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YEDITEPE UNIVERSITY
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“INVESTIGATION OF P53 GENE POLYMORPHISMS IN COLORECTAL CANCER”

DOCTOR OF PHILOSOPHY THESIS

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APPROVAL

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APPROVAL
This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 23.09.2021 and numbered .....

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

DEDICATION

I dedicate my thesis to my precious mother and sister.

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LIST OF SYMBOLS AND ABBREVIATIONS

CRC	Colorectal kanser
APC	Adenomatous polyposis coli
KRAS	Kirsten rat sarcoma virus
TP53	Tumor Protein P53
CIN	Chromosomal instability
MSI	Microsatellite instability
MMR	Mismatch repair
WT	Wild- type
HIV	Human Immunodeficiency Virus
FAP	Adenomatous Polyposis
CEA	Carcinoembryonic antigen
CT	Chemotherapy
FU	5-Fluorouracil
EGFR	Endothelial growth factor receptor
VEGF	Vascular endothelial growth factor
CIMP	CpG Islet Methylation Phenotype
GSK3	Glycogen synthase kinase 3
CpG	Guanine-cytosine content
DNB	DNA Nanoball
cPAS	Combinatorial probe anchor sequencing
HB	Hepatoblastoma
DLBCL	Diffuse Large B-Cell Lymphoma
SNP	Single nucleotide polymorphisms
LRC	Low rectal cancer

ABSTRACT

Ayhan H. Investigation p53 gene polymorphisms in colorectal cancer. Yeditepe University, Institute of Health Science, Department of Molecular Medicine. Doctorate Thesis. İstanbul, 2021.

Globally, colorectal cancer ranks third in incidence, and it's also the leading cause of cancer-related death. When colorectal polyps form, they are benign and do not cause cancer. However, they can evolve into advanced adenomas with high-grade dysplastic lesions and distant organ metastases. Oncogenes and tumor suppressor genes are believed to play a role in colorectal tumor progression. Genome-wide studies have identified APC, KRAS, SMAD4, and TP53 as genes that are often mutated in human colorectal tumors in 10 to 80 percent of non-hypermutated cases. The TP53 gene has been related to a poor prognosis in a range of malignancies, including colorectal tumors, according to new research. People with advanced colorectal cancer who had metastasized were found to have an 80 percent increase in TP53 mutations. However, This mutation is uncommon in benign colonic polyps, and the conversion rate to malignant polyps is 15-30 percent. It is believed that DNA damage and telomere erosion as well as hypoxia and malnutrition as well as oncogenic signals trigger the activation of p53. This results in cell cycle arrest and death, depending on the level and content of cellular stress During our research, we included six individuals with colorectal cancer in the study. Genotyping and polymorphism areas were determined when the patients' blood was isolated and DNA sequenced. Other than frequent polymorphisms, only three unusual polymorphic areas were found in Patient 3.

Key Words: Colorectal cancer, p53, polymorphisms, apoptosis

ABSTRACT (TURKISH)

Ayhan, HA. Kolorektal kanserde p53 gen polimorfizmlerinin araştırılması.Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Moleküler Tıp Anabilim Dalı. Doktora Tezi. İstanbul, 2021.

Kolorektal kanser, dünya çapında en fazla görülen üçüncü kanser ve kansere bağlı ölümlerin önde gelen nedenidir. Kolorektal kanser, yüksek dereceli displazi, invaziv adenokarsinom ve uzak organlara metastaz ile ilerlemiş adenoma olarak ilerleyen iyi huylu bir adenomatöz bağırsak polipi olarak başlar. Kolorektal kanserin böyle kademeli ilerlemesi kavramı, 'çok aşamalı tümörijenez' olarak adlandırılır ve her adımın, tümör baskılayıcılar veya onkogenlerdeki spesifik genetik değişikliklerle ilişkili olduğu düşünülmektedir. Son zamanlarda genom çapında yapılan araştırmalar, hipermutasyona uğramamış kolorektal kanserlerin yaklaşık %10-80'inde APC, KRAS, SMAD4 ve TP53 dahil olmak üzere insan kolorektal kanserlerinde sıklıkla mutasyona uğramış genleri tanımlamıştır. TP53 mutasyonları, kolorektal kanserler de dahil olmak üzere farklı kanser tiplerinde kötü prognoz ile ilişkilendirilmiştir. Tutarlı bir şekilde, metastaz geçiren ilerli kolorektal kanser hastaları incelendiğinde, TP53 mutasyon oranı %80'e kadar artmıştır. Diğer bir yandan ise , iyi huylu kolonik adenomatöz poliplerde TP53 mutasyonları pek yaygın değildir ve malign poliplere dönüşüm oranı yaklaşık %15-30'dur. DNA hasarı, telomer erozyonu, hipoksi ve açlık gibi hücresel stres ve ayrıca onkojenik sinyalleşme, p53'ü aktive ederek, stresin kapsamına ve içeriğine bağlı olarak hücre döngüsünün durması, yaşlanma ve apoptozun teşvik edilmesine yol açmaktadır. Çalışmamıza kolorektal kanserine sahip altı hasta dahil edilmiştir. Hastaların kanından DNA izolasyonu gerçekleştikten sonra DNA dizilemesi yapılarak hastaların polimorfik bölgeleri ve genotipleri belirlenmiştir. Diğer hastalar ile kıyaslandığında Hasta 3'te yaygın görülen polimorfizmler dışında 3 nadir polimorfik bölge tespit edilmiştir.

Anahtar Kelimeler: Kolorektal Kanser, p53, polymorphisms, apoptoz

1. INTRODUCTION AND PURPOSE

Colorectal cancer is one of the most common forms of cancer and accounts for most of the death from cancer-related causes in the globe (1,2). Fewer than 1 in 7 people who have metastases live for 5 years (3). To design a new type of treatment, it is vital to comprehend the ways that cancer spreads. Colorectal cancer starts as a benign adenomatous polyp, which gradually becomes more serious over time, becoming an aggressive adenocarcinoma that can spread to distant organs. Colorectal cancer is thought to develop in a number of stages, each characterized by the occurrence of distinct genetic changes (4-6).

Genome-wide studies have discovered genes that are often mutated in human colorectal cancers, such as APC, KRAS, SMAD4, and TP53, in approximately 10-80% of non-hypermutated colorectal tumors (7,8). Genetic alterations in these genes are expected to promote the acquisition of stemness, accelerated cell proliferation, suppression of differentiation, and impaired genomic DNA maintenance, which may contribute to 'cancer hallmarks' as significant components of cancer cell signaling pathways (9,10). Furthermore, the same genetic changes that alter these pathways are found in numerous cancer types, showing that they are critical driver pathways for oncogenesis regardless of tissue type (11).

There is a relationship between TP53 gene mutations and poor cancer prognoses in diseases such as colorectal cancer. (12). People with advanced colorectal cancer who had metastasized experienced up to an 80 percent rise in TP53 mutation rates when they were studied (13). Despite the low prevalence of TP53 mutations in benign colonic adenomatous polyps, they still convert to malignant polyps in roughly 15-30% of cases (14,15, 16). The discovery of TP53 mutations' importance in later carcinogenesis steps, such as when adenomas morph into invasive adenocarcinomas or metastasize to remote organs, backs up the assertion that these mutations are needed at those stages. DNA damage, telomere erosion, hypoxia, malnutrition, and oncogenic signaling, along with cellular stresses, such as excessive p53 activation, can cause cells to cycle arrest, become senescent, or even undergo apoptosis, depending on the level of stress that is applied (17,18). These stress responses are each handled by a different set of molecules that respond to different kinds of p53 activators, including p21, Puma, Tiger, and PAI-1 (19). TP53 mutations lead to the development of tumors in different organs, including as the

colon, and the mechanism via which this occurs is considered to be a lack of transcriptional activity by p53, resulting in a failure to activate its targets.

In my thesis, we aimed to determine the polymorphic changes in the TP53 gene in patients diagnosed with colorectal cancer and its malignant or benign effects as a result of the changes. Considering the vital functional properties of TP53, our study is planned to bring useful information to the literature.

2.GENERAL INFORMATION

2.1. Anatomy of the Colon

The large intestine, which stretches from the ileum to the anus, is around 1.5 to 2 meters long and makes up about one fifth of the digestive tract. On the surface and in terms of volume and structure, it's different from the small intestines. As a result of this,

1. The large intestine is larger in diameter than the small intestine.

2. The secondary retroperitoneum is the major part and adheres to its posterior wall.

3. In certain places, the longitudinal muscle fibers condense to form strips of Taenia coli.

The large intestine is made up of the cecum, the ascending colon, the transverse colon, the descending colon, the sigmoid colon, and the rectum. (20). The cecum is separated into three parts: the colon, the rectum, and the anus. The cecum is the first section of the large intestine and is located at the base of the stomach. It is almost 6 cm in length and 7.5–8.5 cm in diameter, making it the widest portion of the colon. It is also the most narrow. The colon is the longest and first section of the cecum, and it is located at the base of the stomach. The colon is divided into four sections: the ascending colon, the transverse colon, the descending colon, and the sigmoid colon (Figure 2.1). During digestion, the cecum connects the colon to the ileum of the small intestine, while it connects the colon to the rectum through the sigmoid colon (21)

2.2. The Role of the Large Intestine

The large intestine functionally mediates the absorption of water and electrolytes from the faeces, compresses the faeces for transfer to the rectum, and provides controlled evacuation of faeces from the body (22,23). In principle, the first parts perform the absorption, the last parts carry out the transmission and storage functions. Gram (-) anaerobic bacteria in the lumen of the large intestine form vitamins K, B1, B2 and B12, which are not produced in the human body (24).

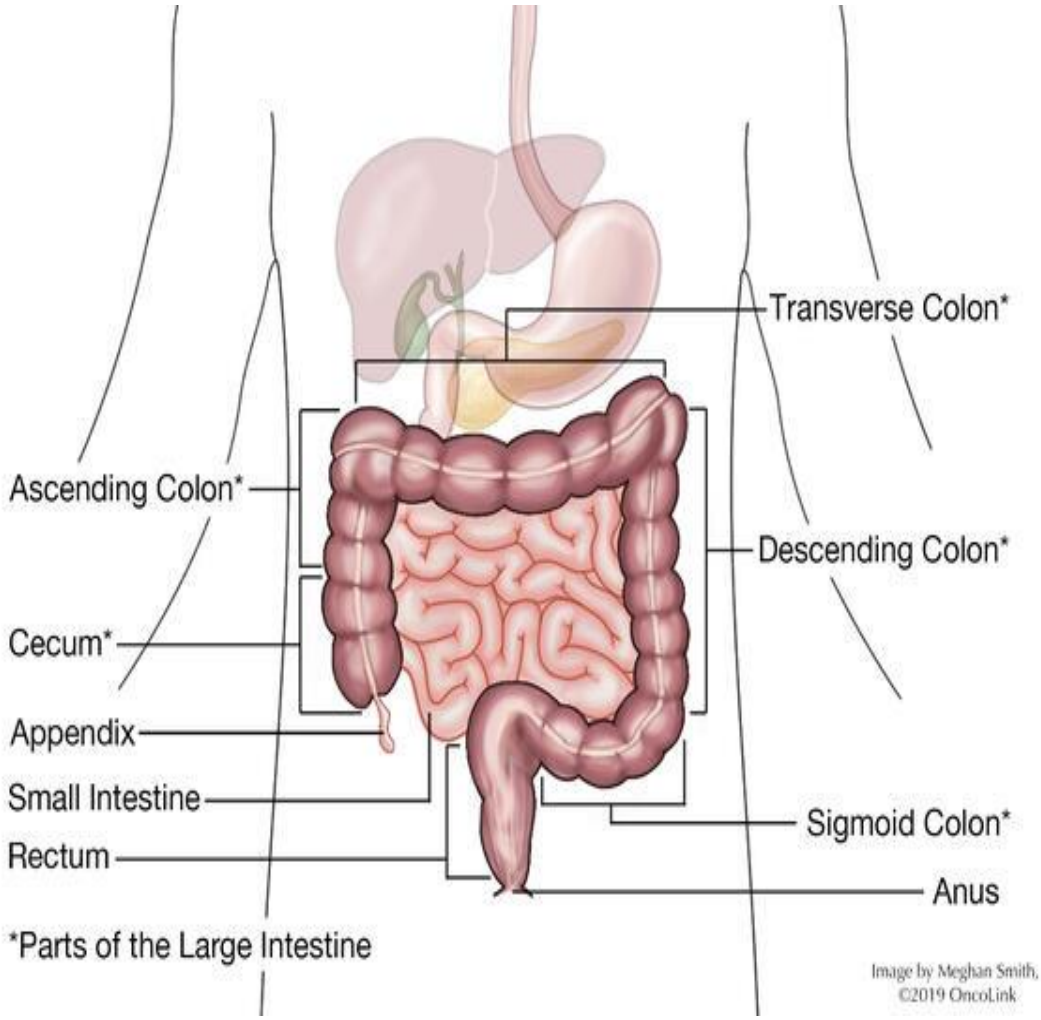

Figure.2.1. Anatomy of colon

2.3. Colorectal Cancer (CRC)

Colorectal cancer (CRC) is one of the types of cancer that starts in the gastrointestinal tract, the rectum or colon of the large intestine (25). CRC, which is a colon and rectum epithelial cancer, is formed as a result of accumulating genetic and epigenetic factoral changes in the cells (colonocytes) lining the colon and rectum. CRC includes a wide spectrum of neoplasms, from benign tumors to invasive cancers (26). Although existing cancers are thought to have both hereditary and acquired genetic components, CRCs are divided into 2 groups as familial (or hereditary) and sporadic (or non-hereditary) (27). Although there is a familial basis in 20% of CRC cases (28), the majority are sporadic and almost three-quarters of these patients have a negative family history (29).

