Molecular characterization of TP53 tumor suppressor gene in colorectal cancer

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Abstract

Introduction: Colorectal cancer (CRC) is one of the most common malignancies in the world, especially in developed countries. In Colombia, the incidence of CRC ranks fourth in men and women. CRC has great genetic heterogeneity. **Purpose:** The purpose of this study was to determine the presence of mutations in exons 5 to 8 of the TP53 gene in colorectal tumors by direct sequencing. **Patients and Methods:** Samples with histopathological diagnoses of sporadic CRC were divided into two groups. Group I included 30 tumor samples from fresh biopsies and Group II included 46 tumor tissue samples embedded in paraffin blocks. Mutational analysis was performed for exons 5 through 8 of the TP53 gene using PCR and direct sequencing. **Results:** The frequency of TP53 mutations was only 4.4%, and mutations that were detected were nonsense mutations. In addition, two polymorphisms that segregate together were identified. All mutations and polymorphisms were detected in samples from Group I. Most of the samples were in advanced stages of cancer. **Conclusions:** The low frequency of mutations in TP53 suggests the existence of alterations on other related genetic pathways in colorectal carcinogenesis. These could include MSI pathways, CIN and epigenetics. Such alterations could not be excluded in the samples tested. Molecular studies of tissue samples embedded in paraffin are difficult to analyze genetically. Molecular characterization of CRC is important for determining the spectrum of mutations and molecular variants present in our population.

Keywords

Colorectal cancer, TP53 gene, genetic heterogeneity, carcinogenesis, genetic instability.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common neoplasms in the world. This is especially true in developed countries where it is considered the third leading cause of cancer and the second leading cause of death in cancer patients. (1) Globally, more than 850,000 people develop CRC annually, and more than 500,000 die from this disease. (2) CRC represents 9.4% of all cancers in men and 10.1% in women, but its frequency varies geographically. (2) In Colombia, incidence of CRC and deaths due to CRC increased between 1962 and 2005. (3) According to data from GLOBOCAN 2008, CRC ranks sixth in cancer incidence and fourth in mortality in Colombia for both men and women. There are 4,107 new cases per year most of which are detected in advanced stages. (1)

Multiple genetic and environmental factors including race, diet, obesity, and smoking have strong impacts on risks and stimulate the emergence of CRC. (4) About 80% of CRC cases occur sporadically while the remaining 20% are related to family history and germline mutations with hereditary predispositions to CRC in APC, MLH1, and MSH2 genes. (5, 6, 7)

CRC has a high degree of genetic heterogeneity with various molecular pathways such as the suppressor pathway explaining its development. The most common pathway is

initiated by mutations in the sequence of APC, K-RAS, and TP53 genes. (8) A second route is microsatellite instability (MSI) which is associated with mutations in genes of the mismatched repair system which are mainly in MLH1 and MSH2 genes. These mutations occur though expanding repeated short nucleotide sequences. (9) Other routes are related to CRC epigenetically. (7) These are characterized by methylation of the promoter of several genes which leads to the inactivation of gene expression and growth factors. (7, 10, 11) All these mechanisms have great significance in the predisposition, initiation, and progression of CRC.

On the other hand, the TP53 tumor suppressor gene is located at 17p13.1 region, and encodes a nuclear phosphoprotein, p53, which acts as a transcription factor. It is involved in various essential cellular functions such as cell cycle control, DNA repair, apoptosis, and response to damage to the genome. As a result, it is called the "guardian of the genome". (12) Mutations in this gene are common in CRC and other neoplasms. It is important to identify them because they are associated with poor prognoses, advanced stages of cancer, and resistance to certain treatments. (13) Consequently, TP53 has been proposed as a useful genetic biomarker for diagnosis and prognosis of diverse neoplasms. (14)

OBJECTIVE

The objective of this study was to determine the presence of mutations in exons 5 to 8 of the TP53 gene in colorectal tumors by direct sequencing.

