Original Article

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Running Title: P53 Mutations in the Tumor and Stool of Colorectal Cancer Patients

Detection of Mutations in Exons 5, 6, and 7 of the *TP53* Gene in the Tumor Tissue and Stool Samples of Patients with Colorectal Cancer from Northwest Iran

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Abstract

Background: Colorectal cancer (CRC) is the third most prevalent cancer with approximately 9,000 annual deaths worldwide. However, early detection can provide a high survival rate. The fecal DNA, as a non-invasive method for detecting the genetic markers, such as the *TP53* gene, can be conducive todisease diagnosis . In this study, we aimed to investigate the presence of the *TP53* mutations in the stool samples and their relationship with somatic mutations in the tissue samples of CRC patients from northwestern Iran.

Method: In the present cohort study, tumor and stool samples were obtained from 64 CRC patients (mean age of 60) who were undergoing surgery. Total genomic DNA was extracted from the tissue and stool samples, and *TP53* mutations were detected using the PCR-SSCP method, followed by direct sequencing. Differences between mutations were observed in the tumors, and the stools were examined using the McNemar method.

Results: Of the 64 CRC patients, 19 individuals (30%) demonstrated 27 point mutations in exons 5-7 of the *TP53* in the tumor samples. Furthermore, analysis of the stool specimens revealed that the 22 mutations (81.5%) identified in the tumor specimens were also present in the stool of 12 patients (P=0.063).

Conclusion: Based on the results, the DNA from the tissue could be replaced with fecal DNA in the mutation detections for CRC. Given the non-invasive nature of fecal sampling, it can be desirable and acceptable for patients in molecular screening tests as it increases the screening rates and improves timely CRC diagnosis.

Keywords: Colorectal Cancer (CRC), *TP53* gene, Mutation, Tumor, Stool

Introduction

Colorectal cancer (CRC) or colon cancer, as one of the most frequent cancer types, after lung cancer, is the second leading cause of cancer death worldwide.[1, 2] Although the diagnosis and treatment of CRC have improved remarkably over the recent years, the incidence of CRC is increasing annually.[3] In 2018, it accounted for nearly 1.80 million cases and 862,000 deaths around the world.[2] While the Middle East has been considered as a low-risk area for colorectal cancer, recent studies have shown a rise CRC in the Middle Eastern countries.[4, 5] With 8 and 7 in 100,000 incidence rates, CRC is the third and fourth most common cancer in Iranian men and women, respectively.[6, 7]

CRC is a complex malignancy involving multiple interactions among behavioral, environmental, biochemical, and genetic factors.[3] Of these, genetic factors have had significant impacts on the development of colorectal cancer, and disease management can be improved through detecting the mutations in driver genes with non-invasive, sensitive, and easy applicability screening methods in early diagnosis.[8-10] Molecular analysis of certain gene mutations involved in the pathogenesis of CRC using fecal DNA is an excellent approach to screening patients with a low amount of DNA from intestinal tumor cells in the feces.[11] Since oncogenes and tumor-suppressor genes are predominantly related to neoplastic growth prior to the clinical manifestations of the disease, they could be highly useful diagnostic markers for CRC.[12, 13] One of the most important tumor-suppressor genes involved in CRC is the *TP53* gene, which is located on the short arm of chromosome 17 (17p13), consisting of 11 exons. It encodes p53 protein with 393 amino acids that participate in the regulation of the gene expression in response to cellular stress, cell cycle checkpoints, DNA repair, and apoptosis.[14-17]

It is generally accepted that p53 signaling is one of the most critical intracellular signaling pathways routinely dysregulated in CRC.[3] Mutations in the *TP53* gene induce cell proliferation and the acquisition of metastatic features in cancer cells.[18] In different populations, the frequency of *TP53* gene mutations in CRC has been estimated from 25% to 100%.[19-23] Given that more than 90% of the *TP53* gene point mutations in colorectal adenocarcinoma occur in 5, 6, and 7 exons, these exons are of particular importance compared to other exons.[24-26] One of the most widely used and suitable molecular approaches in the detection of such mutations is Single-Strand Conformation Polymorphism (SSCP) analysis of DNA fragments followedby direct sequencing.[12]

In view of the current rising trend in CRC, we aimed to detect *TP53* gene mutations in the tissue samples of CRC patients. We further examined the applicability of detecting the same mutations in the fecal DNA of patients, a non-invasive source of DNA that can be replaced with tissue sampling in search of somatic mutations. To the best of our knowledge, this would be the first study of the *TP53* gene mutations detection in an Iranian Azeri population.