Colorectal adenocarcinomas are genetically; tumors with chromosomal instability (CIN), which covers almost most of the tumors, and tumors with microsatellite instability

(MSI), which constitutes approximately 10-15% of tumors, are divided into two categories (30). Approximately 84% of sporadic CRC is mostly characterized by chromosomal instability. About 13-16 % of sporadic CRCs are hypermutated, resulting in MSI due to damaged DNA mismatch repair (MMR), which is frequently associated with wild-type (WT) TP53 and a near-picture of diploid chromosome instability. (31).

Histopathologically, 95% of CRC is adenocarcinoma (25). CRC begins with the tissue growth called polyp in the epithelial inner membrane of the colon and/or rectum and gradually grows through some or all of its layers (25). Adenomatous polyp, often known as an adenoma, is a benign growth that can become cancerous. This malignant transformation results from mutations and deletions of major regulatory genes. Approximately 95% of sporadic CRCs develop from adenomas (32).

2.3.1. Adenoma-Carcinoma Sequence

We know that colorectal cancer develops as a result of the accumulation of epigenetic and genetic alterations. As a result, normal glandular epithelial cells turn into benign neoplasms (Adenoma) and eventually develop into invasive carcinomas in later stages, as illustrated in the flow diagram (Figure 2.2). It has long been recognized that tubulovillous and tubular adenomas can develop into cancer, but there is now new evidence that serrated adenomas have the potential to develop into cancer. Alternatively, a fraction of hyperplastic polyps can proceed progressively to serrated adenomas and then carcinomas by an alternative carcinogenesis pathway (33-37).

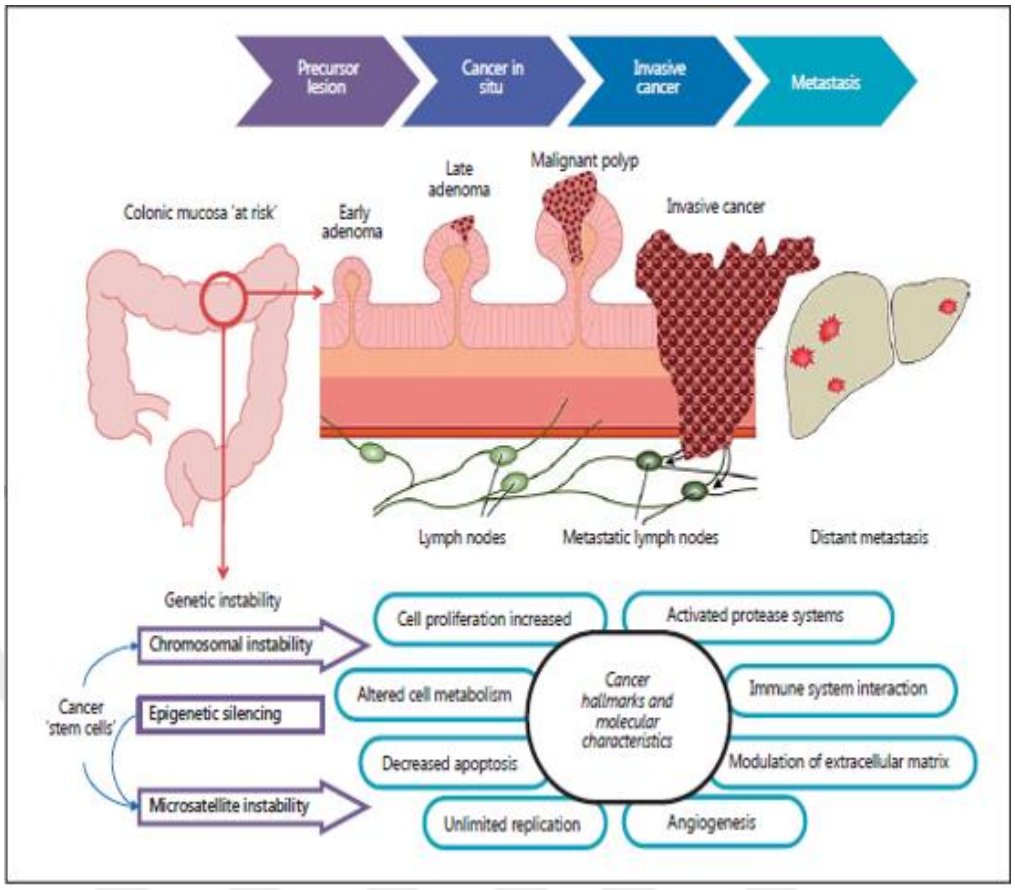

Figure 2.2.: Schematic depiction of the adenoma-carcinoma-metastasis process

2.3.2. Epidemiology of CRC

CRC is one of the leading causes of morbidity and mortality around the world, accounting for approximately 8% of all cancer-related deaths (38). With around 1.36 million new cases diagnosed each year and a death rate of more than 694,000 per year, colorectal cancer is one of the most frequent types of cancer in the world (39). With ongoing growth in emerging nations, the global incidence of colorectal cancer is anticipated to rise to 2.5 million new cases by 2035, according to the World Health Organization. (40,41). CRC is the fourth most common cancer in the world, following breast, prostate, and lung cancers. Males and females have a similar risk of having cancer, which is roughly 10-15 percent (42). Men and women are both at risk for colorectal cancer, and the fatality rates are identical (43). While colorectal cancer (CRC) is the third most common cancer in males after prostate and lung cancer, it is the second most common disease in women after breast cancer (42). The incidence of colorectal cancer has fallen to 45-50 years in the modern era. When compared to women, the progression of the disease accelerates with age in men.

It is estimated that approximately 25% of CRCs are located in the cecum and ascending colon, another 25% are found in the sigmoid colon, another 25% are found in the recto and another 25% are found in other areas of the large intestine (23). It is estimated that 40% of colorectal cancer (CRC) cases are discovered in the right colon (cecum), 31% in the left colon (splenic flexure, descending colon, and sigmoid colon), and 29% in the rectum (rectum is the area between the two intestines) (22). CRC is also one of the most frequent cancer kinds in developed countries, particularly in the United States (29).

For example, the incidence of colon cancer varies greatly between locations or people (27). It is more common in affluent countries such as Northern Europe, Australia and the United States while it is less common in various parts of Asia, Africa, and Latin America (44). CRC cases are greater than half the world's population in more developed countries (29). Dietary patterns and environmental factors are most likely to be responsible for these disparities in occurrence (27).

2.4. Colorectal Cancer Risk Factors

2.4.1. Nutrition and Diet

Diet has a significant role in the colorectal cancer development. In recent epidemiological and experimental studies, consumption of different foods and nutrients affects the risk of colorectal neoplasia (45). Calcium, milk, fiber and whole grains reduce the colorectal cancer risk, while red meat and processed meat increase this risk (46). Consumption of red meat and high-calorie foods, diet without antioxidant, antimutagen, and fiber content creates a risk of tumor formation (47,48). At the same time, nutrients and foods can influence the risk of colorectal cancer as a dietary pattern (49). It may also have a direct effect on inflammation and the immune system response. Therefore, dietary change is likely to reduce the incidence of colorectal cancer (50). Because of the fat in the diet; While cholesterol synthesis increases in the liver through bacteria in the colon, primary bile acids are converted to cholesterol metabolites and secondary bile acids, causing damage to the colonic mucosa (51). With the damage that occurs, protein kinase C and ornithine decarboxylase in the colon increase with the effect of secondary bile acids and prostaglandins are synthesized from arachidonic acid. Following these stages, cellular proliferation occurs and proliferating cells are sensitive to the effects of carcinogens (52).

2.4.2. Cigaret

Cigarette smoking has been linked to a higher incidence of CRC as well as a higher mortality rate (53). It has been demonstrated that beginning to smoke at a young age and continuing to smoke for a long period of time (30-40 years) increase the risk of colon cancer in people (54). Men (21 percent) and women (14 percent) have different risks of colorectal cancer associated with cigarette use (12 percent). The carcinogens found in tobacco smoke include polycyclic hydrocarbons, heterocyclic amines, and nitrosamines, to name a few (55).

2.4.3. Alcohol

There is no clear relationship between alcohol consumption and increased risk of CRC, but the decrease in folate absorption is thought to be the reason why alcohol increases the risk of developing cancer (53). Alcohol itself is not a carcinogen, but has been shown to promote tumorigenesis (56). Acetaldehyde is an important alcohol metabolite and its interaction with smoking can lead to specific mutations in DNA. Alcohol; may affect lipid peroxidation, prostaglandins and free oxygen radical production (57).

2.4.4. Physical Activity and Obesity

Consumption of greasy and fiber-deficient foods, as well as obesity, are significant risk factors for colorectal cancer (58). Inactivity and a sedentary lifestyle both raise the risk of CRC. It is more severe, particularly in men. In one investigation, it was revealed that physical activity had a lesser preventive impact against colorectal cancer in women (59).

2.4.5. Diabetes

A meta-analysis to demonstrate the link between CRC and diabetes showed that people with diabetes have a 30% higher risk of CRC than those without. Due to the slow intestinal regulation in people with diabetes, the fact that the intestinal mucosa is more exposed to irritants may be due (60).

2.4.6. Radiotherapy

Within 15 years, those who get radiation therapy for vaginal, cervical, or prostate cancer are at risk of acquiring rectal cancer (61).

2.4.7. HIV (Human Immunodeficiency Virus):

Some types of HIV pose the risk of cororectal neoplasia (62).

2.4.8. Age

CRC is most common in people between the ages of 20 and 49 in the United States, and it affects both sexes (63). With advancing age, the likelihood of acquiring colorectal cancer grows, and the likelihood of developing the disease is greater than 90 percent in those over the age of 50. People between the ages of 65 and 85 are at six times greater risk of developing this condition than people under the age of 50. (64). Racially and ethnically diverse populations have varying cancer incidence and mortality rates. When more in-depth investigations are conducted, it has been found that the incidence and mortality rate of colorectal cancer (CRC) in African-Americans is greater than in other groups in the United States between 2004 and 2008 (65).

2.4.9. Inflammatory Bowel Diseases

People with Crohn's disease or chronic ulcerative colitis tend to have a higher risk of colon cancer. The rate of cancer development, which is 3-8% in the initial period of the disease, increases to 10% after 10 years and to 30% after 25 years (66).

2.5. Familial Factors and Syndromes

2.5.1. Polyposis Coli (Familial Adenomatous Polyposis-FAP)

Polyposis coli, a hereditary disease, shows autosomal dominant inheritance. It develops from adenomas and occurs at a rate of 1% in CRC (67). These adenomas begin in childhood and their symptoms appear at about 16 years of age. The age of onset of colon cancer in 90% of clinically unfollowed patients is 45 years (68). It is known that the responsible genetic defect is the APC gene located at the q 21 locus on the 5th chromosome (69).

2.5.2. Hereditary Non-polyposis Colon Cancer (HNPKRC) Syndromes (Lynch syndrome I and II)

Around 1-5% of the population is affected by it, and while it is far more common than FAP, it also demonstrates that it has a tendency to be passed on from parent to child. HNPKRK is caused by mutations in mismatch repair genes, which include hMSH2, hMSH6, hMLH1, and PMS2. HNPCR is most often seen in the right colon, where the average age of the patient is 48 when they are diagnosed. Lynch I syndrome only shows up as colon cancer (70). Cancers of the stomach, breast, ovarian, uterus, small intestine, ureter, renal pelvis, and ileum often feature Lynch II syndrome (71)

2.6. Diagnosis

In the diagnosis of CRC, a tumor biopsy is performed by colonoscopy or rectosigmoidoscopy in the patient who presents with rectal bleeding, changes in defecation habits, a feeling of fullness in the abdomen, abdominal pain, signs of obstruction in the intestine, and signs and symptoms of iron deficiency anemia (72). The simple non-invasive diagnostic examination is the stool occult blood test. This test is done to show if there is hemoglobin in the stool. The presence of blood in the stool indicates bleeding in the gastrointestinal tract (73). Tumor markers are chemicals generated in response to a tumor by tumor cells or healthy cells. Tumor markers CA 19.9, CA 72.4 and especially CEA (carcinoembryonic antigen) are useful in the follow-up of the disease but cannot be used in diagnosis (74).