MATERIAL AND METHODS

Patients and samples

Colorectal tumor samples analyzed in this study were divided into two groups: 30 Group I samples, obtained from fresh tumor biopsies, and 46 Group II samples from paraffine embedded tumor tissues. Group I samples were obtained from patients undergoing surgical resections or biopsies of a primary tumor as the Hospital Universitario San Vicente de Paúl in Medellín. This group of patients had 19 women and 11 men with a mean age of 69 years (age range: 25 to 86 years). Each patient's voluntary participation was requested with his or her approval indicated by signed informed consent. None of the patients had a history of cancer, or cancer treatment. Group II samples were selected from the histopathology archive in the department of Pathology at the University of Antioquia from the years 2004 to 2006. These samples came from 27 women and 19 men with a mean age of 61.4 years (age range: 23 to 88 years). A pathologist checked the histopathological diagnoses of all tumor samples. In addition, personal history information was obtained for all of these cases. Five ml of peripheral blood from five healthy individuals stored in EDTA tubes were used as controls.

DNA Extraction

DNA was obtained from fresh tumor tissue biopsies from Group I, and peripheral blood lymphocytes were obtained from the controls using the QIAamp Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions.

For Group II tumor tissue samples embedded in paraffin blocks were cut into 4um pieces with a microtome prior to DNA extraction. Plate tissue cuts were stained with eosin/hematoxylin to identify and demarcate the tumor area. Subsequently, tumor cells were isolated by microdissection, and their DNA was extracted using QIAamp ® DNA Mini Kit (Qiagen). Several cuts were transferred to an eppendorf tube containing 1 ml of xylene for deparaffinization of samples, then 1 ml of 100% ethanol was added, and the samples were dried at 37°C. Samples were then digested with proteinase at 55°C for 24 hours. Following digestion, the isolated DNA was stored at 4°C in TE buffer.

DNA Amplification

The extracted DNA was amplified by PCR in a 9700 thermocycler (Applied Biosystems, USA) using two primer pairs to amplify exons 5 to 8 of the TP53 gene. One pair was used for the amplification of exons 5 and 6, and the other was used for exons 7 and 8. PCR was performed in a 35 ul volume containing 300ng of DNA, or 100ng of DNA from peripheral blood in a reaction mixture concentration of 1X to 10X reaction buffer, 1.0 mM $MgCl_2$, 200 uM dNTPs, 0.4 uM of each primer, and 1.4 U of Taq DNA polymerase (Invitrogen). PCR conditions were: 94°C for 3 min, then 35 cycles of 94°C for 1 min, 1 min at 56°C for primers corresponding to exons 5 and 6, 59°C for primers corresponding to exons 7 and8, 72°C for 40 seconds, and finally 7 min at 72°C. Agarose gel electrophoresis was used to determine the size and quality of the amplified fragments which were then stained with 2% of 8 ul of ethidium bromide at a concentration of 1 ng/ml. The amplified products were stored at -20°C until sequencing.

Sequencing

Amplicons were purified before sequencing. Both strands of the amplified fragments were sequenced using the same PCR primers. Direct sequencing was performed on a 3730xl DNA Analyzer (Applied Biosystems). Chromatograms

were edited and analyzed with Chromas Pro Plus program. Then they were compared alignment with reference sequences reported in GenBank (N-T010718.16.67).

Whether or not the protein had changed was determined using Spidey (NCBI). Positions at which changes occurred were determined with Expasy. Using ClustalW, a multiple sequence alignment program, these protein sequences were compared with a reference sequence published in GenBank. The reference sequence used was p53 protein gi | 23491729.

RESULTS

Group I consisted of 30 colorectal tumor samples obtained from fresh biopsy specimens. It was observed that 26 (87%) were from colon samples, and 4 (13%) from the rectum. Five of these samples were from patients younger than 50 years old.

