

# Mutation Analysis of TP53 in Colorectal Cancer, Peshawar, Khyber Pakhtunkhwa, Pakistan.

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## Research Article

**Keywords:** Cancer, Mutation, TP53 Gene, PCR, Blasting

**Posted Date:** December 18th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-131329/v1>

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# Abstract

**Background** Colorectal cancer is a kind of solid tumor and third most common of cancer which leads to death. It is a heterogeneous disease characterized by genetic and epigenetic aberrations. For this purpose, a total of 50 blood samples in EDTA tubes was collected from Peshawar with complete information of patients using questionnaire

**Result** The results of the present study indicate that the ratio of CRC in males is higher 62% than females (38%). Histopathological results indicate that moderately differentiated adenocarcinoma is 25 (50%) followed by poorly differentiated adenocarcinoma 15 (30%), Well-differentiated adenocarcinoma 8 (16%), and Metastatic adenocarcinoma 2 (4%) in 50 CRC patients. Grading distribution indicating 34% of patients were having a high grade of carcinoma while 66% had low-grade carcinoma. The nucleotide sequence of the human *TP53* gene of CRC patients was aligned with the human wild-type *TP53* database sequence with NC\_191170 (NCBI) using CLUSTALW. After BLASTING, the mutation was found in exon 5 and exon 7 of *TP53*, while no mutation was found in exon 8 of *TP53*. That patient in which point mutation was found in exon 5 and 7 respectively showed less survival as compared to that of colorectal cancer patients having no point mutation in the *TP53* gene.

**Conclusion** The present study concluded that point mutations were found in exons 5 & 7 of the *TP53* gene and patients having *TP53* gene mutations shown less survival rate compared to CRC patients having no mutations.

## Background

Nowadays, cancer is one of the leading disease in human population that can spread among all living cells with the ability to segregate (Rehman et al., 2020), and uncontrollable and unchecked division (Qadir and Ghalia, 2018). Among all types of cancer, colorectal cancer is one of very common type of cancer in all over the world. There are approximately 500,000 per year cases of CRC cases. Among all type of cancer, CRC is third most common type (Nejad and Yaghoobi, 2012). Colorectal cancer is the second most common cancer and leading cause of death worldwide (Moghimi-Dehkordi and Safaee, 2012), and 5<sup>th</sup> leading cancer type in P. R. China (Gao et al., 2020). The *TP53* gene is mutated in all most 50% of all types of cancer regarding human (Olivier et al., 2010). The highest risk of CRC occurrence is mainly linked to certain factors that leads to CRC i.e., high intake of preserved eatables, smoking, alcohol abuse, and inflammatory bowel disease (Haggar and Boushey, 2009).

Pakistan, like many other South Asian countries, have been placed in low-risk zone CRC. Though, a latest study have reported in CRC cases for patients above the age of 50 years (Hasan et al., 2017). From global perspective, Pakistan falls into a high-risk region for colorectal cancer. According to the collective cancer registry from 1994 up to 2014 published by Shaukat Khanum Memorial hospital Lahore (SKMH), CRC is ranked third in number (4.4% in males and 2.8% in females) among top 10 malignancies in both sexes of Pakistan's population (Siegel and Miller, 2015). CRC have been appeared as uncommon type of cancer in

Pakistan that have effected 5.9% men and 5.0% women (Anwar et al., 2008). The risk factor such as, smoking and high intake preserved food are being expensively extended in Pakistan. Hence, there is a chance of rapid rise of CRC in Pakistan in coming few decades (Bhurgri et al., 2011).

The frequency of CRC is 22.8% per 100,000 people aged between 40 and 49 and 6.8% in 30 t 39 years (Ghorbani et al., 2020). The comparative risk of emerging CRC is lower in those people who have more physical activities as compared to that who are not involve in physical activities (Zhang et al., 2018). In colorectal cancer patients, the liver is the most primary site for metastasis (93.1%), followed by lungs, and lymph nodes (Osumi et al., 2019). Rectum (51.3%), sigmoid (14.2%), and descending colon (4.7%) are the most common sites for CRC (Sharma et al., 2020). Many technologies have now been emerged to treat cancer and types of cancer e.g., (Rehman et al., 2020) have used the ethanolic extract of *Allacanthos crab* against cancer cells and concluded that this extract might be potential source of curing cancer cells. Nanotechnology is one of the important and emerging field of science that act as drug delivery, medicine, and anti-cancer agent (Ghorbani et al., 2020). The nanoparticles are small nano-sized materials 1-100 nm in size (Kakakhel et al., 2019), that might act as potential source for cancer cells (Brigger et al., 2012).

Peshawar is the capital of Khyber Pakhtunkhwa. Most of the CRC patients come to Peshawar for their treatment. Therefore, we conducted our study with objective that *TP53* is a significant tumor suppressor, which is mutated in further stages of many cancers and leads to resistance to chemotherapy. The current study was aimed to conduct to reveal mutations of *TP53* in colorectal cancer in Peshawar, Khyber Pakhtunkhwa, Pakistan.

## Methods

### Ethical approval

The present study was ethically approved by the committee of “Department of Zoology, Kohat University of Science and Technology, Kohat 26000, Khyber Pakhtunkhwa, Pakistan”

### Study Area

Peshawar is the capital city of Khyber Pakhtunkhwa. It is a very congested city in the whole province. The population of Peshawar is 1.7 million (2017). Peshawar lies between 33° 44', 34° 15' north latitude, 71° 22', and 71° 42' east longitude (Warwick, 2007).