2.7. Treatment of Colorectal Cancer

Treatment planning related to many diseases, especially cancers, is carried out in a multidisciplinary approach. Treatment options for CRC are related to the location of the tumor (colon or rectum) as well as how far the tumor has spread (or how much it has invaded the intestinal wall and other tissues). Surgery is curative in CRC without metastatic spread. Surgical options can also be considered in the presence of liver-specific and isolated metastases, but only 20-25% of patients are suitable for surgery at the time of metastasis (75). In 10-30% of patients who cannot undergo surgery, the application of chemotherapeutic agents (neoadjuvant chemotherapy) aims to reduce the tumor burden and makes the patient suitable for surgery. There are two groups of chemotherapy (CT) used to achieve this:

1) Cytotoxic chemotherapy: Agents that target all rapidly dividing cells and block DNA synthesis of cells such as 5-Fluorouracil (FU), irinotecan, oxaliplatin, capecitabine (oral 5-FU analogue) are in this group..

2) Targeted agents (cytostatic therapy): Agents in this group act by binding to mechanistically dividing cells, specific receptors and proteins. Examples of these are cetuximab and panitumumab, which target EGFR (endothelial growth factor receptor), regorafenib, which is a tyrosine kinase inhibitor, and bevacizumab and aflibercept, which target VEGF (vascular endothelial growth factor). After surgery, cytotoxic agents are used alone or in combinations in chemotherapy regimens applied at various stages. Targeted agents bevacizumab and cetuximab can also be included in these single or combination uses (76).

2.8. Molecular Mechanism of Colorectal Cancer

Cancers that occur as a concequence of the cumulative accumulation of genetic and epigenetic changes occurring in the normal colonic mucosa are called CRCs. In these cancers, transformation of normal colonic epithelium into adenomatous polyp and then into invasive cancer is in question (77,78). Since it is one of the most prevalent cancers in the world, its molecular mechanism is one of the most researched cancers for diagnosis and treatment (79).

There are three different genetic pathways in the development of colorectal cancer. These;

1. Chromosomal Instability Pathway (CIN)
2. Microsatellite Instability Pathway (MSI)
3. CpG Islet Methylation Phenotype (CIMP)

It is known that CRCs mostly progress through the chromosomal instability pathway. In addition, 15-20% of CRCs develop via the microsatellite instability pathway, while others develop through the CpG islet methylation phenotype (80).

2.8.1. Chromosomal Instability Pathway (CIN)

First suggested by Fearon and Vogelstein in 1990, this pathway is also known as the adenoma-carcinoma process, and 70-80% of CRCs proceed through this pathway. On this road; Chromosomal rearrangements occur with the accumulation of a series of mutations that cause inactivation of tumor suppressor genes and activation of oncogenes (81,82).

Initially, a mutation in the APC gene, which is a tumor suppressor gene, results in the initiation of this process. Because this gene is responsible for the control of the Wnt/beta-catenin signaling system, it is possible that a mutation will result in the pathway being activated (83). It has been shown that activation of the Wnt/-catenin pathway, which has a significant role in cell proliferation, is a key element in the development of colorectal cancer (84,85). The Wnt/-catenin signaling pathway is activated when a stimulus from the outside of the cell is received by the cell skeleton, which then transmits the stimulus to the nucleus. When the Wnt/-catenin pathway does not receive a cell proliferation signal, the APC protein attaches to the glycogen synthase kinase 3 (GSK3) and the axin proteins, resulting in the formation of a cell-damaging complex.. A destructive complex (which prevents β-catenin from transcribing) stops β-catenin from attaching to ubiquitin, preventing it from moving to the nucleus. Mutation in the APC

gene results in a pathway disruption caused by the gene's two alleles. Because the β-catenin gene can't be removed, β-catenin stays in the cytoplasm, moving to the nucleus to start transcription, leading to the formation of an early adenoma. In patients with both the APC and K-ras gene mutations, polyps in the colon epithelium will expand over time, passing the intermediate adenoma stage as they do. At a later point, a later stage of adenoma is attained with the loss of 18q. Mutations in both p53 alleles led to the cancerous tumor (85-88).

2.8.2. Microsatellite Instability (MSI)

Microsatellites are repeated short nucleotide sequences found throughout the genome. This twenty percent of CRCs are caused by the mutator intermediate pathway, a mutator which occurs due to mutations in DNA mismatch genes. When mutations in DNA mismatch genes lead to an increase or decrease in DNA replication, microsatellite instability is the result, with more or fewer microsatellites being created (81,89). DNA polymerase uses its 3'-5' exonuclease activity to fix mismatches during DNA replication. The DNA mismatch repair system (MMR) stabilizes the genome by fixing the sections ignored by the repair mechanism. Mutations in MMR genes will prevent the repair from occurring, causing MSI (81). Seven common MMR genes are recognized; hMLH1, hMLH3, hMSH2, hMSH3, hMSH6, hPMS1 and hPMS2 (81, 90). MSI, which affects around 20% of CRCs, has been found in around 15% of them. MSI is a genetic mutation seen in nearly all hereditary colorectal cancer tumors, and is found in nearly all instances. Nearly two-thirds of HNPCC cases are shown to have mutations in the MMR genes' MSH1 and MSH2. The individuals were found to have additional hMSH6, hPMS1, and hPMS2 mutations, even though these mutations were less frequent (91)

2.8.3. CpG Islet Methylation Phenotype (CIMP)

The most prevalent epigenetic changes are DNA methylation and histone modifications occur in gene expression and function without causing a difference in the DNA sequence. DNA methylation occurs mostly in regions with high guanine-cytosine (CpG) content. The methylation of these areas, which are mostly located in the gene’s promoter region, causes that gene to be silenced. Excessive methylation in the promoters of tumor suppressor genes causes cancer development. CpG islet methylation phenotype is observed in 10-15% of CRCs (79,92).

2.9. P53 GENE

The p53 gene was first described as a nuclear phosphoprotein in 1979 by Lane and Crawford, followed by Linzer and Levine. David Lane, however, pointed to the p53 gene in human tumors by immunohistochemical methods (93-95). The p53 gene is a tumor suppressor gene located at position 17p13-1 on the short arm of the 17th chromosome and is nuclear stained immunohistochemically. The P53 gene, which is 16.20 kb long, consists of 11 exons. The DNA part in exons 2 and 11 is 8-10 kb long and cannot encode (96). There are two types of the p53 gene; 1) Wild type p53 is the normal functional type. It is short-lived, lasting about 6-20 minutes. It is difficult to detect in tissues. 2) The other type is the mutant type, with a lifespan of 3-6 hours and a longer lifespan than the wild type. Easily demonstrated in tissues immunohistochemically

Mutation of this gene, which encodes a 363 amino acid protein called tumor protein 53, is seen in approximately 50% of all cancers worldwide (97,98). The p53 protein consists of four parts;

1. Transcription activation site
2. DNA binding site
3. Oligomerization site
4. Controlling site

p53's DNA-binding region is frequently mutated in cancer instances. In addition to losing tumor-suppressing characteristics, some of these mutations also display carcinogenic tendencies in the p53 protein (99). In 75 percent of CRC cases, the p53 gene has been found to be dysfunctional. In colorectal adenomas, p53 function is lost in 44.9 percent of cases; in multiple CRCs, it's lost in 43.7 percent of cases (100).

The p53 gene is the most commonly altered gene in human malignancies (101,102). At first, it was believed that p53 was solely an oncogene, but it was later determined that only its mutant version plays any role in aberrant cell proliferation whereas the normal form of p53 prevents tumor formation. Numerous mesenchymal, neuronal and lymphoid malignancies have been shown to have homozygous deletion of the p53 gene (101-103).

First, Volgelstein showed that the p53 gene was mutated on chromosome 17 in colorectal cancers (104,105). As a result of various mutations, one of the two alleles of the p53 gene expressed in normal cells is lost. P53 gene loss occurs by point mutation, deletion, rearrangement, insertion. The largest mutation observed in the p53 gene is the

point mutation. Gene mutations develop especially between 100-300 amino acid regions, which are specific binding sites to DNA (105).

The most important feature that makes the p53 gene different from other tumor suppressor genes is that inactivation of a single allele is sufficient for the loss of its tumor suppressor function. This is because some mutant p53 proteins bind with the cellular heat shcok protein “hsc 70”, and the normal p53 protein binds to them, forming complexes with a long half-life that do not function. As a result, the mutant p53 protein inactivates the normal p53 protein.

A cell carrying a mutant and a normal allele behaves as if it has no functional p53 protein. This type of mutation is called "dominant negative". This is because the mutant allele acts dominantly and inhibits the function of the normal allele (105). The half-life of p53 protein with conformational change as a result of point mutation is extended up to 3-6 hours, and therefore, unlike normal p53, it is detected immunohistochemically (104-106). P53 is found at basal level and inactive in the cell under normal conditions. Under physiological conditions, its lifespan is very short, such as 20 minutes, and it undergoes proteolysis via ubiquitin. It does not interfere with cell division or the survival of the cell (107-108). Various stress conditions rapidly activate p53. Examples of p53 activating signals are hypoxia, DNA damage, dysregulated growth signals, oncogenes, heat shock, and exposure to nitric oxide. In particular, p53 can react rapidly to DNA damage and activate mechanisms that will enable DNA repair. P53 has taken the name of "guardian of the genome" because it ensures that the DNA and the information contained in the genome in general are preserved as they are. Under stress conditions, the amount of p53 increases rapidly and p53 becomes active. Increasing the amount of p53 is achieved both by increasing its half-life and by translating protein from a high rate of p53 mRNA.

2 main effects of the activity of p53:

1-Blocking the cell cycle and stopping the growth

2-Apoptosis

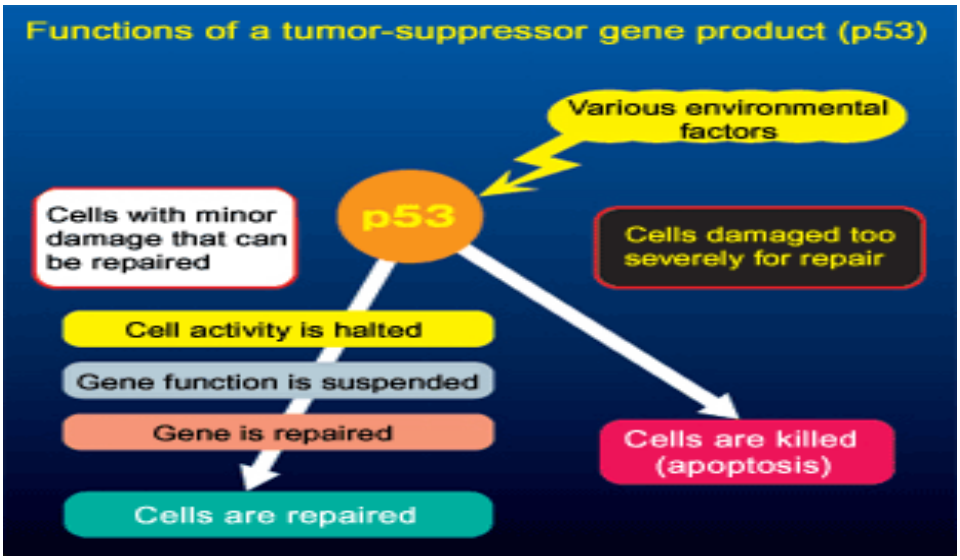

Figure 2.3. Functions of tumor-suppressor gene product p53

The main mechanism by which p53 acts is the process that controls the transition from G1 to S phase during cell division. After all; P53 senses DNA damage by unknown mechanisms and performs DNA repair by blocking the G1 stage of the cell cycle and activating DNA repair genes. In other words, it ensures that the cell cycle stops at the G1 stage. It activates DNA repair genes, so that the damage occurred at the time of creation is repaired and allows the cycle to continue from the stage in which it was left (109).

A cell with damaged DNA and unable to repair it is sent to apoptosis by P53. Because of this activity, P53 is called the "genome guard". In the case of homozygous loss of p53, damaged DNA cannot be repaired and mutations are fixed within the cell. As a result, the cell enters a one-way path towards malignant transformation. An inhibitory role of p53 in tumorigenesis was later identified. Besides DNA damage, hypoxia can also activate p53. Tumor angiogenesis is critical for the growth of tumor cells. Tumor cells undergoing hypoxia go to apoptosis if they have normal p53 genes. But if p53 genes are mutated then hypoxic tumor cells become resistant to apoptosis (110).

3. MATERIALS AND METHODS

3.1. Genomic DNA isolation from blood

Venous blood samples taken from the patient in Medical Park Goztepe in 5 cc EDTA tubes were kept in the refrigerator at +4C° until DNA isolation. DNA isolation from samples was obtained using iPrep DNA extraction robot (Invitrogen) and iPrep genomic DNA isolation kit from blood (iPrep gDNA Blood kit, Invitrogen, Thermo Fisher Scientific Inc). This system is capable of isolating DNA from 13 samples in one run. DNA was isolated by using 350µl of peripheral blood for each sample in the robotic system called magnetic bead-based technology depending on the surface charge over the pH of the buffer in the environment. At low pH, the positively charged CST® (ChargeSwitchTechnology®) binds to the negatively charged nucleic acid backbone. Therefore, proteins and other contaminants cannot bind and are washed with liquid wash buffer. To clean nucleic acids; The charge of the bead surface is neutralized by raising the pH to 8.5 using a low salt elution buffer. The isolated nucleic acid passes into the wash buffer without delay and is ready to be used in studies. After the isolated DNA samples were taken into appropriate tubes, they were stored in a +4C° refrigerator (110).