According to histopathological studies all samples of this group had advanced stages of cancer, they were classified as adenocarcinomas in 93.3% (28/30), and neuroendocrine carcinomas 6.7% (2/30). Regarding the histological type of tumors, adenocarcinomas were well differentiated in 56.7% (17/30) of the samples, 23.3% (7/30) moderately differentiated, and 6.7% (2/30) mucinous. In two of the cases it was not possible to determine the degree of differentiation. Table 1 represents clinical histopathological description of CRC population evaluated.

Group I samples were mainly located in the descending colon in 53.3% (16/30) of the samples, 20% (6/30) in the ascending colon, 10% (3/30) in the transverse colon, and 13.3% (4/30) in the rectum. In one case the initial tumor site could not be established. In addition, it was found that 46.7% (14/30) of the samples were in stages III and IV (Table 1).

On the other hand, all tumor samples from Group I were successfully analyzed for exons 5 to 8 of the TP53 gene with a mutation frequency of 6.7% (2/30). The two identified mutations were located in exon 6 generating a stop codon (Table 2, Figure 1). Particularly in the case 032, a mutation by two neighboring base substitutions, which generated a stop codon changing the trinucleotide CTG to TAG. That translation corresponded to a G > T transversion, and a C> T transition (Table 2) In addition, genetic analysis of TP53 by direct sequencing allowed identification of two polymorphisms which are presented simultaneously in 16.7% of samples (5/30), and reported in the database of the International Agency for Research on cancer (IARC) as g.13491 C >T, and g.13511 T>G (15) (Table 2, Figure 1). It is noteworthy that these two polymorphisms were also identified in two healthy individuals.

Table 1. General description of the clinicopathological information from CRC samples evaluated in both groups.

	Group I Fresh tissue n (%)	Group II Paraffin-embedded tissue n (%)
Number of patients	30	46
Age (mean)	62	60
Gender		
Female	19 (63.3)	27 (58.7)
Male	11 (36.7)	19 (41.3)
CRC stage		
I	3 (10)	4 (8.7)
II	10 (33.3)	22 (47,8)
III	2 (6.6)	16 (34.8)
IV	10 (33.3)	4 (8.7)
Unclassified	5 (16.7)	0
Histological type		
Well differentiated adenocarcinoma	17 (56.7)	32 (69.6)
Moderately differentiated adenocarcinoma	7 (23.3)	10 (21.7)
Poorly differentiated adenocarcinoma	0 (0)	1 (2.1)
Mucinous adenocarcinoma	2 (6.6)	1 (2.1)
Neuroendocrine tumor	2 (6.6)	0 (0)
Others	2 (6.6)	0 (0)
Location of tumor		
Colon	26 (87)	32 (69.6)
Ascendent colon	6 (20)	10 (31.25)
Transverse colon	3 (10)	2 (6.25)
Descendent colon	16 (53.3)	20 (62.5)
Rectum	4 (13.3)	14 (30.4)
Unclassified	1 (3.3)	0 (0)

Group II sample tissues, embedded in paraffin blocks, 32 (69.6%) were colon samples, and 14 (30.4%) rectum samples. Nine of the 46 samples were from patients younger than 50 years old.

Regarding histopathology samples in this group, we observed that all had an advanced stage of cancer, 100% (46/46) were classified as adenocarcinomas. With respect to the histological type of tumors, 69.6% (32/46) of cases were classified as well-differentiated adenocarcinoma, followed by 21.7% (10/46) of moderately differentiated, and a case (2.1%) poorly differentiated and mucinous. In two cases it was not possible to determine the degree of differentiation. Histopathologic and clinical description of the population with CRC is shown in Table 1.

The colorectal tumor samples in Group II were found most frequently in the descending colon (62.5%), followed by the ascending colon (31.25%), 6.5% in transverse colon, and 30.4% (14/46) in the rectum. 5.1% of 43 samples were classified in stages III and IV (Table 1).