### Study population

CRC patients from five major hospitals, Institute of Radiotherapy and nuclear medicine (IRNUM), Lady Reading Hospital (LRH), Khyber Teaching Hospital (KTH), Hayatabad Medical Complex (HMC), North West General Hospital of Peshawar were selected, to carry out the present study.

### Sample Collection:

Total 50 blood samples were obtained from CRC diagnosed patients aseptically in EDTA tubes, along with complete information of the patients: age, gender, the onset of first symptoms, dietary habits, treatment, tumor location, time of first surgery, patients follow up and staging & grading were obtained on “patient info. Sheet”.

#### DNA extraction:

Genomic DNA was extracted from blood plasma by taking 250µl of blood in Eppendorf tube and 750µl of solution A were mixed by inverting the tubes 4-6 times and kept at room temperature for analysis.

#### TP53 amplification

Three oligonucleotide primer pairs (**Table 1**) were designed for *TP53* to amplify fragments of 193, 202 and 158 bp corresponding to exons 5, 7 and 8 of the human *TP53* gene. DNA concentration, cycling time and other amplification conditions were optimized for each primer pair on blood DNA.

**Table 1:** Sequences of primers used for amplification of exons 5, 7 and 8 of *TP53* gene [*TP53* gene ID: 7157]

Sequence of Primer		Length of PCR product
EXON 5	F: GTACTCCCCTGCCCTCAACA R: CTGCTCACCATCGCTATCTG	193 bp
EXON 7	F: GGCTCTGACTGTACCACCAT R: GGAAGAAATCGGTAAGAGG	202 bp
EXON 8	F: GGTAATCTACTGGGACGGAAC R: GCTTCTTGTCCTGCTTGCTT	158 bp

#### PCR master mixture conditions for *TP53* Gene

The fragments were amplified in 25-µl reaction volume containing 1-µl of genomic DNA; 2.5 µl of each primer; 2.5µl dNTP –Mix; 2.5µl of buffer 10X; 0.4µl of Taq-polymerase and 13.6 µl of distal water. The data is given in **Table 2**.

**Table 2:** PCR master mixture conditions for TP53

PCR – Master Mix	25 µl Vol.	Primers Pairs
DNA	1µl	<b>Tp53 -Exon 5</b>
F' Primer (1:100)	2.5µl	<b>Tp53 -Exon 7</b>
R' Primer (1:100)	2.5µl	
dNTP – Mix (2.5mM)	2.5µl	<b>Tp53- Exon 8</b>
Buffer (10X)	2.5µl	
<i>Taq</i> -polymerase (1U)	0.4µl	
dist H2O	13.6µl	

### PCR conditions for Exon 5&7 *TP53*

PCR was performed in the Master cycler (Eppendorf) machine under the following conditions; initial denaturation was performed at 94 °C for 5min, which was then followed by 35 cycles each at 94 °C for 30 second; annealing at 58 °C for 30 sec and initial extension at 72 °C for 30 sec and then there was a final extension at 72 °C for 5min and then the end temperature was set at 4°C.

### PCR conditions for exon 8 *TP53*

PCR was performed in the Master cycler (Eppendorf) machine under the following conditions; initial denaturation was performed at 94 °C for 5min, which was then followed by 35 cycles each at 94 °C for 30 second; annealing at 57 °C for 30 sec and initial extension at 72 °C for 30 sec and then there was a final extension at 72 °C for 5min and then the end temperature was set at 4°C

### Impact of *TP53* mutations on the prognosis of CRC patients

To find out the impact of mutations in the *TP53* gene on colorectal patient survival rate PCR product were sequenced.

### *TP53* Sequencing

PCR products were run on 1.5 % agarose gel & recovered the amplified product with thermos Scientific Genomic DNA Purification Kit. The recovered DNA was quantified a ratio of the 260/280nm spectrophotometer. To find mutations in *TP53* gene, DNA was sent Agha Khan University, Karachi [3µl PCR product + 27µl water = 30µl].

## Results

### Demography of the Study

The present study was based on 50 colorectal cancer diagnosed patient of age (16-74 years) were comprises of 31 males (62%) and 19 females (38%), from different areas of District Peshawar. The demographic information of the participants is presented in **Table 3**. The patients were divided into different age groups with different percentages, patients between age group of 16-20 years were 2 (4%), patients in age groups 21-25 years were 4 (8%), those in between age group of 26-30 years were 7 (14%), likewise in age group 31-35 years had 5 (10%) patients, while age groups 36-40 years 2 (4%), 41-45 years 3 (6%), 46-50 years 8 (16%) 51-55 years 6 (12), 56-60 years 6 (12%), 61-65 years 5 (10%), 66-70 years 1 (2%), 71-75 years 1 (2%) had patients respectively.

**Table 3:** Socio- demographic characteristics of participants / patients

Sex	Number of Patients	Percentage (%)
Male	31	62
Female	19	38
Age group		
16-20	2	4
21-25	4	8
26-30	7	14
31-35	5	10
36-40	2	4
41-45	3	6
46-50	8	16
51-55	6	12
56-60	6	12
61-65	5	10
66-70	1	2
71-75	1	2

### Histopathology of colorectal cancer

In our study we found patients with high rate of moderately differentiated adenocarcinoma that is 25 (50%), secondly followed by poorly differentiated adenocarcinoma which is 15 (30%), while Well differentiated adenocarcinoma is 8 (16%) and Metastatic adenocarcinoma is 2 (4%) in 50 CRC patients as given below in **figure 1**.

### **CRC patients' distribution on basis of grading**

We distributed 50 patients on basis of grading i.e. differentiation of cancerous cells from normal cells according to which 34% patients were having a high grade of carcinoma while 66% were having low-grade carcinoma as mentioned in **Figure 2**.