3.2. DNA Purity Measurement

It was determined that 50ng/µl (µg/ml) double-stranded DNA content gave 1 optical density (OD) at 260 nm wavelength. The purity of DNA samples was measured with the Nanodrop2000 instrument. The purity of DNA samples was determined by analyzing the OD260/OD280 ratio. Samples with an OD260/OD280 value between 1.7 and 1.9 of DNA, which were accepted as suitable for genotyping, were accepted as clean (111).

Spectrophotometer (NanoDrop 2000, Thermo Scientific Inc) device;

1) After the computer to which the Nanodrop is connected is turned on, the spectrophotometer program is started.

2) When the program is opened, the DNA analysis section is selected from the table on the screen to measure the DNA in the sample.

3) Routine wavelength verification test is done and after the process is finished, it is ensured that there is no contaminant in the Nanodrop sensor.

4) The Nanodrop lever is opened and the sensor is cleaned. After the procedure is finished, the arm is slowly closed.

5) Before measuring the concentrations of the samples, a blank is taken. For this, the Nanodrop arm is lifted and 1.5 µl of the blank solution is placed on the Nanodrop sensor.

6) After the blank solution is loaded, the arm is lowered and the blank section at the top left of the computer program is selected.

7) After the process is completed, the Nanodrop sensor is cleaned and 1.5 µl of sample is added to the Nanodrop sensor and the arm is closed.

8) DNA concentration is analyzed by selecting the measure tab in the upper left part of the computer program.

9) Before closing the program, the blank is taken and the sensor is thoroughly cleaned.

3.3. Library Construction Protocol

3.3.1.Reagent Preperation

3.3.1.1. First, we prepared 1x Elute Enhancer at room temperature in a sterile centrifuge tube according to Table 3.1. Also, 1x Elute Enhancer has a shelf life of 7 days.

Table 3.1. 1x Elute Enhancer Preparation

Componenets	**Volume**
20x Elute Enhancer	1µL
Nuclease Free Water	19 µL
Total	20µL

3.3.1.2. We prepared the En-TE buffer in a sterile centrifuge tube according to Table 3.2 as in the preparation of the Elute Enhacer.

Table 3.2. En-TE buffer preparation

Componenets	Volume
1x Elute Enhancer	2μL
1x TE Buffer	998 μL
Total	1000μL

3.3.1.3. We prepared En-Beads according to Table 3.3 in a sterile microfuge tube.

Table 3.3 Preparation of En-Beads

Componenets	Volume
1x Elute Enhancer	15μL
DNA Clean Beads	1485 μL
Total	1500μL

3.3.2. Magnetic Beads and Cleaning Procedures

We used DNA Clean Beads, which were included in the MGIEasy DNA Clean Beads Kit, to purify DNA from one bead to another using a magnetic field (MGI, Cat. No. 1000005278).

1) We took the DNA Clean Beads out of the refrigerator and let them sit at room temperature for 30 minutes before use. We mixed thoroughly by vortexing before use.

2) We transferred 45 μL of En-Beads to the tube containing Fragmentation Products. We pipet it up and down 10 times to mix well and make sure all liquid and beads are completely flushed from the pipette tip into the tube before continuing.

3) Incubated for 10 minutes at room temperature

4) Following a 2-5 minute wait to allow the liquid to clear in a Magnetic Separation Rack, the liquid is briefly centrifuged and placed back in the rack to achieve clarity. Once the supernatant was obtained, we gently poured it into a new PCR tube.

5) We transferred 15 μL of En-Beads to the tube from step 4 containing 120 μL of supernatant. We mixed it well by vortexing to mix it well.

6) We then incubated at room temperature for 10 minutes.

7) After brief centrifugation, we placed the tube in a Magnetic Separation Rack for more than 5 minutes until the liquid was clear. We discarded the supernatant by carefully removing it with a pipette.

8) A freshly produced ethanol solution of 80 percent was added to the tube while it was still on the Magnetic Separation Rack. Following two inversions on our magnetic platform, we gently collected the supernatant and threw it away.

9) We ran through Step 8 again and attempted to drain the liquid from the tube. We ran a few quick centrifugations, then used a magnetic separation to get rid of the excess liquid and to capture the substance's pure fluid under it..

10) In order to dry the beads, we placed the tube in a Magnetic Separation Rack and left the top open.

11) The tube was removed from the Magnetic Separation Rack and 45 L of En-TE Buffer was added to separate the DNA and vortex well before reattaching it.

12) For the next five minutes, we incubated it at ambient temperature.

13) Having completed a brief centrifugation, we returned the tube to the Magnetic Separation Rack for another 5 minutes, or until the liquid was clear. We transferred 43 µL of the supernatant to a fresh 0.2 mL PCR tube and set the tube aside.

14) We then measured the purified products with the Qubit® dsDNA HS Assay Kit.

3.3.3. End Repair and A-tailing

3.3.3.1. We transferred the appropriate amount of sample (80-200 ng) to a new 0.2 mL PCR tube and added En-TE Buffer to a total volume of 40 µL. Table 3.4. We prepared End Repair and A-tailing Reaction Mixture on ice as in

Table 3.4. End Repair and A-tailing Reaction Mixture

Componenets	Volume
ER Buffer	7µL
ER Enzyme Mix	3 µL
Total	10µL

3.3.3.3. We added the contents of a 0.2 mL PCR tube that was filled with 10 µL of the End Repair and A-tailing Reaction Mixture that we had created in step 3.3.3.1. We were done in under a minute after doing three rounds of about three seconds each with some short centrifugation. We then used a PCR tube to follow the procedure outlined in Table 3.5. We started with a 0.2 mL PCR tube, which was placed in the thermocycler, and conducted the reaction until the final volume was 50 µL. We centrifuged the samples once the allotted time had passed.

Table 3.5. End Repair and A-tailing Reaction Conditions

Temperature	Time
Heated Lid (70°C)	On
14°C	15 min
37°C	25 min
65°C	15 min
4°C	Hold

3.3.4.Adapter Ligation

3.3.4.1. MGIEasy PF Adapters-16 (Tube) Kit Instruction

Adapters should be used in certain batches on the basis of balancing the base composition. To use the adapters in the appropriate combination, we followed the steps below:

Kit includes 16 Adapters divided into 3 sets

• 2 sets of 4 Adapters: (01-04) and (13-16)

• 1 set of 8 Adapters: (97-104)

Assuming that the data output requirement is the same for each sample on a given strip, we used the table below to organize your Barcode Adapter combinations (Table 3.6.)

Table 3.6. Barcode Adapter combinations

Samples/Lane	Instruction
1	A minimum of one set of adapters is required. We then placed two Adapters together (adapters 01-02 and 03-04) and included both of them into the sample.
2	It is necessary to have at least one set of Adapters: We used four Adapters (01-04), mixed them in equal amounts in pairs, and got two mixtures of identical volume as a result. Each sample received 1 combination, which we divided equally. (For example, we combined numbers 01 and 02 and then added them to example 1; we combined numbers 03 and 04 and then added them to example 2)
3	Adapters are required for at least two sets of Adapters For instances 1 and 2, we used the two-sample-per-strip approach. When it came to sample 3, we followed the same procedure as before (1 sample/strip). It was necessary to utilize a different set of adapters for Examples 1,2 and 3.
4	A minimum of one set of Adapters is required. 4 Adapters (01-04) were used, with each sample receiving one Adapter. The Adapters 01, 02, 03, and 04 were added to Example 1, 2, 3, and 4.
5	It is necessary to have at least two adapter sets. For samples 1-4, we used the same procedure as described above (4 samples per strip). For example 5, we utilized the one sample/strip method described above. Adapter sets for Examples 1-4 and 5 were created using separate adapters.
6	It is necessary to have at least two adapter sets: For samples 1-4, we used the same procedure as described above (4 samples per strip). As an example, we utilized the following

	procedure (2 samples per strip) for samples 5 and 6. For Examples 1-4 and 5-6, we used distinct Adapter sets from one another.
7	That means you'll need all 3 adapter sets. We utilized the four-sample-per-strip approach for samples 1 to 4. First adapter set (used) We utilized the two-sample-per-strip technique for instances 5 and 6. There were two adapter sets in use: On sample 7, we used the same procedure as before (1 sample per strip). (We utilized the third pair of adapters)
8	Adapters are a must for a single set. We used 8 standard adapters (97-104), mixing a single standard adapter with each sample.

As soon as the centrifuge had stopped, we added five microliters of the MGIEasy PF Adapters solution. To collect the mixture, we vortexed 3 times for 3 seconds each, followed by a quick centrifugation. Adapter Ligation mixture was then made on ice (Table 3.7.)

Table 3.7. Adapter Ligation Reaction Mixture

Components	**Volume**
Ad-Lig Buffer	18 µL
Ad Ligase	5 µL
Ligation enhancer	2 µL
Total	25 µL

Since Ad-Lig Buffer is very viscous, we mixed it well before use. We slowly transferred 25 µL of Adapter Ligation Reaction Mix into the 0.2 mL PCR tube obtained from step 3.3.1. We vortexed 6 times and centrifuged briefly to collect the mixture at the bottom of the tube. Then the thermocycle was adjusted according to the conditions specified in table 3.8 and the final volume was 80 µL. After the desired time, we performed a short centrifugation. We added 20 µL of En-TE Buffer so that the total volume was 100 µL.

Table 3.8. Adapter Ligation Reaction Conditions

Temperature	Tıme
Heated Lid (30°C)	On
25°C	30 min
4°C	Hold

3.3.5. Cleanup of Adapter-ligated DNA

Prior to usage, we removed the En-Beads from the refrigerator and left them to sit at room temperature for 30 minutes before vortexing them to ensure that they were thoroughly mixed before use. 3.4.2 En-Beads 50 L into the tube from step 3.3.7 and pipet at least 10 times to mix well. Before to continuing, we ensured that all liquid and beads had been flushed from the pipette tip into the tube. We incubated at room temperature for 10 minutes. A quick centrifugation was followed by 2-5 minutes in a Magnetic Separation Rack to clear the liquid phase. Using pipettes, we discarded the supernatant. So, we added 160 L of freshly made 80 percent ethanol to wash the bead and the tube walls while the tube was still on the Magnetic Separation Rack (MSR). The 0.2 mL PCR tube would be inverted twice on the magnetic platform, and the supernatant would be gently removed. Our beads were air-dried at room temperature in a tube on the Magnetic Separation Rack with the top left open. En-TE Buffer and 50 L En-TE Buffer were used to separate the DNA from the tube, and the mixture was pipingd up and down at least 10 times. We incubated at room temperature for 5 minutes. This was followed by a quick centrifugation, followed by a 2-5 minute soak in the Magnetic Separation Rack. Using a new 0.2 mL PCR tube, we put the supernatant of 49 L into it. Using Qubit, we took 1 L of the supernatant and tested the concentration. Adapter-ligated DNA was kept at -20°C after cleanup.

3.3.6. Denaturation

Using a thermocycler, we inserted the mixture in the 0.2 mL PCR tube, followed by a 50 µL reaction volume in the table 3.9. We executed the following software. This was followed by some short-term centrifugation

Table 3.9. Denaturation Reaction Conditions

Temperature	**Tıme**
Heated Lid (30°C)	On
95°C	3min
4°C	10

3.3.7. Single Strand Circularization

We were required to keep the Circularization Reaction Mixture iced, as per protocol (Table 3.10.). We took our PCR tube and loaded it with 12 µL of the Circularization Reaction Mix. We were done in under a minute after doing three rounds of about three seconds each with some short centrifugation. The next step was to use table 3.11 and to load the PCR tube with a 60-µL total reaction volume. We quickly followed this by chilling the PCR tube and moving on to the next step.

Table 3.10. Circularization Reaction Mixture

Components	**Volume**
Cir Buffer	11.5 µL
Cir Enzyme Mix	0.5 µL
Total	12 µL

Table 3.11. Circularization Reaction Conditions

Temperature	**Tıme**
Heated Lid (42°C)	On
37°C	30 min
4°C	Hold

3.3.8. Exo Digestion

While on Thermocycle, we prepared Exo Digestion Reaction Mixture on ice. (Table 3.12)

Table 3.12. Exo Digestion Reaction Mixture

Components	Volume
Exo Buffer	1.4 µL
Exo Enzyme Mix	2.6 µL
Total	4 µL

The Exo Digestion Reaction Mix was poured into the PCR tube from previous steps, with 4 µL going in. We used a vortex machine to do a 3-second rotation three times to collect the fluid combination at the bottom of the tube. We started by putting the liquid in the PCR tube and placing it in the thermocycler. Then, we ran the 64 µL reaction using the information in Table 3.13. We ran a high-speed centrifuge. In order to keep our PCR running smoothly, we have included 3 µL of Exo Stop Buffer in our PCR tube. The mixture was gathered from the bottom of the tube after we performed 3 3-second vortex rotations and a brief centrifugation.