The overall results of the 76 samples analyzed in groups I and II showed that 58 (76.3%) were originated in the colon, and 18 (23.7%) in the rectum (Table 1). 97.4% (74/76) were classified as adenocarcinomas, and from these the most common histologic type observed was the well-differentiated adenocarcinoma with 64.5% (49/76), followed by 22.3% (17/76) of moderately differentiated. As for the location of tumors in the colon, it was found that most of the cases, 62.1% (36/58) were in the descending colon, 27.6% (16/58) in the ascending colon, and 8.6 % in the transverse. Furthermore, 50% of the samples (38/76) were classified into advanced stages III and IV.

Furthermore, the results of the sequences for the mutation analysis of TP53 in the 46 samples of group II showed no results in 8 samples. From the remaining 38, only 15 samples (39.5%) succeeded the analysis of mutation for exons 5-6 and 7-8. In 21 samples only exons 7 and 8 sequences were found (no results for exons 5 ad 6), and in two samples only exons 5 and 6. From the entire group II sequences analyzed no mutations or polymorphisms were detected in exons 5 to 8 of TP53 gene (Figure 1). In summary, the overall frequency of TP53 mutations in 45 samples in which it was possible to make a complete analysis of mutations for exons 5 to 8 was 4.44% (2/45).

DISCUSSION

CRC is characterized by a great genetic heterogeneity. Different genetic pathways have been described where mutations are frequent events in the TP53 gene. In this work, molecular characterization was performed in order to identify mutations in the TP53 gene, in two groups of sporadic colorectal tumors, one from fresh tissues, and the other comprised paraffin embedded tissue.

Table 2. Mutations and polymorphisms identified in the TP53 gene from CRC samples

	Exon/ Intron	Position	Codon	Mutation	Aminoacid changes	Types of change
Mutations	Exon 6	c.562C>T c.563T>A	188	CTG→TAG	p.L188STOP	Substitution
	Exon 6	c.595G>T	199	GGA→TGA	p.G199STOP	Substitution
Polimosphisms	Intron 7	c.782+72C>T	-	rs12947788	-	Substitution
	Intron 7	c.782+92T>G	-	rs12951053	-	Substitution