### **Grouping of CRC patients on basis of staging**

In our study, we found patients of CRC belong to four stages of cancer but with a different percentage. Patients of stage I was 6%, 22 % were of stage II while Stage III and Stage IV were 36% respectively given in below **Figure 3**.

### **Presence of tumor in a different location**

CRC patients have a different location of the tumor from one another on the colon and rectum part of large intestine. Tumors were not present on the same location in all the patients instead they were on different positions and sites. The site that comprises the highest number of the tumor was rectum in 50% patient, the second is a colon in 22% patient and 10% were on rectosigmoid. Colon-sigmoid, sigmoid, and cecum had 4 % of tumors respectively while rectum and liver marts, ascending and CRC was of 2% each as shown in **Figure 4**.

### **Regime gave to CRC patients**

CRC patients of different cancer stages were treated differently; some were treated with chemotherapy while some were given radiation. Those, which were not operated yet were grouped in others. These include 84% were under chemotherapy, while 12% were given Radiation and only 4 % were under other treatment as shown in **Figure 5**.

### **PCR results of Exon-5 (*TP53*) run on 1.5% gel electrophoresis**

Amplification of exon-5 of tumor suppressor protein 53, bands were obtained through polymerase chain reaction shown in **Figure 6**.

Agarose gel electrophoresis (1.5%) results of exon 5 of the human *TP53* gene. L1-L16: amplified bands (193bp) of exon 5, L17/M: DNA-marker (1000bp).

### **PCR results of Exon-7 (*TP53*) run on 1.5% gel electrophoresis**

On amplification of exon-7 of *TP 53*, following bands of exon 7 were obtained through polymerase chain reaction shown in **Figure 7**.

Agarose gel electrophoresis (1.5%) results of exon 7 of the human *TP53* gene. L1-L8: amplified bands (202bp) of exon 7, L9/M: DNA-marker (1000bp).

### **PCR results of Exon-8 (*TP53*) run on 1.5% gel electrophoresis**

On amplification of exon-8 of *TP53*, following bands of exon 8 were obtained through polymerase chain reaction. The **Figure 8** below shows the Exon-8 (*TP53*)

Agarose gel electrophoresis (1.5%) results of exon 8 of the human *TP53* gene. L1,L7,L9,L10: amplified bands (158bp) of exon 8, L12/M: DNA-marker (1000bp).

### ***TP53*-mutation analysis**

The nucleotide sequence of the human *TP53* gene of CRC patients was aligned with a human wild-type *TP53* database sequence with NC\_191170 (NCBI) using CLUSTALW [26].

### ***TP53*-mutations within Exons 5, 7 & 8**

We sent DNA samples of 20 colorectal cancer patient to Agha Khan University Karachi for sequencing of *TP53* gene. Sequencing results that we obtain were BLAST with wild-type sequence of *TP53*, available at NCBI. After BLASTING, we found mutations in exon 5 and exon 7 of *TP53* while no mutation was found in exon 8 of *TP53*. Here is the summary of all mutation we found. We found 3-point mutations in exon 5 of *TP53*, these mutations were in Patient number 7, 20 and 28. Patient number 7 the point mutation was at bp 150 and Guanine was replaced by Adenine, when the impact on protein level was checked it was at amino acid 44 alanine was replaced by threonine. The point mutation in patient 20 was at bp 160 at this point cytosine replaces thymine and having impact at 48 amino acid and replaces threonine to Isoleucine, while the point mutation was seen at 169 bp in patient number 28; replacing Cytosine by Thymine and having impact on protein level at 58 amino acid places Alanine instead of Valine. A silent mutation is found at exon 7 of *TP53* in patient number 5 as given below in **Table 4**.

### **Table 4: *TP53*-mutations within Exons 5, 7 & 8**

Exon #	Patient #	Wild type codon	Mutant codon	Mutation	Impact on DNA level	Impact on protein level
5	T7 (Female)	GCT(Ala)	ACT(Thr)	Point mutation	bp 150 G→A	Ala→Thr
	T20 (male)	ACC(Thr)	ATC(Ile)	Point mutation	bp 160 C→T	Thr→Ils
	T28 (male)	GCT(Ala)	GTT(Val)	Point mutation	bp 170 C→T	Ala→Val
7	T5 (male)	GCA(Ala)	ACA(Thr)	Point mutation	bp 130 C→A	Ala→Thr
8	Non-mutations found					

### Impact of *P53* mutations in exon 5 and 7 on patient`s survival

That patient in which point mutation was found on exon 5 and 7 respectively they showed less survival as compared to that of colorectal cancer patients having no point mutation in the *TP53* gene. The data regarding *P53* mutations in exon 5 and 7 is given below in **Table 5** and **6** respectively.