Table 3.13. Exo Digestive Reaction Conditions

Temperature	Time
Heated Lid (42°C)	On
37°C	30 min
4°C	Hold

3.3.9. Cleanup of Exo Digestion Product

We set the En-Beads out at room temperature for 30 minutes after removing them from the refrigerator. We vortexed it well to ensure even distribution. In order to begin the Exo Digestion procedure, we injected 120 µL of En-Beads into the product. We make careful to mix everything well by alternating gentle up-and-down pipetting 10 times to remove solution and beads from the tip of the pipette and then into the tube. For 10 minutes, we let it sit at room temperature. We centrifuged the tube, and then put it in a magnetic rack for two to five minutes to allow the liquid to settle. Using a pipette, we got rid of the supernatant.

We then poured in 160 µL of freshly made 80% ethanol to flush the beadler and the inside of the tube, which we kept in the Magnetic Separation Rack. Once we turned the 0.2 mL PCR tube upside down on the magnetic platform twice, we retrieved the supernatant, removed it, and cleaned the tube out. We had our Magnetic Separation Rack open, and we put our PCR tube in it and waited for the beads to dry, which took about an hour. We took the tube from the Magnetic Separation Rack and added 25 µL of En-TE Buffer to wash the DNA. This was done to rinse off the DNA after Magnetic Separation. We incubated the solution for 10 minutes at room temperature after mixing by gently pipetting it up and down at least 10 times. We held the tube on the Magnetic Separation rack for 2-5 minutes after centrifuging until the liquid cleared. To spin down the culture, we moved 24 µL of liquid to a 1.5 mL tube. Stored at -20°C, purified digestion products are easy to find. The Qubit ssDNA Assay Kit was used to determine the levels of purified Exo Digestion Products.

3.4. Sequencing

After patient samples were ready for sequencing, DNA sequencing was performed using the DNBSEQ-G400RS High-throughput (Rapid) Sequencing device. Then the genotypes of the patients were determined.This sequencing set utilizes DNBSEQTM technology. A sequencing run starts with the hybridization of a DNA anchor, then a fluorescent probe is attached to the DNA Nanoball (DNB) using combinatorial probe anchor sequencing (cPAS) chemistry. Finally, the high-resolution imaging system captures the fluorescent signal. After digital processing of the optical signal, the sequencer generates high quality and high accuracy sequencing information.

4.RESULTS

Six patients diagnosed with colorectal cancer were included in our study, and the TP53 gene, a tumor suppressor gene, was sequenced using the next generation sequencing method. Comprehensive demografik results of 6 colorectal cancer patients and can be seen in Table 4.1. The rs1042522 polymorphism occurred in the exon, and all the remaining polymorphisms occurred in different introns. In addition, in the third patient, the polymorphism defined by rs191918079 occurred in the non-coding region of the 3 ends of the gene and its effect was determined as benign.

Table 4.1.Demographic results of working groups

	Average	**Patient 1**	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**	**Patient 6**
Gender (Male/Female)	%66.6/ %33.3 (n:4/n :2)	Female	Female	Male	Male	Male	Male
Age (year)	50,6	39	62	42	54	46	61
Smoking (Active / None)	%33.3/ %66.6 (n:2/n :4)	Active	None	None	Active	None	Active
Family History (Yes/No)	%50/%50 (n:3/n :3)	No	Yes	No	Yes	Yes	No
Survival (Survive / Mortality)	%16.66/ %83.3 (n:1/n :5)	Ex	Survive	Survive	Survive	Survive	Survive

4.1. Sequence alignment map for patient

4.1.1. Sequence alignment map for patient 1

The polymorphic regions of the patient 1 and their characteristics are shown in Table 4.2. Also, the sequence alignment map of the first patient is shown in figure 4.1.1-1 As can be seen, in the first patient, homozygous TT genotype defined by rs1625895, heterozygous CG genotype defined by rs1042522, heterozygous CG genotype defined by rs1642785, homozygous TT genotype defined by rs2909430 and deletion in the polymorphic region defined by rs59758982 were observed. The effects of the changes in the 5 polymorphic regions of the patient were found to be benign.

Table 4.2. Polymorphic regions and features of the first patient

ID	Genotyping	Effect	Region	Nucleotide Taransition
rs1625895	Homozygote	Benign	Intron 6	C/T
rs1042522	Heterozygote	Benign	Exone 4	C/G
rs1642785	Heterozygote	Benign	Intron 2	C/G
rs2909430	Homozygote	Benign	Intron 4	C/T
rs59758982	Heterozygote	Benign	Intron 3	Deletion

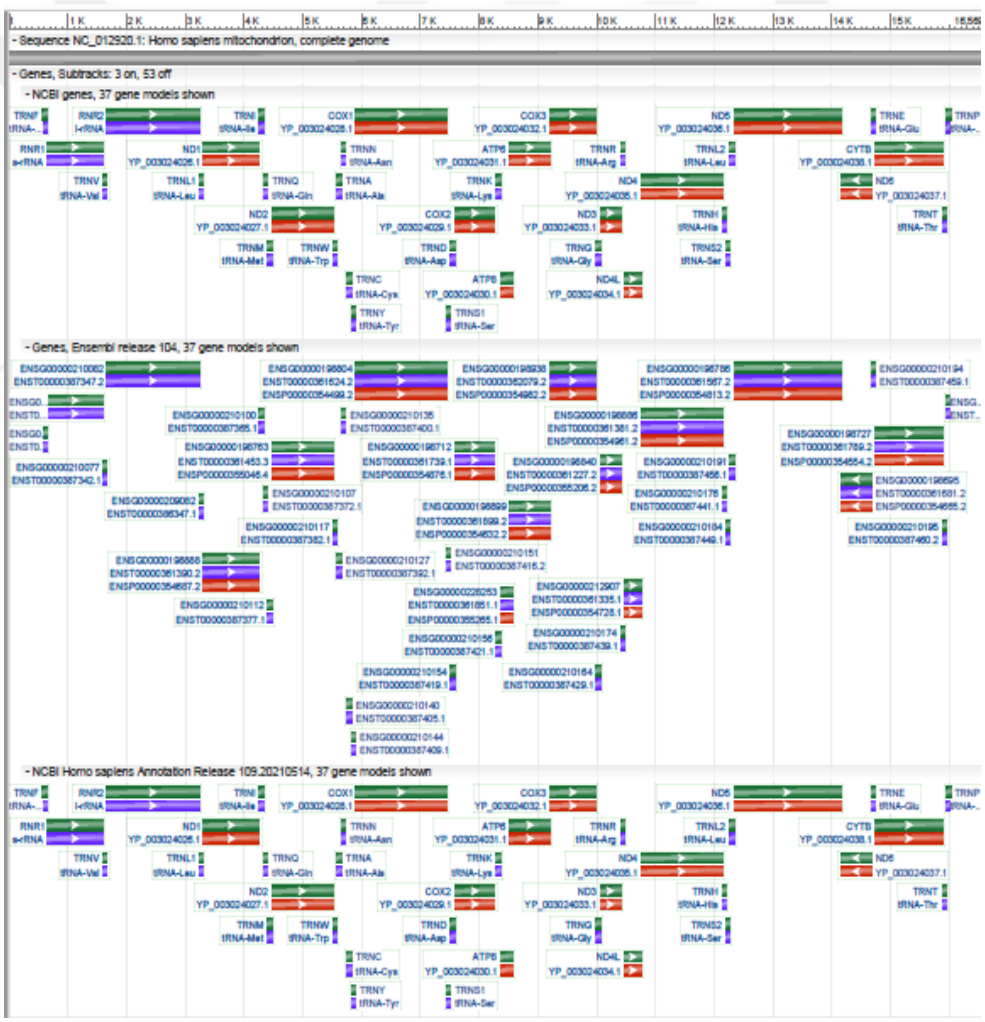

Figure 4.1.1-1. Sequence Alignment map for first patient

4.1.2. Sequence alignment map for patient 2

The polymorphic regions and features of the second patient are shown in Table 4.3. Also, the sequence alignment map of the second patient is shown in figure 4.1.2.-1 As indicated in Table 4.2, homozygous TT genotype defined by rs1625895, heterozygous CG genotype defined by rs1042522, heterozygous GG genotype defined by rs1642785, homozygous TT genotype defined by rs2909430 and deletion were observed in the polymorphic region defined by rs59758982. The effects of the changes in the 5 polymorphic regions of the patient were found to be benign.

Table 4.3. Polymorphic regions and features of the second patient

ID	Genotyping	Effect	Region	Nucleotide Taransition
rs1625895	Homozygote	Benign	Intron 6	C/T
rs1042522	Heterozygote	Benign	Exone 4	C/G
rs1642785	Homozygote	Benign	Intron 2	C/G
rs2909430	Homozygote	Benign	Intron 4	C/T
rs59758982	Heterozygote	Benign	Intron 3	Deletion

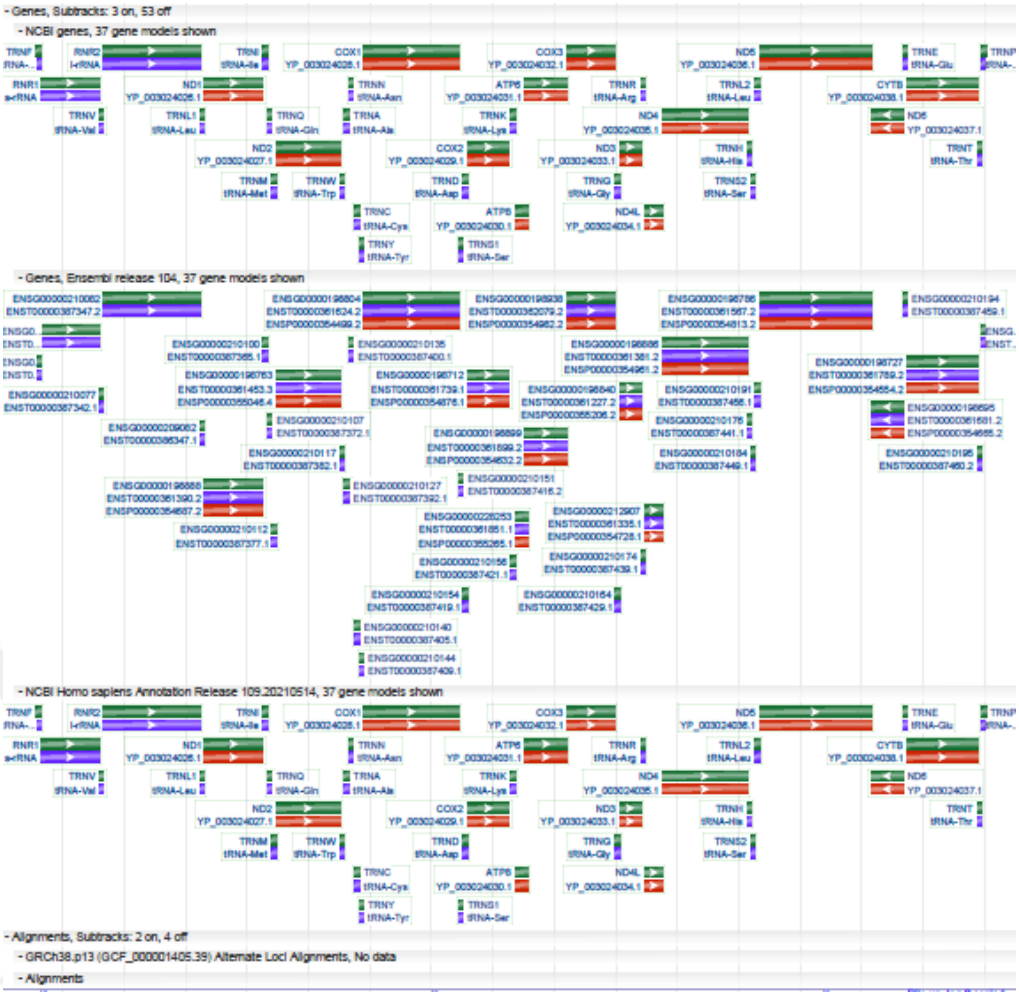

Figure 4.1.2.-1. Sequence Alignment map for second patient

4.1.3. Sequence alignment map for patient 3

The polymorphic regions and features of the third patient are shown in Table 4.4. Also, the sequence alignment map of the second third patient is shown in figure 4.1.3-1 While common polymorphic regions were observed in other patients, more polymorphic regions were detected in patient 3. As can be seen, in patient 3, homozygous TT genotype defined by rs1625895, heterozygous CG genotype defined by rs1042522, heterozygous CG genotype defined by rs1642785, and homozygous TT genotype defined by rs2909430 were observed, in the same patient, deletion in the polymorphic region defined by rs59758982, heterozygous CA genotype defined by rs191918079, heterozygous AT genotype defined by rs145153611, and GA heterozygous genotypes defined by rs540683791 were detected.