Materials and Methods

Study population and sample collection

In the present cohort study, a total of 64 specimens of colorectal carcinomas were obtained from29 Iranian Azeri men and 35 women with an average age of 60 years and the same ethnicity and geographical residence; the patients were proven as CRC in previous clinical examinations. Those with a history of chemotherapy and/or radiotherapy were excluded from the study. Written informed consents were signed by every participant who was completely informed as to the study procedures. Ethical protocols of the study were approved by the Ethics Committee of Tabriz University of Medical Sciences, and the IR.TBZMED.REC.1394.871 registry number was obtained. These specimens were obtained from patients admitted to the surgical department of Imam Reza hospital, Tabriz, Iran, between 2015 and 2017. Stool samples were collected from every patient prior to any medical interventions, including surgery or recent colonoscopy. Tumor samples were also obtained during surgery as a routine procedure of treatment protocols. Fifty-one colon samples (79.68%) and 13 rectum samples (20.31%) were referred to the laboratory under certain conditions with complete patient information, including clinicopathological and demographic data. Fresh tumor tissue samples were immediately snap-frozen in liquid nitrogen. All samples were stored at -80 °C until further analysis.

DNA extraction and mutation detection

Total genomic DNA was prepared from tissue samples using the CinnaPure-DNA kit (Cinna Colon, Iran), and fecal DNA was also extracted from the stool samples using QIAamp® DNA Stool kit (Qiagen co. USA) according to the manufacturer's recommended protocols. DNA concentrations and specifications were analyzed using Micro-Volume Spectrophotometer (Maestrogen, Taiwan) and excluded from further analysis if the final concentrations were <100 ng/µl or A260/A280 was outside the range of 1.7-1.9.

Exons 5, 6, and 7 of the *TP53* gene were amplified by conventional polymerase chain reaction (PCR) using previously reported primer pairs.[27] PCR reactions were performed under the following conditions: 10 µL master mix (Ampliqon, Denmark), 1 µL of each primer set, and 2 µL genomic DNA in the total volume of 20 µL for each reaction. Thermal cycler programs were 5 min at 94 °C for initial denaturation followed by 35 cycles comprised of 30 sec at 94 °C, 30 sec at appropriate annealing temperature, and 30 sec at 72. In each program, we also applied 10 min at 72 °C for the final extension .

To analyze any probable mutation in the amplified regions, the PCR products were subjected to Single-Strand Conformation Polymorphism analysis. Afterwards, 10 µL of PCR mixture was heated for about 20 min at 95 °C with 12 µL of formamide dye mixture (95% formamide, 20 mM EDTA, 0.05% xylene cyanole, and 0.05% bromophenol blue); subsequently, it was incubated at -20 °C for almost 20 min to prevent further renaturation and stabilize the spatial conformation of the single-stranded PCR products. Following incubation, 7.5 µL of the mixture was directly applied to a 28% polyacrylamide gel containing 50 mM Tris-borate. Glycerol (2%) was further added. Electrophoresis was performed at 160 volts for 3 hours at room temperature. Thereafter, PCR products were subjected to direct Sanger Sequencing using the DNA sequencing system (ABI Corporation, Wisconsin, USA), and the results were analyzed using Chromas Pro 2.1.3. (Technelysium Pty Ltd, South Brisbane, AU).

Statistical Analysis

Statistical analysis was performed using IBM Statistical Package for the Social Sciences (IBM SPSS Statistics®, version 25, Chicago, IL, USA). Non-parametric data were assessed using the Mann-Whitney U-test, and the associations between the mutations and the histological parameters were assessed by Pearson's chi-square and Fisher's exact tests (if required). Using McNemar method, the difference between the observed mutations in the tumor and the stool was explored. To analyze the meaningfulness of the findings, probability values of 0.05 or less were regarded as statistically significant.