**Table 5:** Impact of *P53* mutations in exon 5 on survival

Exon-5 <i>TP53</i> -Exons	Mt- <i>TP53</i> (Survival)
T7	↓ (Compared to CRC patients having no mutations)
T20	↓ (Compared to CRC patients having no mutations)
T28	↓ (Compared to CRC patients having no mutations)
T5	↓ (Compared to CRC patients having no mutations)

**Table 6:** Impact of *P53* mutations in exon 5 on survival

Exon-5 <i>TP53</i> -Exons	Mt- <i>TP53</i> (Survival)
T7	↓ (Compared to CRC patients having no mutations)
T20	↓ (Compared to CRC patients having no mutations)
T28	↓ (Compared to CRC patients having no mutations)

## Discussion

Colorectal cancer with many similar characteristics, is compact cancer that develop in various parts of the colon or rectum and commonly known as cancer of large bowel or intestine. CRC emerged as one of the most common cause of death among cancer-related deaths. Approximately, one million people each year develop colorectal cancer around the world. Most people with colorectal cancer die within the time period of five years from the day of first diagnosis (Frebourg and Friend, 1992). According to the genetic model which is proposed for development of colorectal cancer, the progressive acquisition of activity or loss of function is because of accumulation of sequential mutations in oncogenes and tumor suppressor genes such as adenomatous polyposis coli (APC), Kristen-ras (K-ras) and tumor suppressor protein 53, that drives healthy colonic epithelia through increasingly dysplasia adenoma to carcinoma transition (George et al., 2007). *TP53* is one of the most frequently mutated genes in human cancers and loss of *TP53* function through mutation in a gene is the most important event in colorectal cancer development. The most common mutations are single base substitutions that alter protein function. Some of the mutations being oncogenic confer gain-of-function properties (Tominaga et al., 2010). In the present study, we searched for mutations in the *TP53* gene in colorectal cancer patients. Previously no such study has been conducted in Khyber Pakhtunkhwa nor in Pakistan. In this study, we extracted genomic DNA from 50 CRC patients and amplified exons 5, 7 and 8 of *TP53* through PCR. We for the first time investigated and screened for mutation exons 5, 7 and 8 from genomic DNA which were extracted from the blood of CRC patients by polymerase chain reaction (PCR). The PCR products were then purified and sequenced directly and obtained nucleotides sequences were compared with *TP53* database sequence NC-191170 using CLUSTALW (Yu et al., 2015). In our study we screened 50- CRC patients for a mutation in *TP53* exons 5, 7 and 8 using PCR direct sequencing. *Tp53* status and mutations were then correlated with the survival rates of CRC patients. Out of 50 CRC patients, the *TP53* mutation was found in 4 of the analyzed patients. Data published regarding *tp53* mutations showed that majority of these mutations were reported in exon 5, 6, 7 and 8 (DNA binding domain), and mainly in some hotspot codons, such as 175, 245, 248, 273 and 282, comprising of G to A, C to T transition and leading to the substitution of a single amino acid in p53 protein. In patient number 7 (T7) a point mutation was found at bp150 leading to GCT→ACT transition resulting in Threonine instead of Alanine. Similarly, in patient number 20 (T20) a point mutation was found at bp160 leading to ACC → ATC transition resulting in Isoleucine instead of Threonine. Another mutation was found in patient 5 exon 7 at bp 130 leading to GCA→ACA, resulting in Threonine instead of Alanine. No mutation was found in exon 8 in any other patient. Based on mutational data from 9000 colorectal tumors the most frequent mutations observed in both adenocarcinomas and carcinomas have been at codons 175 and 273. However, the observed frequency of mutation at codon 248 has been reported approximately three times higher in adenocarcinomas than in adenomas (Iacopetta et al., 2006). Many studies have reported the association of somatic mutations in *TP53* or abnormal protein expression with poor survival or lack of response to therapy. However, the clinical significance of *TP53* status still remains controversial (Roth, 1999). We also found those patients have point mutations in their *TP53* gene shows low prognosis as compared to that of those colorectal cancer patients having with *TP53* gene. Most of the studies on the clinical relevance of *TP53* polymorphisms

mainly focused on the role of Arg72Pro. The Arg72 variant has been reported to be a more potent inhibitor of chemotherapy-induced apoptosis than the corresponding Pro72 variant (Naccarati et al., 2012). Thus, TP53 genotyping may be useful for stratification and selection of patients that will most benefit from certain molecularly targeted therapies.

## Conclusion

Extraction and amplification of DNA even with old ages can be analyzed. The mutation is a sudden change in a gene. A point mutation was mostly found in exon 5 and 7 of the TP53 gene. Patients having *TP53* gene mutations shown less survival rate compared to CRC patients having no mutations. Most of the work is needed in the future.

## List Of Abbreviations

PCR: Polymerase chain reaction

CRC: Colorectal Cancer

EDTA: Ethylenediaminetetraacetic acid

P. R. China: People republic of China

IRNUM: Institute of Radiotherapy and nuclear medicine LRH: Lady Reading Hospital

KTH: Khyber Teaching Hospital

HMC: Hayatabad Medical Complex

NWGHP: North West General Hospital of Peshawar

## Declarations

### Ethics consent to participate

All procedures performed in studies involving human participants were following the ethical standards and the study was approved by the institutional committee of Department of Zoology, Kohat University of Science and Technology, Kohat 26000, Khyber Pakhtunkhwa, Pakistan according to 1964 Helsinki declaration. The participant voluntarily participates in the present study. The overall data was conducted in the form of questionnaire.

### Consent for publication

Not applicable

### Availability of data and materials

Not applicable

### Competing interests

All the authors declared that there is no competing interest

## Funding

No funding received

## Author contribution

Contributions to the conception **IK**; design of the work; **MAK**; analysis, **ZA** interpretation of data; **IS**; have drafted the work **IA**; Substantively revision **MJ**; Experimental **AF**.

## Acknowledgment

Authors are also very grateful to “Department of Zoology, **Kohat** University of Science and Technology, Khyber Pakhtunkhwa, Pakistan” “School of Life Sciences, **Lanzhou** University, Lanzhou, Gansu, China” for providing a sound environment for laboratory work.