Tablo 4.4. Polymorphic regions and features of the third patient

ID	Genotyping	Effect	Region	Nucleotide Taransition
rs1625895	Homozygote	Benign	Intron 6	C/T
rs1042522	Heterozygote	Benign	Exone 4	C/G
rs1642785	Heterozygote	Benign	Intron 2	C/G
rs2909430	Homozygote	Benign	Intron 4	C/T
rs59758982	Heterozygote	Benign	Intron 3	Deletion
rs191918079	Heterozygote	Benign	3’ UTR	C/A
rs145153611	Heterozygote	Benign	Intron 4	A/T
rs540683791	Heterozygote	Benign	Intron 3	G/A

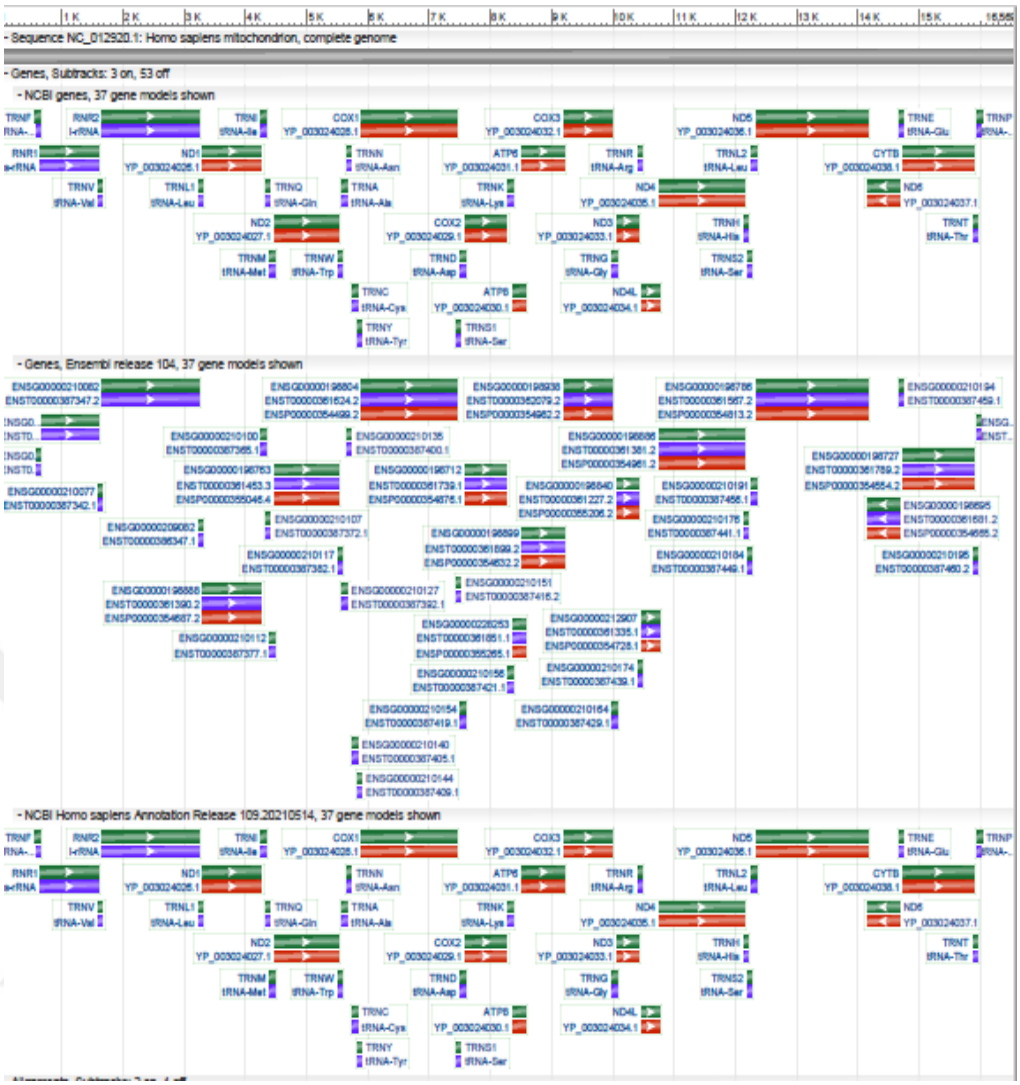

Figure 4.1.3.-1 Sequence Alignment map for 3.patient

4.1.4. Sequence alignment map for patient 4

The polymorphic regions of the 4. patient and their characteristics are shown in Table 4.5. Also, the sequence alignment map of the 4. patient is shown in figure 4.1.4-1. As can be seen, in patient 4, homozygous TT genotype defined by rs1625895, homozygous GG genotype defined by rs1042522, homozygous GG genotype defined by rs1642785, homozygous TT genotype defined by rs2909430 and deletion were observed in the polymorphic region defined by rs59758982. The effects of the changes in the 5 polymorphic regions of the patient were found to be benign.

Tablo 4.5. Polymorphic regions and features of the fourth patient

ID	Genotyping	Effect	Region	Nucleotide Taransition
rs1625895	Homozygote	Benign	Intron 6	C/T
rs1042522	Homozygote	Benign	Exone 4	C/G
rs1642785	Homozygote	Benign	Intron 2	C/G
rs2909430	Homozygote	Benign	Intron 4	C/T
rs59758982	Heterozygote	Benign	Intron 3	Deletion

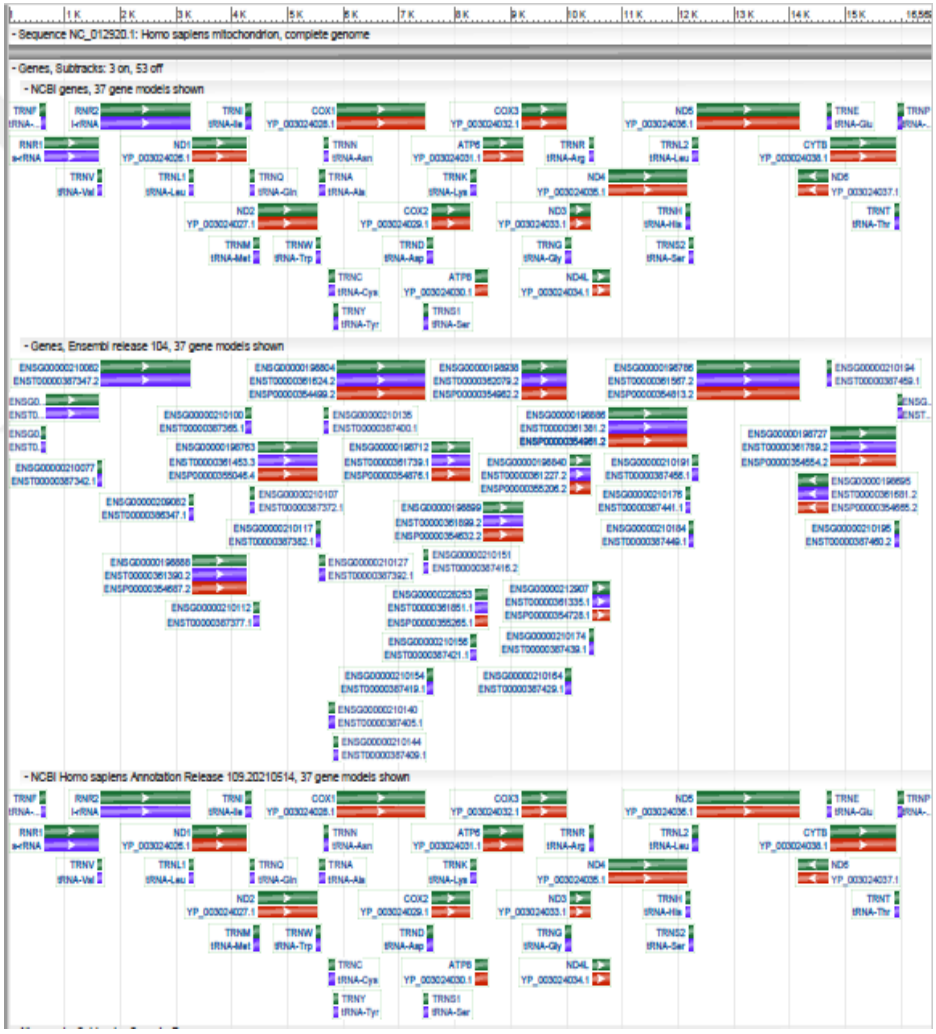

Figure 4.1.4.-1 Sequence Alignment map for 4. patient

4.1.5. Sequence alignment map for patient 5

The polymorphic regions of the 5. patient and their characteristics are shown in Table 4.6. Also, the sequence alignment map of the 5.patient is shown in figure 4.1.5-1. As can be seen, in patient 5, homozygous TT genotype defined by rs1625895, heterozygous CG genotype defined by rs1042522, heterozygous GC genotype defined by rs1642785, homozygous TT genotype defined by rs2909430 and deletion in the polymorphic region defined by rs59758982 were observed. The effects of the changes in the 5 polymorphic regions of the patient were found to be benign.

Tablo 4.6. Polymorphic regions of the fifth patient and their characteristics

ID	Genotyping	Effect	Region	Nucleotide Taransition
rs1625895	Homozygote	Benign	Intron 6	C/T
rs1042522	Heterozygote	Benign	Exone 4	C/G
rs1642785	Heterozygote	Benign	Intron 2	C/G
rs2909430	Homozygote	Benign	Intron 4	C/T
rs59758982	Heterozygote	Benign	Intron 3	Deletion

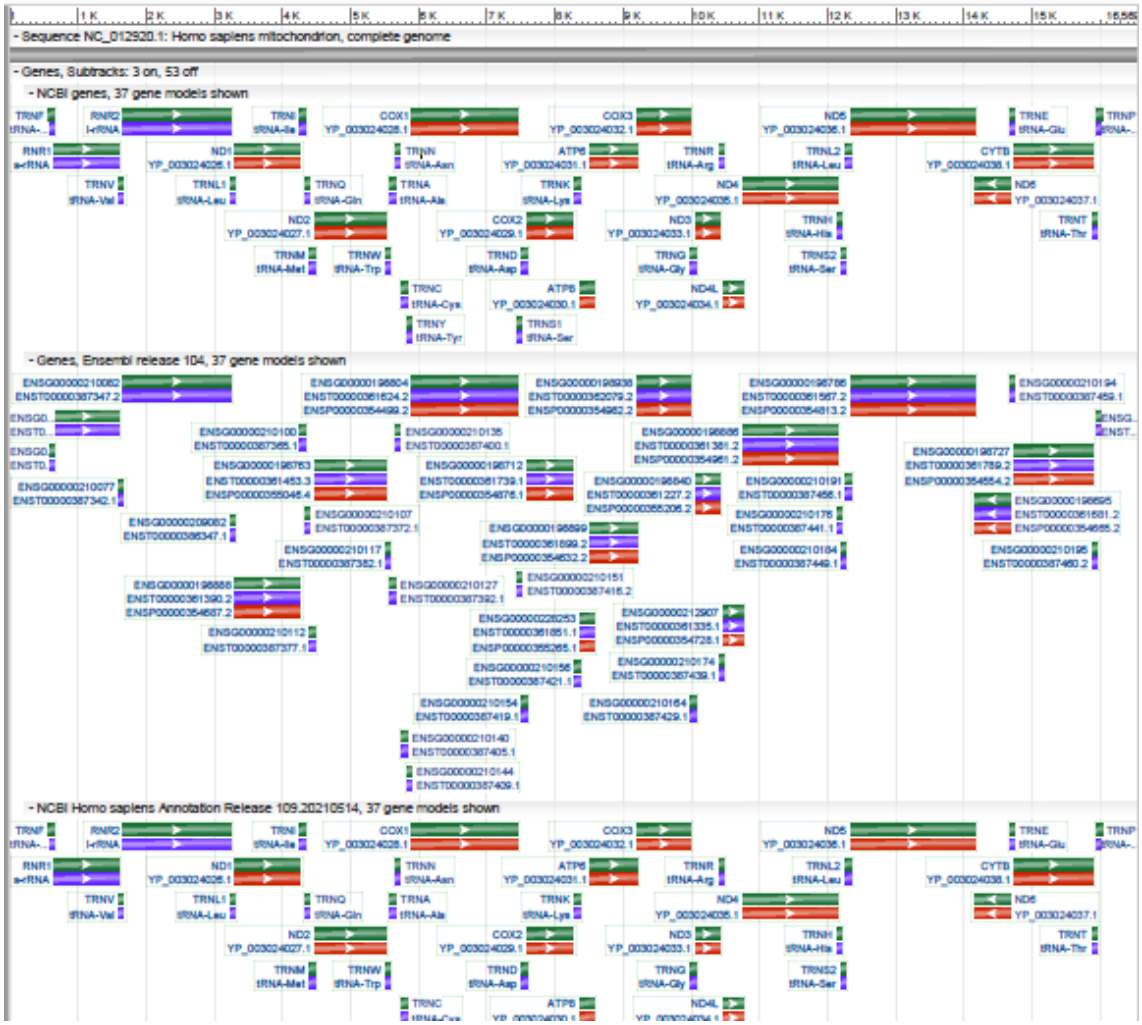

Figure 4.1.5-1 Sequence Alignment map for 5. patient

4.1.6. Sequence alignment map for patient 6

The polymorphic regions of the 6. patient and their characteristics are shown in Table 4.7. Also, the sequence alignment map of the 6.patient is shown in figure 4.1.6-1. As can be seen, in patient 6, homozygous TT genotype defined by rs1625895, heterozygous GC genotype defined by rs1042522, homozygous GG genotype defined by rs1642785, homozygous TT genotype defined by rs2909430 and deletion in the polymorphic region defined by rs59758982 were observed. The effects of the changes in the 5 polymorphic regions of the patient were found to be benign.