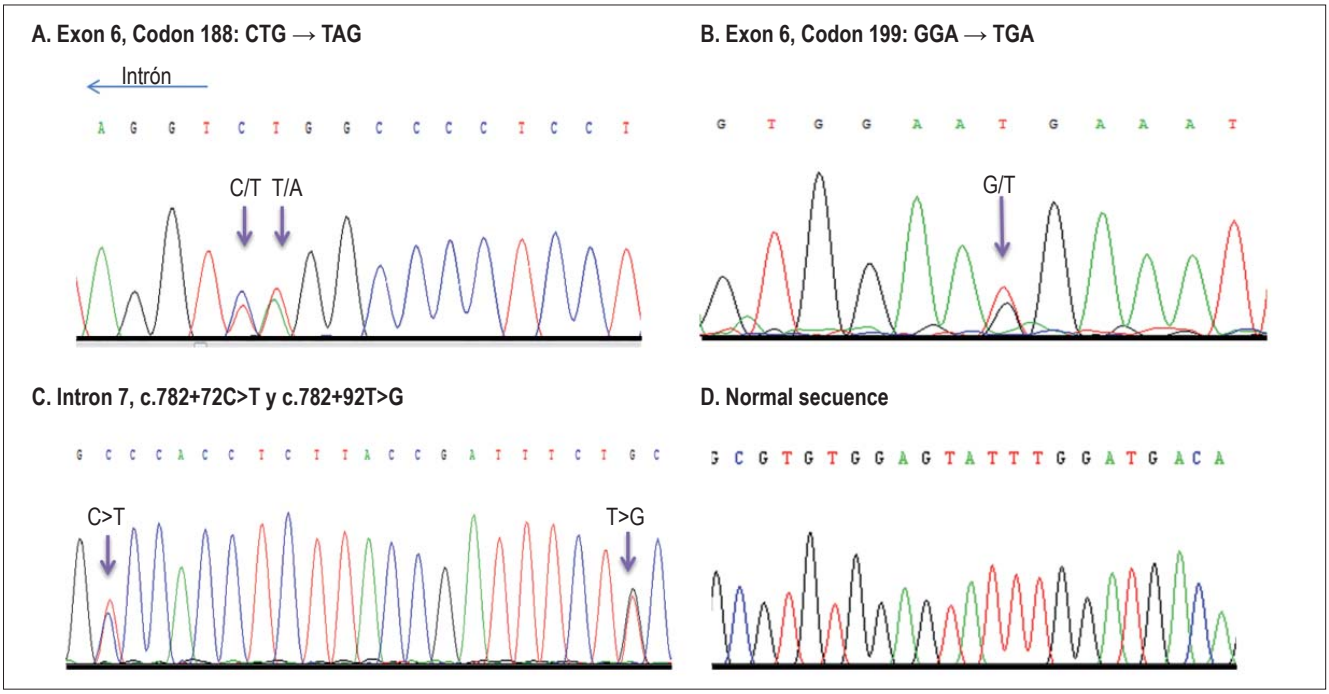

Figure 1. Chromatograms obtained from direct sequencing of TP53 gene in CRC samples. A. Mutation at codon 188 of exon 6. B. Mutation at codon 199 of exon 6. C. Polymorphisms simultaneously identified in intron 7-8. D. Normal sequence of exon 5 in a paraffin-embedded tumor sample tissue.

Histopathological studies of the analyzed samples from the two groups showed a very high frequency of adenocarcinomas (97%), from which 50% of the samples were in an advanced stage of cancer. Also, more than half of the colon tumor samples examined was located in the descending colon. These findings are similar to those reported in the literature for sporadic CRC. Thus, this study corroborates the case of Colombia where cancer is diagnosed at an advanced stage.

Regarding the molecular characterization, a low frequency (6.7%) of mutations of the TP53 gene was found in fresh tumor samples, and no mutations were identified in the paraffin embedded tissue samples (Group II). Neither in the 15 samples in which it was possible to completely analyze exons 5 through 8 or in the 21 samples in which it was possible to analyze only exons 7 and 8. In summary, overall mutation frequency was 4.4% in 45 samples from the two groups, in which exons 5 to 8 were completely analyzed. The frequency obtained was significantly lower than other studies with sporadic CRC in different populations. (12,16) Frequency differences may be explained by the following reasons: first, in this study all exons of TP53 gene were not sequenced, only 5 to 8 in which we have identified more than 80% of the mutations. Thus, one could not exclude the possibility of mutations in other exons that were not tested. The great genetic heterogeneity, evidenced by multiple genetic pathways, occurring in colorectal carcinogenesis must also be considered. It is noteworthy that the absence of mutations in key genes of CRC carcinogenesis, as observed in the present study, may be due to independent molecular pathways of TP53 mutation such as microsatellite instability (MSI). This condition occurs in 20% of all sporadic CRC, besides, it has an inverse relationship with TP53 gene mutations. (17) MSI pathway is also associated with methylation during CRC development, specifically in tumors with high MSI. These are related with MLH1 methylation, and BRAF gene mutation (18). In a previous study by our group, 41 samples of CRC presented a MSI frequency of 34.1% (19). In addition, in cases of tumors with high MSI, no mutations were detected in repair genes MLH1 and MSH2, which led us to conclude that MSI occurs as an independent and common molecular pathway in colorectal carcinogenesis. From this conclusion, one could suggest that tumor samples analyzed in this work, without TP53 mutations, the presence of MSI cannot be ruled out, which will be evaluated in the next phase of the study.