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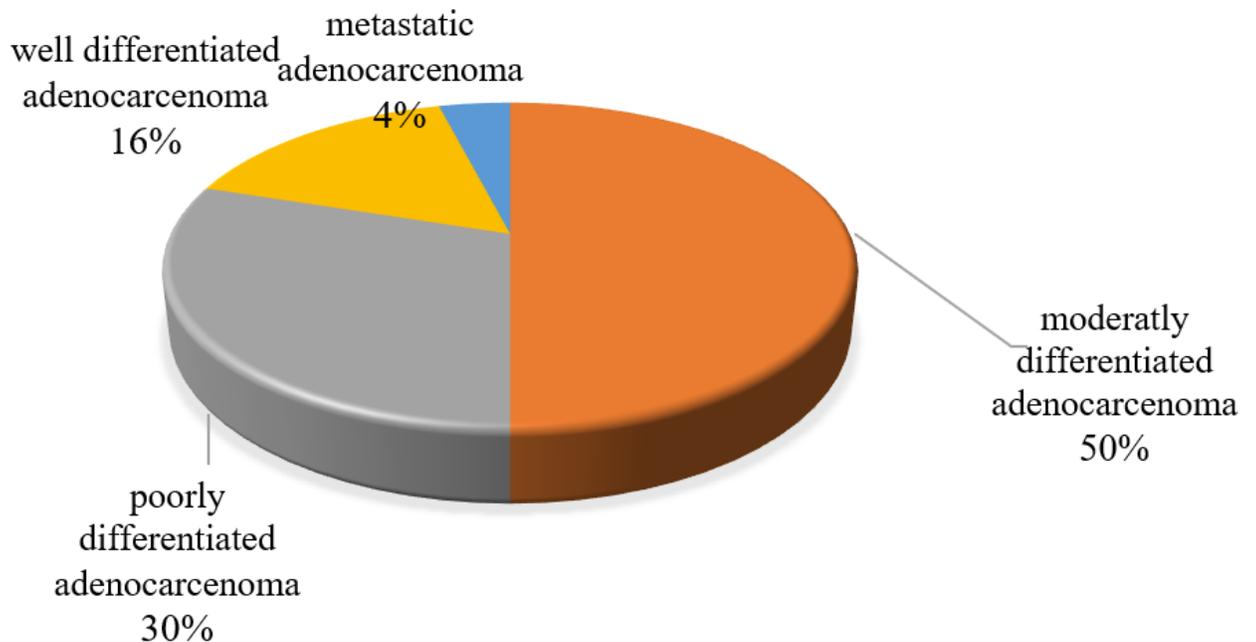
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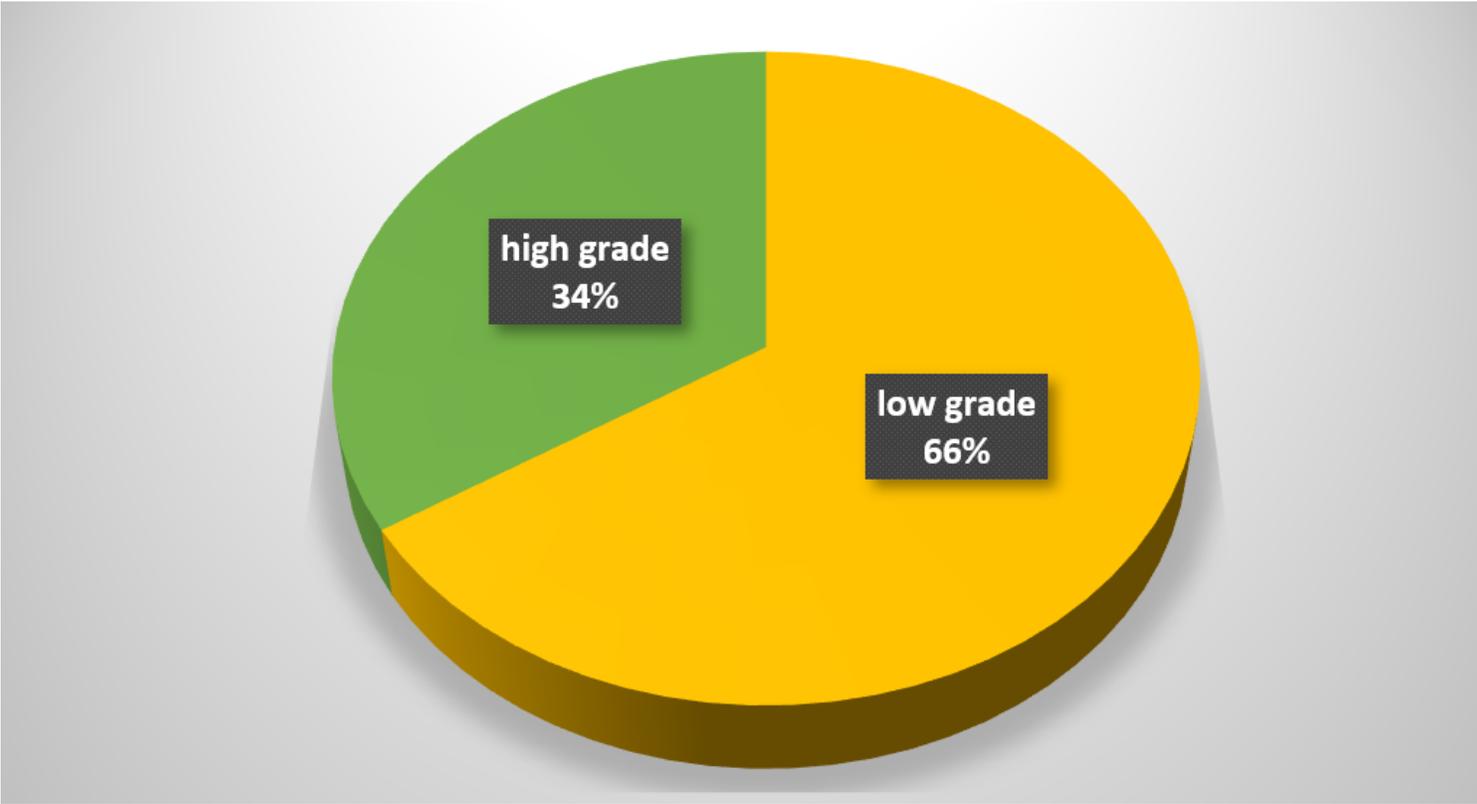
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## Figures