Results

Detection of TP53 gene mutations

Tumor and stool specimens were collected from 73 CRC patients, and 64 DNA samples were qualitatively competent for the final analysis of mutations in exons 5–7 of the *TP53* gene. There were 30 (46.9%) males and 34 (53.1%) females with colorectal cancer. The mean age of the patients was 60.05 (range 45-80 years). Nineteen patients (29.7%) had mutations in exons 5-7 of the *TP53* gene, and five cases had more than one mutations in their stool and/or tumoral tissue.

TP53 gene status and clinicopathologic characteristics

Males were observed to be dominant, though not significantly, in terms of the rate of *TP53* mutations . The histological characteristics of the tumors in patients with and without mutations in the *TP53* gene were examined; based on the results, the frequency of mutation increased in the B, C, and D stages of the tumor, and it was statistically significant throughout all cancer stages (P=0.016). Further analysis of the TNM staging system was also performed in patients with and without mutations in the *TP53* gene, in which stages III and IV showed higher frequencies of mutations with significant differences in all stages (P=0.041). There was also a significant association between the presence of the mutation and the histological differentiation of tumor (P=0.025), and most of the tumors with *TP53* mutations were moderately differentiated. However, no significant association was observed between the presence of mutation and tumor location (P=0.559) (Table 1).

Frequencies of TP53 gene mutations in tumor samples

PCR-SSCP analysis of *TP53* gene exons 5-7 of tumor tissue and stool samples resulted in the detection of different patterns in 19 patients (Figure 1-3). After the sequencing analysis of

patients with different patterns, a total of 27 point mutations were found in exons 5-7 of the *TP53* gene, of which 22 were missense (81.5%), 3 were nonsense (11%), and two demonstrated silent mutations (7.4%). Exon 7 was the most involved exon, comprising 40.74% of the *TP53* gene mutations, and codon 245, with 11.11% of *TP53* mutations, reserved the highest mutation rate (Table 2).

Evaluation of TP53 gene mutations in stool specimens

The stool specimens of CRC patients were also examined for the *TP53* gene mutations (Figure 1-3). Of the 27 identified mutations in the tumor tissues, 22 (81.5%) were also detected in the feces of patients, and there was no significant difference between the mutations in the tumor and the stool (*P*=0.063) (Table 2).

Discussion

The current study was the first to assess64 CRC patients from the Iranian Azeri Turkish ethnic group for the 5-7 exons of the *TP53* gene. The overall results showed that 19 out of 64 patients (29.7%) carried at least a *TP53* gene mutation. All of the mutations identified in this study had been previously reported by other authors in different ethnicities. Although most of the 27 point mutations were categorized as nonsense and/or missense, two silent mutations were also detected in exons 5 and 7, possibly affecting the inactivation of the splice sites.[28]

Our findings are broadly consistent with several reports around the world. Yamashita et al., Chang et al., and Piaskowski et al. observed *TP53* gene mutations in 25%, 43%, and 22.5% of patients with CRC in Japanese, Taiwanese, and Polish populations, respectively.[19, 29, 30] In addition, reports from central Iran (Golmohammadi et al.), northern Iran (Mahdavinia et al.), and southeastern Iran (Lohrasbi Nejad et al.), which are ethnically and geographically similar to the Turkish Azeri population, indicated similar mutation rates of the *TP53* gene.[25, 26, 31] Based on the analysis of direct sequencing, there was more than one mutation in the five patients (7.8%) with colorectal cancer, which is analogous to Mahdavinia et al..[31]

Further evaluation of clinical characteristics in this study proposed that the mutation rates in males were more than in females (58% and 42% in males and females). Although the difference did not reach a statistically significant level, complementary studies may have relevant explanations for the male predominance in colorectal carcinogenesis and the *TP53* gene alterations.[32] Moreover, assessment of Duke's stage and TNM stage in patients with *TP53* mutations indicated high rates of gene mutations in B, C, and D Duke's stages (*P*=0.016) as well as the III and IV TNM stages (*P*=0.041), which is in line with the findings of Dong et al., Ghavam-Nasiri et al., and Wang et al.[33, 34, 35] Tumor differentiation was further examined in *TP53* mutated patients, and almost 90% were well and/or moderately differentiated (*P*=0.025), a frequency that is similar to the reports from central Iran.[25] Contrary to Leahy et al., the colon showed a more significant number of mutations in the *TP53* gene compared with the rectum. However, there was no significant difference between mutated andunmutated patients.[27]