Tablo 4.7. Polymorphic regions of the sixth patient and their characteristics

ID	Genotyping	Effect	Region	Nucleotide Taransition
rs1625895	Homozygote	Benign	Intron 6	C/T
rs1042522	Heterozygote	Benign	Exone 4	C/G
rs1642785	Homozygote	Benign	Intron 2	C/G
rs2909430	Homozygote	Benign	Intron 4	C/T
rs59758982	Heterozygote	Benign	Intron 3	Deletion

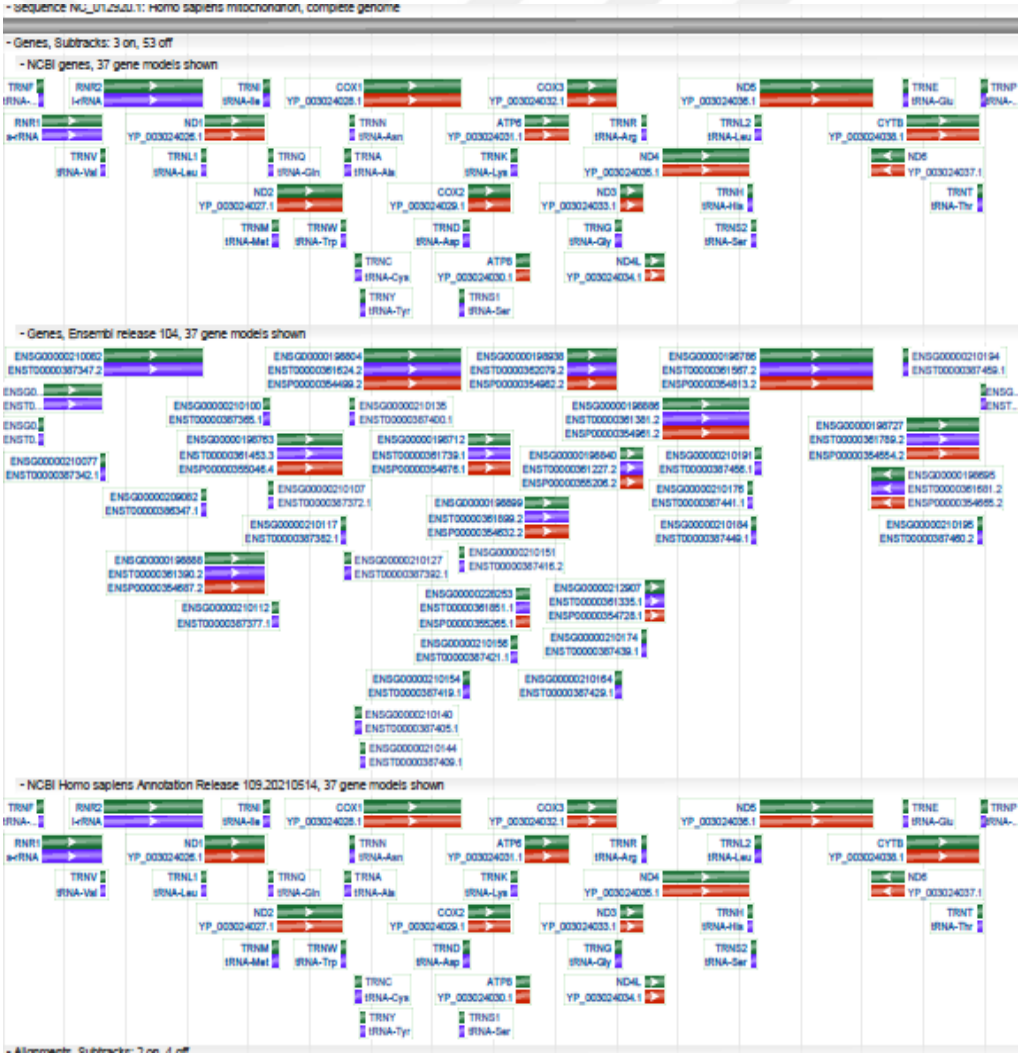

Figure 4.1.6-1 Sequence Alignment map for 6. patient

5. DISCUSSION AND RESULTS

Despite decreasing from 20 years ago, colorectal cancer (CRC) is still the third most frequent cancer with a 9% fatality rate (111). In terms of the hypothesis of CRC carcinogenesis proposed previously, CRC is the end result of a complex chain of events, perhaps meaning that many genes contribute to the appearance of cancer in diverse ways (112,113). A range of genetic mutations and variations are implicated with CRC risk, and are involved in most populations globally. Mutation and variant discovery might lead to the earlier diagnosis and treatment of CRC (114). It's well-known that this polymorphism alteration in the 4th exon of the 72nd codon of the TP53 gene, a replacement of arginine with proline in codon 72 (Ar72Pro), is crucial in cancer susceptibility and progression (115,116). Because of its ability to contact BCL2 (BCL2 antagonist/killer) directly via the mitochondria, it was assumed that the Arg72 allele would be more potent in inducing apoptosis than the Pro72 allele, because of the homozygous state of the Arg72 allele's Arg72 allele, since the presence of the TP53 mitochondrial facilitates direct contact with the pro-apoptotic BAK protein (117). This could be the reason for the previously found association with the Pro72 allele, which is more resistant to inducing apoptosis and so presents a greater risk of cancer. (118,114). The TP53 (rs1042522) variation has been proven by Elshazli et al. to be a potential risk factor for CRC susceptibility using meta-analysis (119).

An embryonic liver tumor, hepatoblastoma (HB), is a malignant tumor that develops from liver precursor cells during infancy. As the most frequent liver cancer in children, nearly 80 percent of all childhood liver tumors are caused by this malignancy (120, 121). Hepatitis B affects predominantly children under the age of 5, with the largest frequency occurring in children aged 6 months to 3 years. A 17-month-old median is used, and males are more at risk (122). HB is characterized by an enlarged liver mass and a high level of alpha fetoprotein (123).

It was also discovered in an earlier research project that the stronger inhibitory function was shared by the stronger functions of the Arg-type TP53 protein. These functions included cell death and damage repair (126). Researchers have found evidence that HPV E6 protein easily degrades the TP53 Arg type protein, whereas the TP53 Pro type protein is not (127). According to Yang et al., this is the largest case-control study to date studying the relationship between Chinese HB risk and the TP53 rs1042522 C>G

polymorphism. As a result of the study, it was determined that the C>G alteration did not increase the risk of HB in the Chinese population, and that it should be tested in other types of juvenile cancer (124).

Researchers Yoon et al. discovered that people with the Pro/Pro TP53 genotype were more likely than those with the Arg/Arg genotype to develop hepatocellular carcinoma, in a study conducted in the Korean population (128). In a study undertaken to determine whether or not 213 sick children and 958 healthy children had hepatoblastoma susceptibility, it was discovered that there was no significant link between the TP53 gene rs1042522 C>G polymorphism and hepatoblastoma susceptibility (hepatoblastoma risk) (129).

The advancement of breast cancer treatment relies on several genetic, epigenetic, and polymorphism variations (130). There are a number of SNP variations with TP53 that could have a considerable impact on breast cancer progression. A number of research have been done that examine the influence of the TP53 rs1042522 polymorphism on premenopausal breast cancer patients (131-134). Until 2020, there was no research on the TP53 rs1042522 polymorphism in Turkish young breast cancer patients. Taşkın et al. investigated the allele frequencies in the control group and in patients with SSc and discovered that the CG genotype was greater in the control group, but that the allele frequencies between the control group and the patients did not indicate a significant difference. Control group C allele rates were 42.2 percent and 41.1 percent, and patient group C allele frequencies were 57.8 percent and 58.9 percent. They also demonstrated that people with the heterozygous genotype were more likely to get breast cancer (with an odds ratio of 0.4196, a 95% confidence interval of 0.1941–0.9067, and a p-value of 0.027). (135). Another study looked at the MDM-2 and TP53 gene polymorphisms' connection to breast cancer in Indian women who were in their premenopausal and postmenopausal years. Participants were found to have either of two genetic variants (Arg/Pro (GC) or Arg/Pro + Pro/Pro (GC + CC)) in the p53 gene (both menopausal) Research has discovered that this treatment helps fight cancer in post- and pre-menopausal women. The similar link was discovered when researchers exclusively considered postmenopausal women, where the variant CG yielded a p-value of 0.009 (and an odds ratio of 0.25), while the variant CG + CC achieved a p-value of 0.013 (and an odds ratio of 0.27). A genetic link between codon 72 gene polymorphism and breast cancer in premenopausal women like Taşkın et al. observed did not exist (136).

Very few research have investigated the effects of TP53 polymorphisms on malignant bone tumor risk, and those that have were unable to consider all factors. The study by Thurow et al. looked at the TP53 Arg72Pro SNP (TP53 rs1042522) and discovered no correlation with Ewing sarcoma (137). Hattinger et al. discovered that the TP53 Arg72Pro SNP (TP53 rs1042522) enhanced the probability of developing osteosarcoma substantially (12). (138). According to Ru et al, the GG genotype of rs1042522 makes osteosarcoma patients (OR = 1.89, 95% CI: 1.16-3.07) significantly more likely than those with the CC genotype (139). Ito et al. included osteosarcoma and Ewing sarcoma (R72P, P=0.958) in their analysis. When the study finished, the number of polymorphisms in benign and malignant tumor groups did not differ (140). Huang et al. investigated internet databases extensively to gather more up-to-date information for their project. Five case-control studies, all of which used a total of 567 patients and 935 controls, and a meta-analysis were all used to investigate if TP53 genetic variations increased the incidence of malignant bone tumors. Four investigations were carried out between Huang et alattempts .'s to associate this SNP with the risk of osteosarcoma, whereas two studies had previously addressed the risk of Ewing sarcoma. The TP53 rs1042522 polymorphism enhanced the likelihood of bone tumor malignancy, as evidenced by their investigation (141).

In the study of Kulmambetova et al., in which 143 gastric cancer patients in the Kazakh population and 355 control groups without this disease were included, it was determined that carrying the rs1042522 SNP G allele was a risk for gastric cancer. The G allele frequency was found to be 0.533 in a sample of 498 individuals, and this rate was observed to be more than African (0.331) and less than European (0.715) and American (0.68) populations (142). The results of the regression studies of Wu et al. (TP53 rs1042522: OR 1.69, 95% CI 1.27–2.24 for CC vs. GG; and OR 1.51, 95% CI 1.17–1.94 for GC vs. GG) are in line with the results obtained by Kulmambetova et al. (143). Moreover, it is thought that the TP53 Arg72Pro variant may be a biomarker in susceptibility to gastric cancer in studies including Japan, Korea and China, which constitute the Asian population (144).

Not only do sedentary lifestyles and excessive alcohol use contribute to cardiovascular disease, but they also contribute to the development of cancer at a similar rate (145). Apart from that, persistent inflammation is responsible for the development of both disorders (146). For example, there are significant disparities between healthy and

sick individuals who have the TP53 Arg72Pro genotype according to Omrani Nava's study. Coronary Artery Disease and proline conversion have been linked (147).

The TP53 gene's intron 6 polymorphism (rs1625895) has been previously connected with cancer patients' white blood cell apoptosis (148). There have been discoveries on the connection between rs1625895 and Li-Fraumeni syndrome as well as the increase in risk for multiple tumor types (149). While introns don't modify the protein structure, it's important to remember that introns can also hold regulatory regions. A variety of polymorphisms connected to phenotypic variation have been discovered (150, 151). In Sauka et alresearch .'s into lung cancer patients, those with the homozygous genotype of rs1625895 (A/A) were found to have quite a high occurrence of apoptosis of white blood cells, compared to people with the heterozygous genotype (148). And evidence revealing that individuals with lung and head and neck malignancies who have G/G genotypes or G allele/G allele combinations indicate reduced survival and changed treatment effectiveness have been discovered (152,153). In Voropaeva et alstudy, .'s they wanted to find out if R-CHOP immunochemotherapy was more effective in Diffuse Large B-Cell Lymphoma (DLBCL) patients with certain genotypes than others. a study determined that the rs1625895 polymorphism (TP53 gene G/G genotype) is related with high likelihood of R-CHOP failure (154).