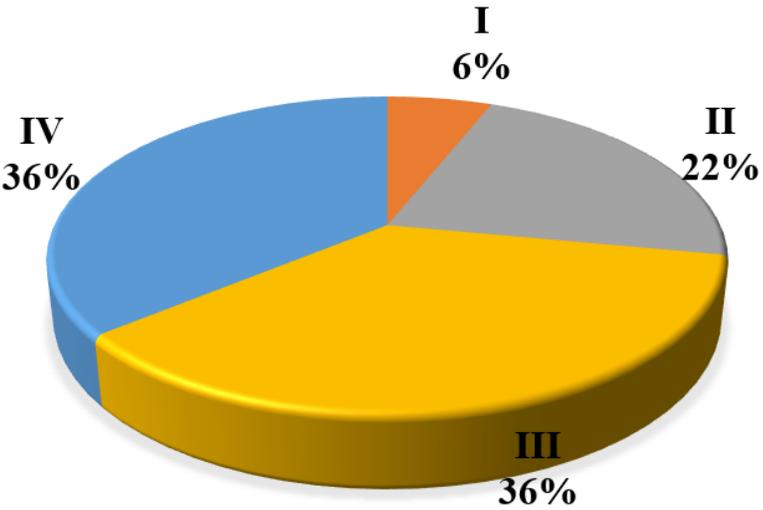
**Figure 1**

Histopathology of colorectal cancer patients



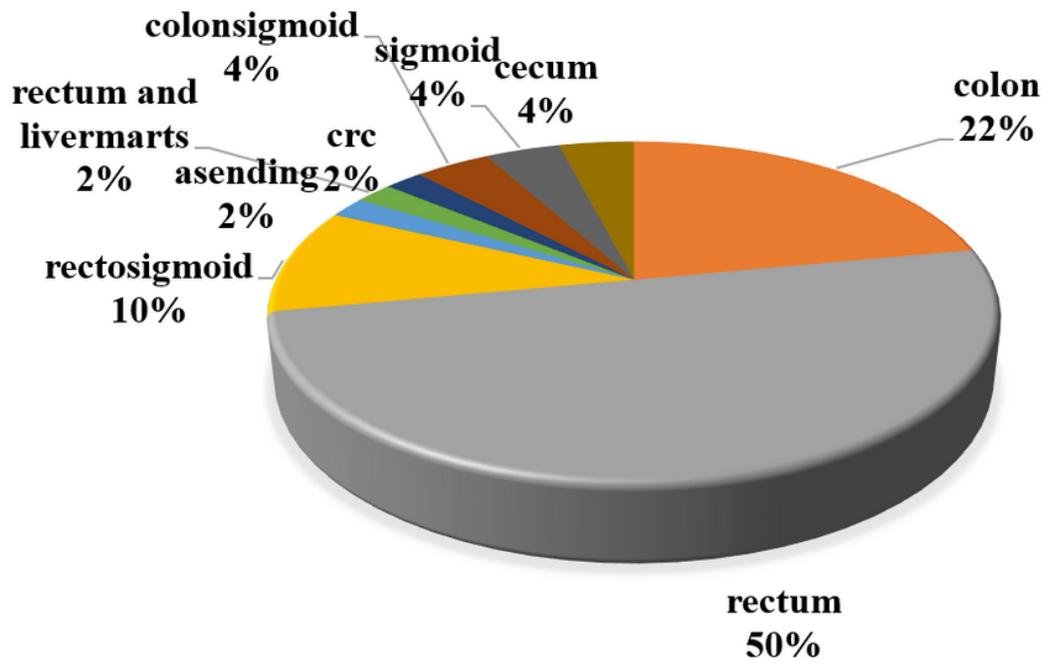
**Figure 2**

Distribution of CRC patients on basis of tumor grading



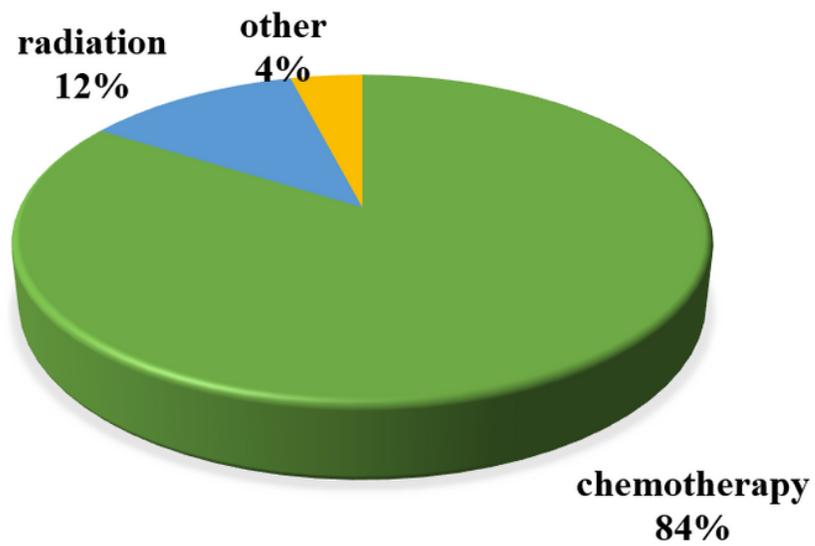