We also tried to investigate exons 5-7 of the *TP53* gene mutations in the stool specimens of patients with colorectal cancer. Analysis of the PCR-SSCP and sequencing data demonstrated that 22 out of the 27 (81.5%) discovered mutations in the tumor specimens were also detected in patients' fecal, whichis far higher than that reported by Eguchi et al.(36%).[36] Based on McNemar method, there was no significant difference between the two groups in terms of the number of mutations observed in the tumor and the stool (*P*=0.063).

Conclusion

This was the first attempt at evaluating the *TP53* gene mutations in tumor and stool specimens of CRC patients from the northwest of Iran. Based on our observations, a significant number of patients with CRC had mutations in one or more loci of exons 5-7 of the *TP53* gene, and there were correlations between mutations in the *TP53* gene and tumor differentiation and Duke's and TNM stages. Also, the rate and type of mutations in the *TP53* gene seemed to be similar to other Iranian and non-Iranian populations. These results may help researchers understand the role of *TP53* gene mutations in CRC among different populations with different lifestyles and genetic backgrounds. In addition, findings from the fecal analysis of the patients confirmed the same mutations in the tumor specimens of most cases; this can be used as a powerful and non-invasive tool for the early diagnosis and prognosis of patients with

genetic susceptibility to colorectal cancer. Finally, it should be noted that the promotion of the fecal DNA purity could increase the accuracy and efficiency of this diagnostic method.

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Conflict of Interest
None declared.

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Table 1. Clinicopathologic characteristics of colorectal cancer patients with and without *TP53* mutations

	***TP53* Gene**		
Variables	**Normal**	**Abnormal**	**P-Value**
Age	60±8	61±6	0.653*
Sex			0.251‡
Male	19 (42.2%)	11 (57.9%)	
Female	26 (57.8%)	8 (42.1%)	
Duke's Stage			0.016‡
A	20 (44.4%)	2 (10.5%)	
B	14 (31.1%)	6 (31.6%)	
C	10 (22.2%)	8 (42.1%)	
D	1 (2.2%)	3 (15.8%)	
TNM Stage			0.041‡
I	24 (53.3%)	5 (26.3%)	
II	10 (22.2%)	3 (15.8%)	
III	10 (22.2%)	8 (42.1%)	
IV	1 (2.2%)	3 (15.8%)	
Tumor Differentiation			0.025‡
Well	28 (62.2%)	8 (42.1%)	
Moderate	7 (15.6%)	9 (47.4%)	
Poor	10 (22.2%)	2 (10.5%)	
Tumor Location			0.559‡
Colon	35 (77.8%)	16 (84.2%)	
Rectum	10 (22.2%)	3 (15.8%)	

* Mann-Whitney U-test

‡ Pearson's Chi-square

Table 2. Features of the *TP53* gene mutations in exons 5-7 in the tumor and the stool of the colorectal cancer patients from Northwest Iran, sorted by the tumor differentiation, exon, codon, mutation, amino acid change, and mutation category