Moreover, it has also been observed in the literature a high frequency of chromosome instability (CIN) in samples from colorectal adenocarcinomas. In a study previously published by our group, a high frequency of aneuploidy of chromosome 17, as well as a high percentage of deletion in the locus 17p13.1 of TP53 gene was found in gastrointestinal tumor samples. (20) From these studies, one can conclude that the above are common chromosomal abnormalities in the development of gastrointestinal tumors. Therefore, these abnormalities cannot be excluded in tumor samples without TP53 mutations.

It should be mentioned that it is interesting that a low frequency of mutations in key genes related to colorectal carcinogenesis was observed in several molecular characterization studies of CRC performed by our group. In more than 110 tumor samples that have been assessed, a low frequency of mutations in key genes related to colorectal carcinogenesis is observed, contrary to those reported in the literature. We have observed a high frequency of MSI and CIN instabilities, which allows us to classify them in the group of tumors that develop in the genetic pathways of MSI and CIN, rather than the route of point mutations. The variability in the frequency of TP53 mutation published in different populations may be explained by ethnic and geographic differences, as well as lifestyle and diet. Molecular epidemiology studies report that exogenous carcinogens, such as exposure to ultraviolet light and various chemicals and infectious agents induce TP53 mutations in this gene.

Fearon and Vogelstein proposed a molecular colorectal carcinogenesis model to explain the transformation of adenoma into carcinoma, in which a sequence of mutations in the APC, KRAS, and TP53 genes was described. (21) It is now proposed that mutations in these genes occur in different pathways given that genetic heterogeneity of CRC not always follows this pattern. Mutations in other genes such as BRAF, Pik3, β-catenin, MLH1, and LOH in the 8q24 region, confirm the existence of multiple pathways of colorectal carcinogenesis (18).

Concerning the two polymorphisms identified in this work, g.13491 C>T and g.13511 T>G, these have been reported in the literature. So far there are no studies associating both polymorphism with the risk of developing CRC. However, in recent years, several studies scan the full genome (Genome Wide Association Studies, GWAS), and they report several SNPs associated with CRC risk.

Furthermore, molecular analysis of exons 5 to 8 of TP53 gene were successful in all fresh tumor samples from Group I, compared with those obtained with paraffin embedded tissue samples from Group II. In these samples greater difficulties occurred during the extraction and DNA amplification, therefore, the genetic results were less successful due to the storage conditions of the samples. From these, only 15 samples (39.5%) had a complete analysis of mutations in exons 5 to 8 affecting the size of the initial sample, thus, lower probability to detect mutations. With respect to this issue, extracting nucleic acids (DNA and RNA) of good quality and purity from paraffin-embedded tumor tissue and formalin-fixed has many difficulties, and it is a challenge for researchers. The main difficulty is the complete paraffin removal, for which several methods exist such as

the heating of deparaffinized sample or the use of organic solvents. However, the integrity and quality of the DNA is affected during this procedure, resulting in a degraded DNA. (22, 23) Besides, the conditions of fixation with formaldehyde facilitate the formation of cross-links between nucleic acids and proteins, also affecting the quality and purity of the extracted DNA. Hence, it is difficult to amplify DNA in these conditions since PCR is inhibited, decreasing genetic results in many pathological specimens. (23) This could explain why it was not possible to obtain genetic results in several samples of our study. (22)

Finally, this study found a low frequency of mutations in the TP53 gene in samples with sporadic CRC, which differs significantly from those reported in the literature, suggesting the existence of several independent genetic pathways of TP53-related CRC carcinogenesis. The results obtained in this study, as the others obtained by our group, highlight the importance of molecular characterization of CRC. It provides important information about the spectrum of mutations and genetic variants in our population for further comparisons with others, and their relationship with clinical pathological feature of patients. The genetic alterations found must be validated in future studies with a larger sample size, and using other genomic techniques, in order to have a better understanding of the molecular etiology of CRC.

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