**Figure 3**

Division of CRC patients` on basis of staging



**Figure 4**

Presence of tumor in a different location



**Figure 5**

Regime gave to CRC patients

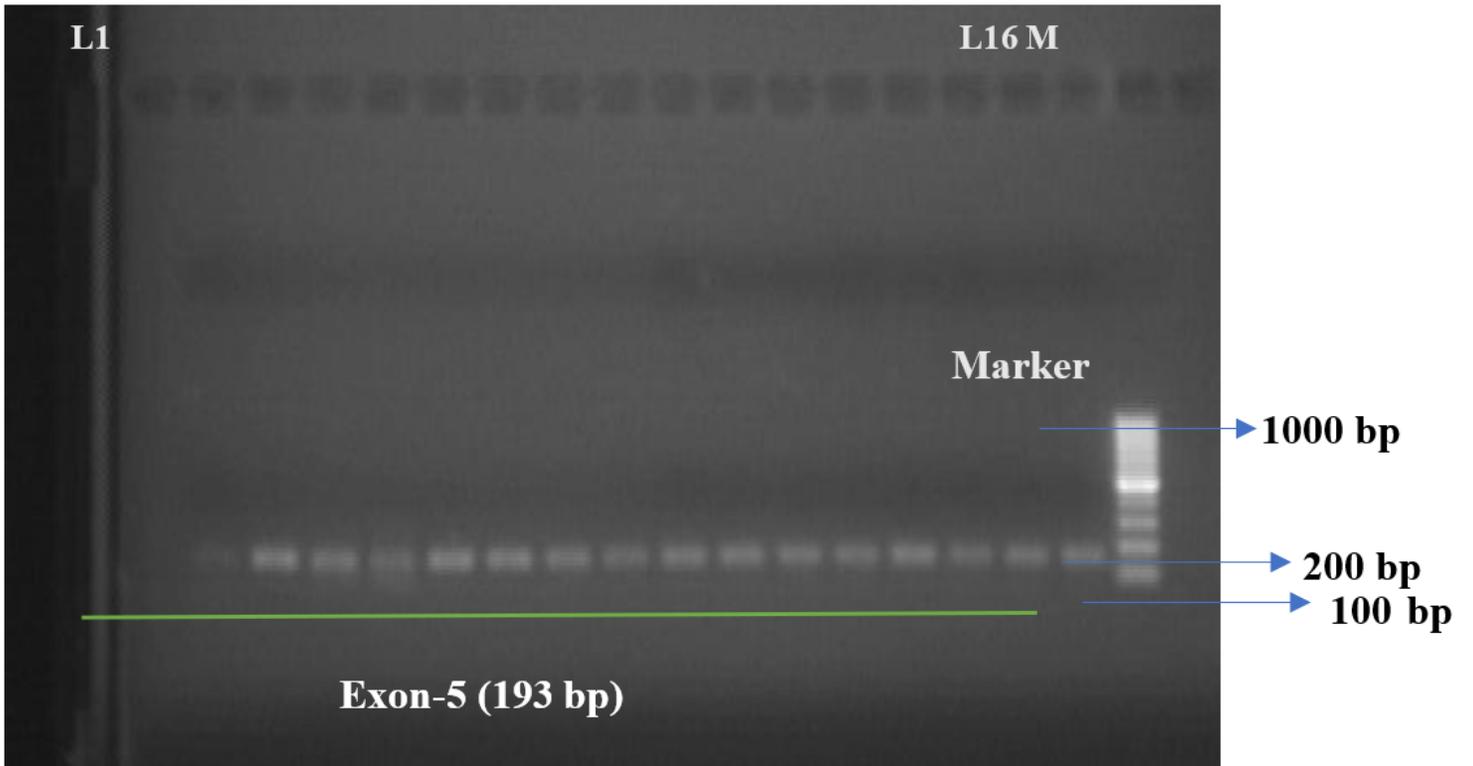
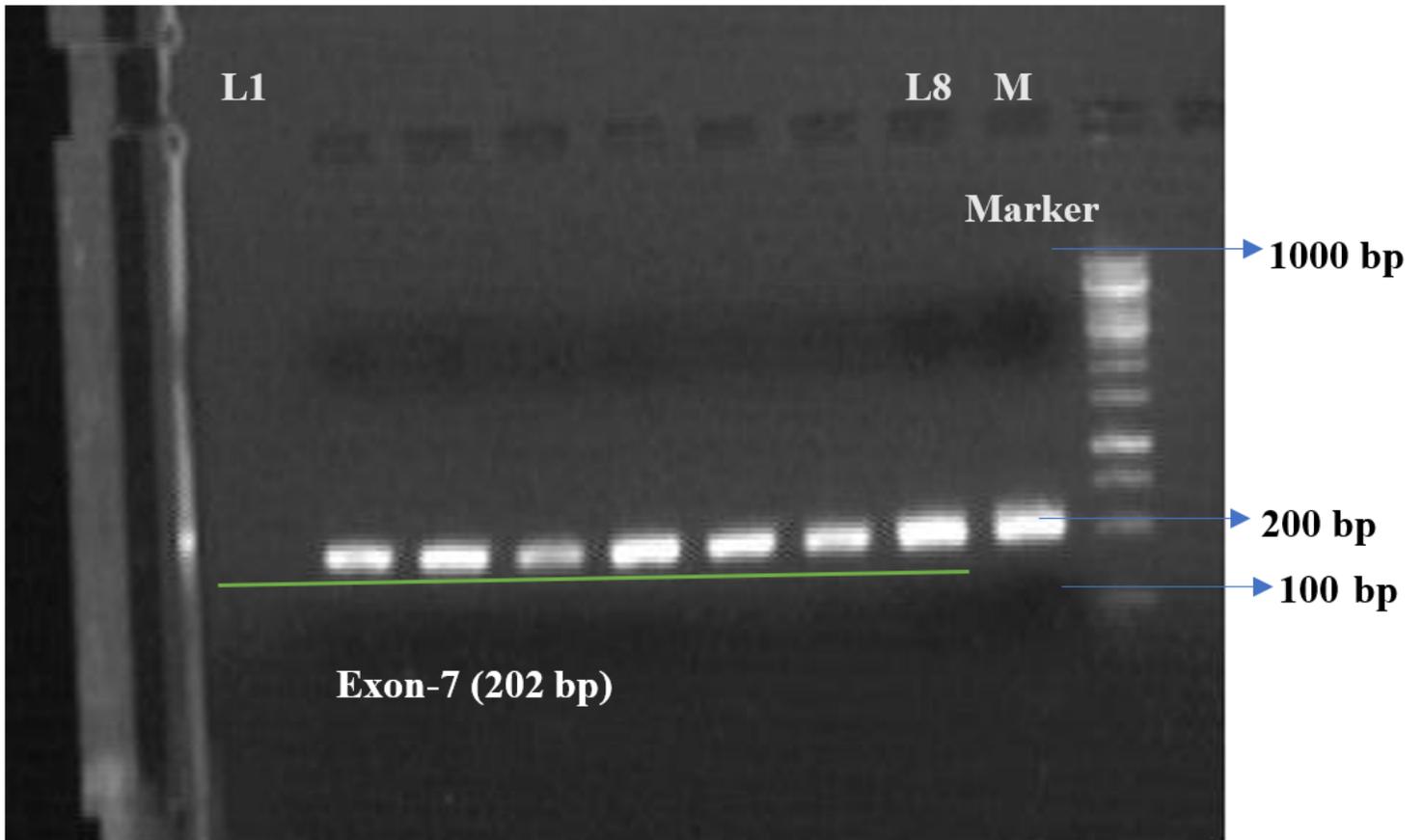