Case No.	Gender	Age	Tumor Differentiation	Exon	Codon	Mutation	Amino Acid Change	Mutation Category	Stool	P-Value
2	Male	61	Moderate	6	212	ACT > CCT	Thr > Pro	Missense	+	0.063*
3	Female	58	Well	5	135	TGC > TTC	Cys > Phe	Missense	+	
10	Female	60	Well	5	181	CGC > CCC	Arg > Pro	Missense	+	
10	Female	60	Well	7	236	TAC > TGC	Tyr > Cys	Missense	+	
10	Female	60	Well	7	242	TGC > TCC	Thr > Ser	Missense	+	
14	Male	52	Poor	5	157	GTC > GTN	Val > Val	Silent	-	
19	Female	55	Well	7	245	GGC > GAC	Gly > Asp	Missense	-	
21	Female	62	Well	5	175	CGC > CAC	Arg > His	Missense	+	
21	Female	62	Well	7	250	CCC > CTC	Pro > Leu	Missense	+	
25	Female	55	Moderate	7	248	CGG > TGG	Arg > Trp	Missense	+	
27	Male	64	Moderate	6	196	CGA > TGA	Arg > Stop	Nonsense	+	
30	Female	51	Well	5	184	GAT > AAT	Asp > Asn	Missense	+	
30	Female	51	Well	6	213	CGA > TGA	Arg > Stop	Nonsense	+	
31	Male	69	Moderate	5	157	GTC > AGC	Val > Ser	Missense	-	
34	Male	78	Moderate	7	245	GGC > AGC	Gly > Ser	Missense	+	
36	Male	66	Well	6	220	TAT > TGT	Tyr > Cys	Missense	+	
37	Male	60	Poor	7	255	ATC > ATA	Ile > Ile	Silent	+	
41	Male	57	Well	5	127	TCC > TTC	Ser > Phe	Missense	+	
41	Male	57	Well	6	195	ATC > ACC	Ile > Thr	Missense	+	
41	Male	57	Well	7	225	GTT > GCT	Val > Ala	Missense	+	
41	Male	57	Well	7	245	GGC > AGC	Gly > Ser	Missense	+	
43	Male	58	Well	5	167	CAG > CGG	Gln > Arg	Missense	-	
43	Male	58	Well	6	213	CGA > TGA	Arg > Stop	Nonsense	+	
46	Female	66	Moderate	6	220	TAT > GAT	Tyr > Asp	Missense	+	
49	Male	57	Moderate	7	248	CGG > CAG	Arg > Gln	Missense	+	
56	Female	64	Moderate	7	255	ATC > TTC	Ile > Phe	Missense	-	
60	Male	59	Moderate	5	175	CGC > CAC	Arg > His	Missense	+	