Obesity is a global concern, and in Saudi Arabia, where it is the third most common condition, its growth has been quite swift. (155). A lifestyle, diet, and genetics all come into play in the disease's development, but it is the behavior and genetics that matter most. When you consume more calories from food, and have less activity in your life, fat begins to build up in your body. Obesity is seen as a condition that has an inflammatory effect but doesn't reach clinical severity; it is connected to a number of other health problems. Obesity often leads to a wide variety of chronic conditions, all of which are caused by inflammation. Multiple mutations have been found to have affected the rate of energy expenditure in body weight regulation through multiple important pathways in obesity development. New genetic research suggests that tumor suppressor gene p53 variations, which are linked to obesity and type 2 diabetes, can be found in many cancers (156).

Polymorphism rs1642785 occurs in the second exon of the p53 gene (intron-2). That this variation raises cancer risk and reduces p53 pre-stability mRNA's has been observed as well (157). According to Sabir et al., p53 gene polymorphism has an impact

on the occurrence and development of obesity among Saudis. So, 136 samples were studied for single nucleotide polymorphisms (SNPs) associated with various clinical characteristics, such as TP53 gene variation rs1642785, rs9894946 or rs1042522. In the study, it was found that the Pro72Arg polymorphism had a substantial connection with the Saudi population. According to their findings, people with the homozygous SNP rs1042522 GG genotype were at a two-fold increased risk compared to those with the CC genotype. The G allele of the SNP rs1042522 variation was found to be significantly more risky than the C allele. There is evidence that the mutant allele in the p53 SNP rs1642785 variation, however, can affect transcription levels by acting in the CA-rich region, which is critical for triggering the destabilization of p53 mRNAs (158).

Despite the fact that gliomas are the most frequent of all brain tumors, the origin of the disease remains a mystery despite decades of intensive research into the condition (159). Wrensch and colleagues hypothesized that gene polymorphisms in the etiology of glioma would play a significant role in the onset of the disease, and they were right. According to their findings, polymorphisms in genes that are involved in processes such as DNA repair and cell cycle control may be connected with glioma susceptibility (160).

The research published by Jha et al. revealed that intron 2 polymorphism (rs1642785) was linked to the codon 72 polymorphic allele, and that a substantial association existed between genotypic status and this polymorphism. Analysis of available data reveals that the rs1642785 polymorphism has a risk of bringing about glioma in Indian patients, in addition to the codon 72 polymorphism. These mutations cause a gradient effect that interferes with gene function and compromises the encoded information (161).

6 to 8 cm away from the rectum, low rectal cancer arises (162). When colorectal cancer develops in a specific anatomical location, it has a distinctive biologic behavior. There are more pathological types, clinical data and surgical options for lower rectal cancer than for middle and higher rectal malignancies (163,164). Over time, we've learned more about LRC's treatment options and biological characteristics, but its high recurrence risk means that LRC is still one of the most serious problems facing human health today (165). A more accurate molecular phenotypic classification can greatly aid in the complete study of LRC's biological behavior, as well as the development of more effective, individualized treatments for the disease.

According to a review of existing research, p53 overexpression has been shown to predict cancer survival independently (166). In contrast, another study determined that p53's predictive usefulness in colorectal cancer was missing (167). A separate investigation found that the expression of p53 protein was linked to short-term outcomes in colon cancer. A substantial correlation between the level of p53 expression and rectal cancer was discovered, as well as a link between the amount of p53-positive cells and clinicopathological characteristics (168).

LRC is yet to be thoroughly studied in terms of TP53 polymorphisms and protein expression, as well as the impact of p53 protein expression on biological behavior and prognosis. Learning about the relationship is crucial for evaluating LRC before surgery, as well as for developing personalized treatment plans. It has been shown that TP53 gene polymorphisms affect the expression of the p53 protein, and that this has a bearing on the biology of LRC (169).

To test this hypothesis, Zhang and colleagues looked at five of the most common SNPs in the gene TP53 (rs1042522,12947788,1625895,2909430 and rs12951053) and the expression of the protein p53. It was shown that mutant carriers of rs1042522 had higher levels of p53 protein production compared to other polymorphisms as a result of the investigation. p53 protein expression was not associated with other TP53 SNPs. There are only one exon polymorphism locus (rs1042522) among the five polymorphic loci selected for this study, while four other polymorphic loci are found within intron regions. A functionally important polymorphism may still exist despite this conclusion. To find out how non-protein coding SNPs can affect gene splicing requires more research (169).

The optic nerve atrophy is a unique condition known as glaucoma. POAG is the most common kind of glaucoma and affects many people. High intraocular pressure (IOP) is, however categorised as high tension glaucoma (HTG) and normal tension glaucoma (NTG), recognized as a major issue (170). In Ressiniotis' study, researchers investigated a total of 223 participants: 139 POAG sufferers and 73 controls. This study found that the insertion polymorphism in intron 3, p53 haplotype, showed a substantial change in distribution with the insertion of 16 base pairs (rs1042522 and rs59758982). hence it was said that in people with a 16 base pair insertion polymorphism, Arg72 is quite common compared to patients (171). Acharya et al. found that no significant genotype frequency difference existed between 67 Indian patients with POAG and 112 healthy controls ($p=0.059$). Additionally, the allele with 16 base pair additions was underrepresented

(p=0.028). Insertions in the p53 intron 3 or at Arg72 may contribute to POAG via the mutations POAG is often the result of ocular neuropathy and other genetic variants in the patients DNA (Slipinska-Pluskiewicz et al., 2009). (172).

Our study is the first to examine the relationship between colorectal cancer and p53 polymorphic regions in the Turkish population. If we look at our studies on a patient basis, homozygous inheritance in all patients in the rs1625895 coded polymorphism, heterozygous inheritance in the patients excluded from the 4th patient in the rs1042522 coded polymorphism, homozygous inheritance in patients 2,4,6 in the rs1642785 coded polymorphism and heterozygous inheritance with 1,3,5 patients. , homozygous inheritance was observed in all patients in the rs29009430 coded polymorphism, and heterozygous inheritance was observed in all patients in the rs59758982 coded polymorphism. In addition, when patient 3 was compared with other patients, 3 different polymorphisms were detected, apart from the common polymorphisms. In the light of our knowledge; Our study is the first to show these 3 polymorphisms in colorectal cancer patients. There is a need to increase the patient population in order to get more detailed results and to determine the effects of these polymorphisms on cancer treatment.

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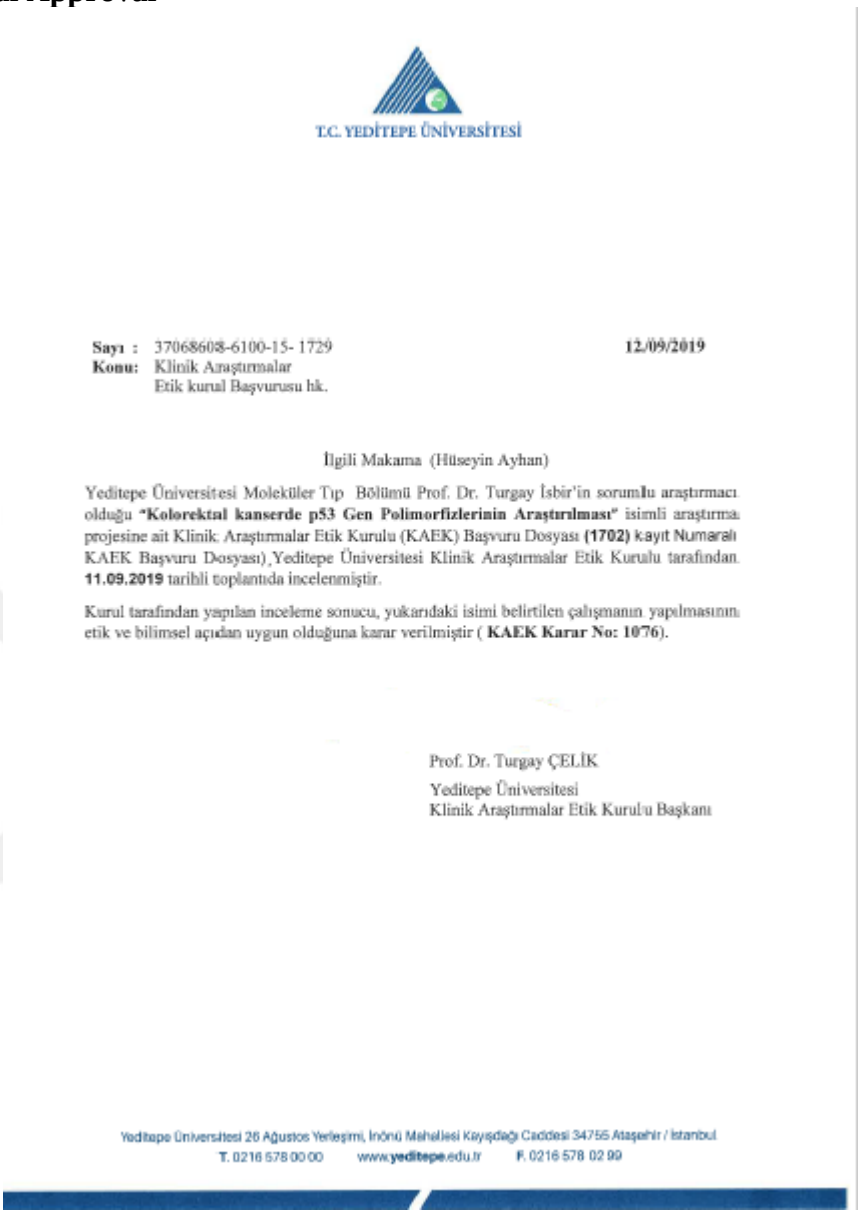
7. APPENDICES

7.1. Ethical Approval



T.C. YEDİTEPE ÜNİVERSİTESİ

Sayı : 37068608-6100-15- 1729 **12/09/2019**
Konu: Klinik Araştırmalar
Etik kurul Başvurusu hk.

İlgili Makama (Hüseyin Ayhan)

Yeditepe Üniversitesi Moleküler Tıp Bölümü Prof. Dr. Turgay İsbir'in sorumlu araştırmacı olduğu **"Kolorektal kanserde p53 Gen Polimorfizlerinin Araştırılması"** isimli araştırma projesine ait Klinik Araştırmalar Etik Kurulu (KAEK) Başvuru Dosyası **(1702)** kayıt Numaralı KAEK Başvuru Dosyası),Yeditepe Üniversitesi Klinik Araştırmalar Etik Kurulu tarafından **11.09.2019** tarihli toplantıda incelenmiştir.

Kurul tarafından yapılan inceleme sonucu, yukarıdaki isimi belirtilen çalışmanın yapılmasının etik ve bilimsel açıdan uygun olduğuna karar verilmiştir (**KAEK Karar No: 1076**).

Prof. Dr. Turgay ÇELİK
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7.2. Curriculum Vitae

Personal Informations

Name	Huseyin	**Surname**	Ayhan
Place of Birth		**Date of Birth**	
Nationality		**TR ID Number**	
E-mail		**Phone number**	

Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Doctorate	Molecular Medicine	**Health Institute**	2021
Master	Molecular Medicine	**Health Institute**	2017
University	Ege University	**Molecular Biology and Genetics**	2014
High school	Ibrahim Bodur Anatolian High School		2007

[#] All the grades must be listed if there is more than one (KPDS, ÜDS, TOEFL; EELTS vs),

Languages	**Grades ([#]))**
English	85(YOKDIL)

Work Experience (Sort from present to past)

Position	Institute	**Duration (Year - Year)**
Postgraduate student	Health	2015-2021
		-

Computer Skills

Program	**Level**
Microsoft Word	Good

***Excellent , good, average or basic**

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

Potential protevtive role of SDF-1 and CXCR4 gene variants in the development of dementia Dalan A, Timirci-Kahraman O, Yılmaz SC, Duman S, Altinkilic M, Ayhan H, İsbir T.**Psychiatria Danubina, 2020;**32(1):92-96
SCARB1 Gene Polymorphisms and HDL Subfractions in Coronary Artery Disease. Ayhan H, Gormus U, Isbir S, Yılmaz SG, Isbir T. **In Vivo**. 2017;31(5):873-876

Articles published in other journals

Proceedings presented in international scientific meetings and published in proceedings book.

22-25 May 2017, Istanbul-Turkey / VI. International Congress of Molecular Medicine. Investigation of MODY 2 (c.7: 44183880G>A) Polymorphism in patients with coronary artery disease. Tugce Gul, Dilara Aydemir, Ezgi Yalbır, Seda Gulec Yılmaz, Huseyin Ayhan, Selim Isbir, Atike Tekeli Kunt, Orhan Fındık, Turgay Isbir
22-25 May 2017, Istanbul-Turkey / VI. International Congress of Molecular Medicine. The polymorphism in the Glucokinase gene is associated with ovarian cancer. Selvi Duman, Seda Güleç Yılmaz , Rukset Attar, Ahmet Büyükören, Emre Murat Altınkılıç, Hüseyin Ayhan, Zerrin Barut and Turgay İsbir