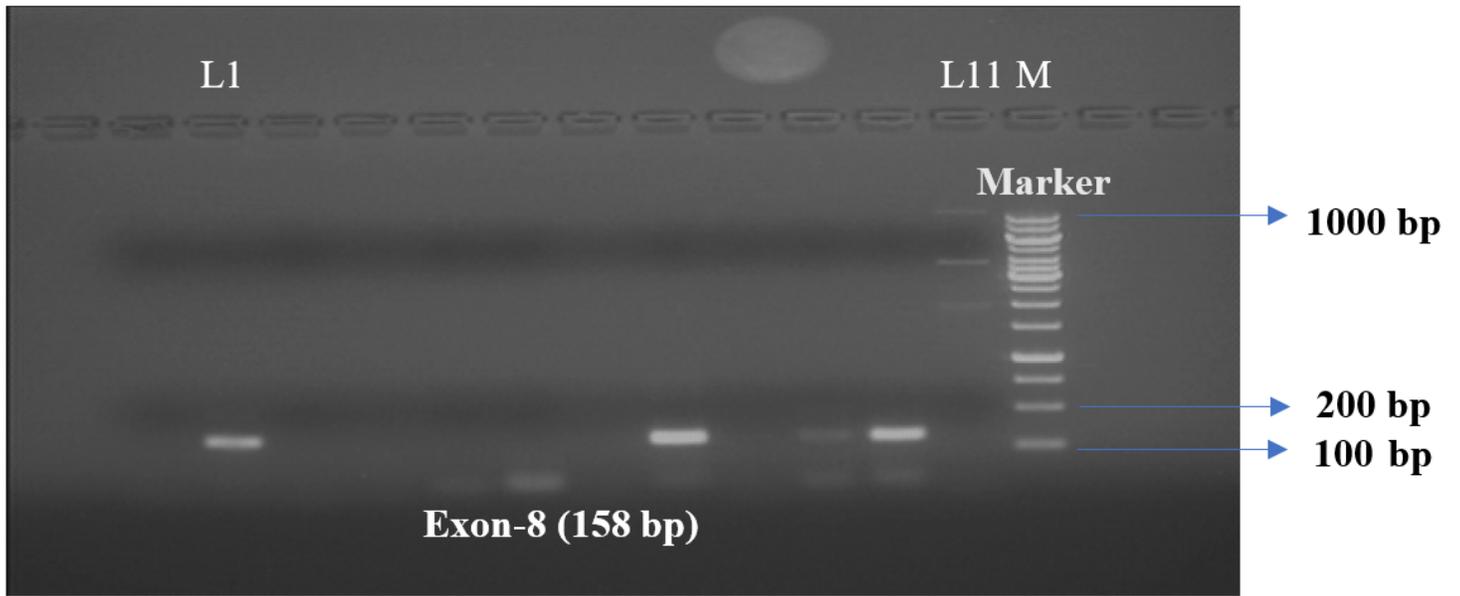
Figure 6

PCR results of Exon-5 (TP53)



**Figure 7**

PCR results of Exon-7 (TP53)



**Figure 8**

PCR results of Exon-8 (TP53)