* McNemar Test

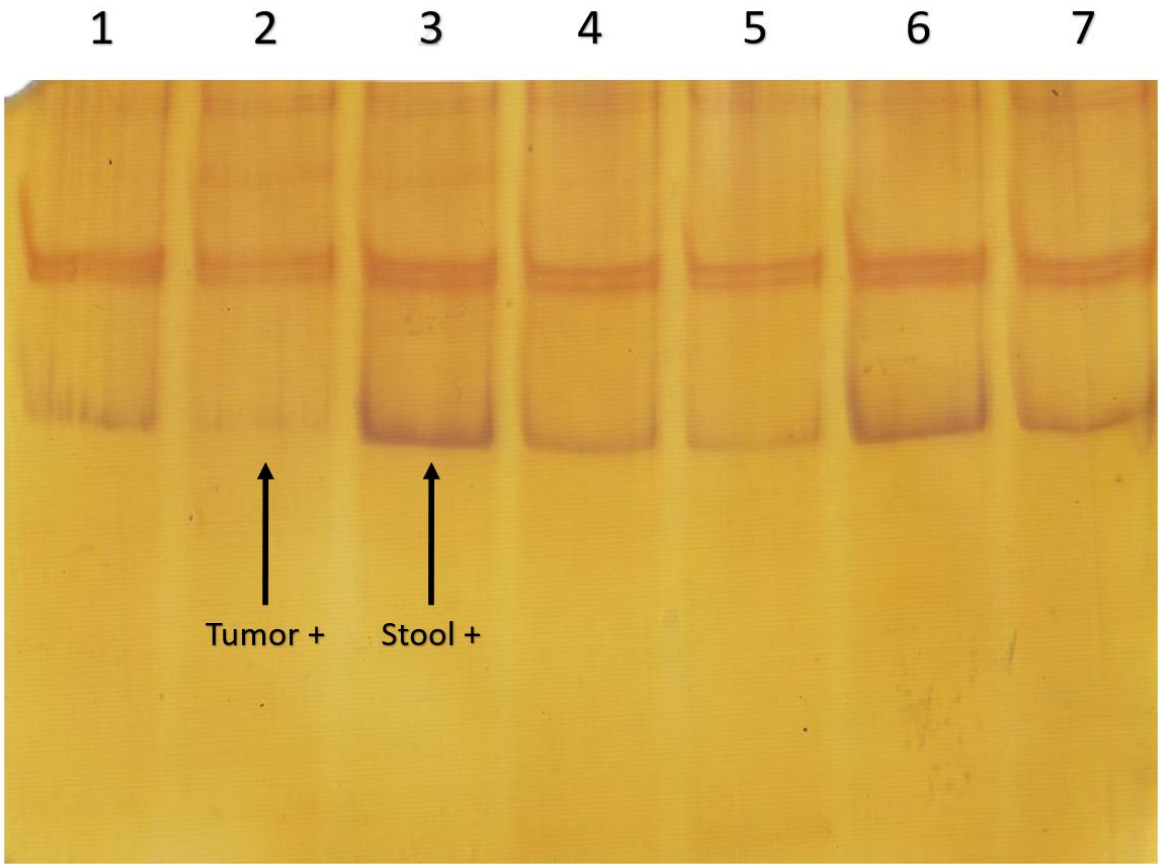

Figure 1. PCR-SSCP analysis of exon 5 of *TP53* gene from colorectal cancer tumor tissue and stool. Arrows are indicating shift bands in lane 2 (FT30) and lane 3 (ST30).

