Dr. Abdurrahman ŞAHİN'in çalışmadan kendi isteği ile ayrılmak istemesi ve Prof. Dr. Mehmet YALNIZ'ın katılması isteğinin uygun olduğuna oybirliğiyle karar verilmiştir.

Kurul Üyeleri:
Prof. Dr. Mustafa KAPLAN, Prof. Dr. Demet ÇİÇEK, Prof. Dr. Engin ŞAHNA (Bulunmadı), Prof. Dr. Erdal TAŞKIN (Bulunmadı), Doç. Dr. Nevzat GÖZEL, Doç. Dr. Funda GÜLCÜ BULMUŞ, Doç. Dr. Fazilet ERMAN, Doç. Dr. Burcu GÜL BAYKALIR, Dr. Öğr. Üyesi Turgay BÖRK, Dr. Öğr. Üyesi Adem GÖK

E-İmzalıdır
Prof. Dr. Mustafa KAPLAN
Kurul Başkanı

Prof. Dr. Demet ÇİÇEK
Doç. Dr. Nevzat GÖZEL
Doç. Dr. Funda GÜLCÜ BULMUŞ
Doç. Dr. Fazilet ERMAN
Doç. Dr. Burcu GÜL BAYKALIR
Dr. Öğr. Üyesi Turgay BÖRK
Dr. Öğr. Üyesi Adem GÖK

Bu belge, güvenli elektronik imza ile imzalanmıştır.

Figure A1.3. Ethical Committee (approval of research ethics committee)

CURRICULUM VITAE

Bahra Radhaa HAMARASHID

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