FT= Fresh Tissue, ST=Stool

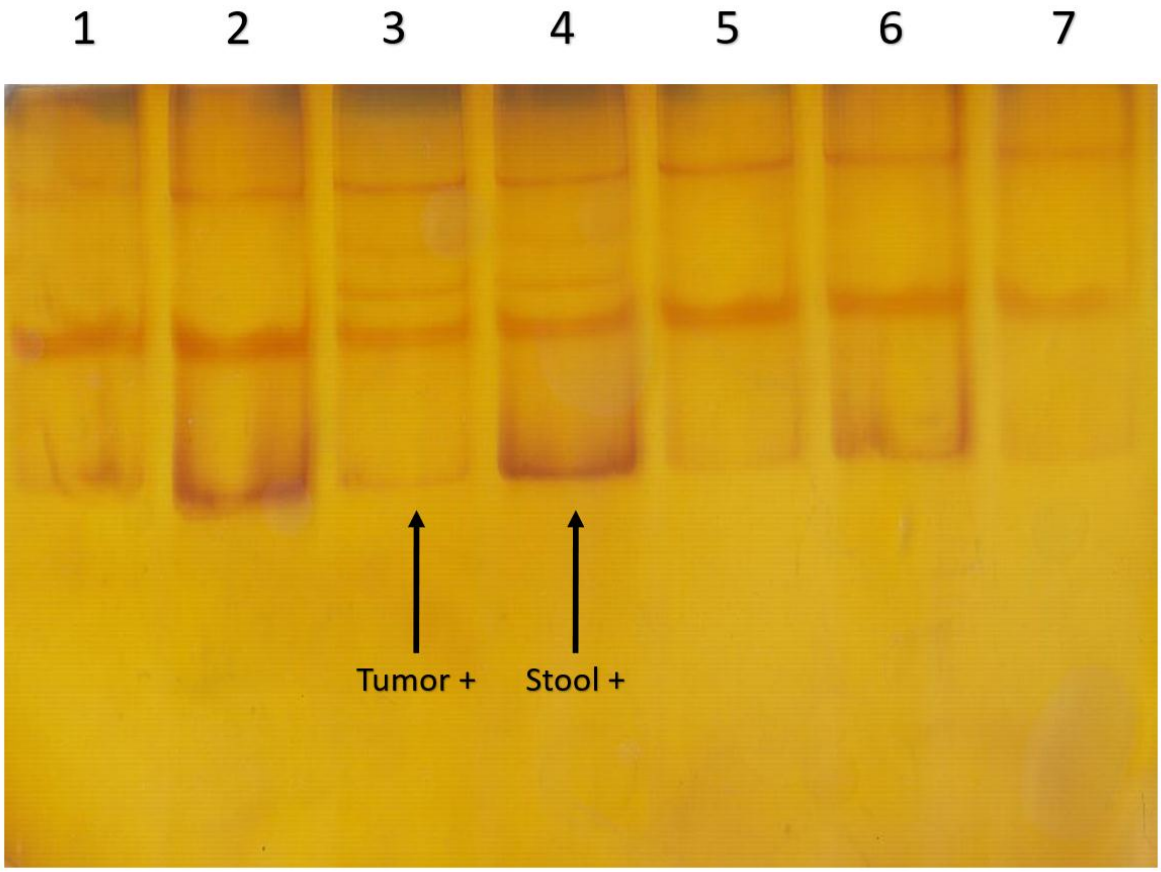

Figure 2. PCR-SSCP analysis of exon 6 of *TP53* gene from colorectal cancer tumor tissue and stool. Arrows are indicating shift bands in lane 3 (FT2) and lane 4 (ST2).

FT= Fresh Tissue, ST=Stool

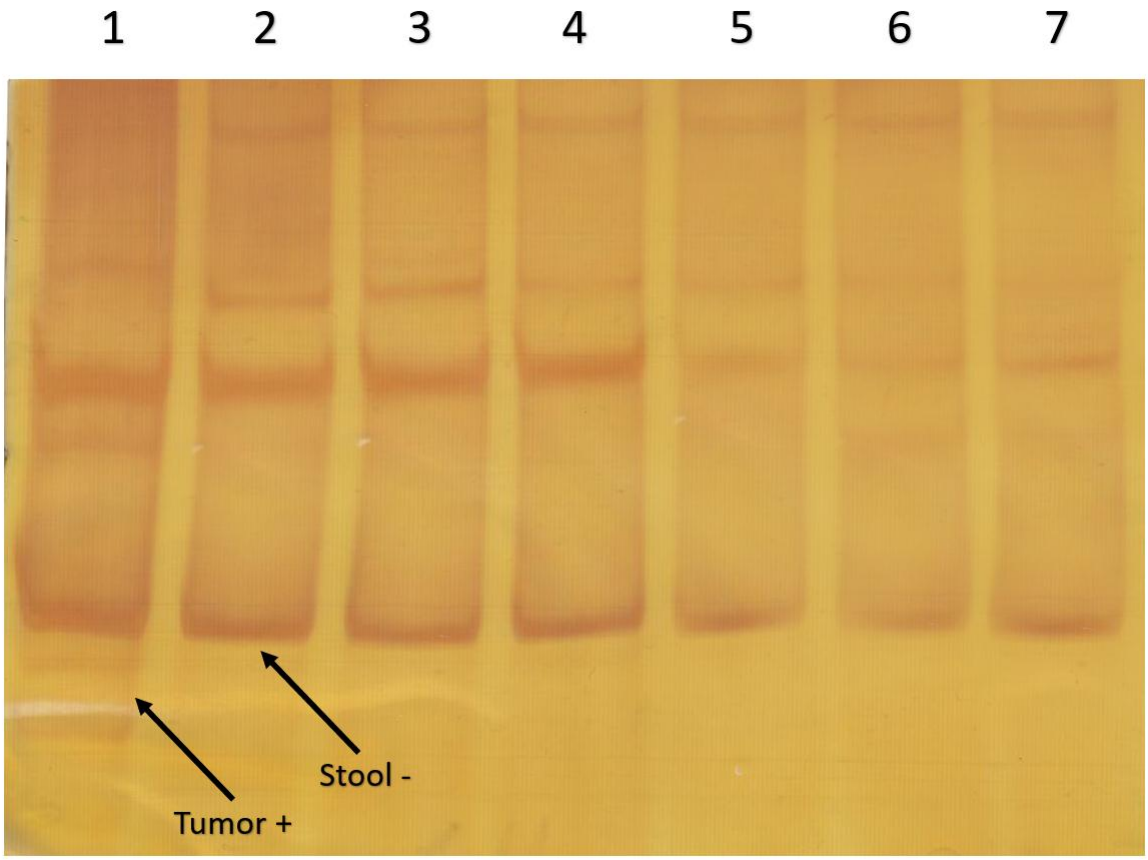

Figure 3. PCR-SSCP analysis of exon 7 of *TP53* gene from colorectal cancer tumor tissue and stool. Arrows are indicating shift band in lane 1 (FT19) but not in lane 2 (ST19).

FT= Fresh Tissue, ST=Stool