Association of intron microsatellite status and exon mutational profiles of TP53 in human colorectal cancer

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Abstract. Microsatellite instability (MSI) and loss of heterozygosity (LOH), which cause genomic instability, contribute to cancer pathogenesis. However, only few studies have evaluated the association of a single microsatellite locus of the TP53 gene with the mutation spectra of TP53 exons. A total of 256 patients with colorectal cancer were enrolled in the present study. MSI/LOH alterations of a microsatellite in the TP53 intron (TP53ALU) were assessed via short tandem repeat scanning. The exon mutation profile was evaluated by direct sequencing. The mutation rate of TP53 exons was significantly higher in tumors with LOH alterations of TP53 introns compared with those in tumors with a microsatellite-stable status in the TP53 intron (P=0.0047). TNM stage II was significantly more frequent in MSI vs. LOH or MSS of the TP53 intron (P=0.027 and P=0.048, respectively). Thus, microsatellite alterations may be valuable predictors of TP53 exon mutation and the TNM stage of colorectal cancers.

Introduction

Microsatellites (MS), short tandem repeat sequences, are composed of 1-6 base pairs (bps). Approximately 1 million microsatellite loci, mostly as $(CA)_n$, are dispersed in the introns and exons of the human genome. Simple DNA repeats are prone to expansion/contraction via the formation of secondary structures during DNA synthesis. Such structures inhibit replication forks and create opportunities for template-primer slippage, making these repeats unstable (1). MS instability (MSI) is characterized by alterations in length within MS, resulting from mutational inactivation or epigenetic silencing of DNA mismatch repair genes [e.g. MutS homolog 1 (MSH1), MSH2, MSH3 and mutL homolog 1]. These mutations include coding region frameshift mutations caused by MSI, which may drive oncogenesis by inactivating tumor-suppressor genes or disrupting other non-coding regulatory sequences (2). Furthermore, as compared with the number of repeats in the germline genome, an abnormal number of repeats, in ≥30% of the microsatellite loci examined is defined as microsatellite instability-high (MSI-H). MSI-H is known to occur in ~10% of sporadic colorectal cancers (CRCs) and 3% hereditary CRCs (3).

CRC is the second and third most commonly diagnosed cancer type among females and males worldwide, respectively (4). CRC is a heterogeneous disease and may be divided into certain molecular subtypes. Molecular changes that occur in CRC may be categorized into three major groups: i) Chromosomal instability, ii) MSI and iii) CpG island methylation phenotype that silences gene function with aberrant hypermethylation (5). The following three major applications have been developed for the MSI phenotype: i) Genetic evaluation of Lynch syndrome; ii) predicting the response to chemotherapy drugs and iii) predicting the prognosis of CRC patients. Thus, the MSI status has an important role in the study of CRC.

Loss of heterozygosity (LOH) is another form of MS alteration, which may be caused by mutation in one allele of a gene. Early studies have demonstrated that 81% of patients with sporadic breast cancer and 93% of patients with sporadic cancer (10 types) exhibit missense mutation LOH (6,7). It was reported that a high frequency of LOH coincided with mutant P53 protein stabilization (8).

The human TP53 gene is located on chromosome 17p and comprises 11 exons and 10 introns (9). The p53 protein is a phospho protein consisting of 393 amino acids. Upon DNA damage, activation of p53 leads to cell cycle arrest, enabling the cells to repair the damaged DNA. Exon mutations in the TP53 gene are the most commonly observed genetic alterations in CRC with a prevalence of 50-70% amongst CRC cases (10). Loss of function of mutant p53 is a critical event in the progression of CRC. Such mutations, which provide clues about the mechanisms of genetic damage, tend to be differentially associated with other cancer-associated genetic alterations and have prognostic and clinical relevance (11).

Although the association of MSI and clinical features of patients with CRC has been widely investigated, differences

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between MSI and LOH alterations of a single MS in CRC have not been previously addressed, to the best of our knowledge, particularly the MS in the tumor suppressor gene TP53. Furthermore, the associations between MS alterations (MSI and LOH) in TP53 introns and mutations in TP53 exons remain elusive. In the present study, patients were stratified using different MS statuses, including MSI, LOH and MS-stable (MSS) on TP53 intron and the mutational profiles of exonsin the TP53 gene were evaluated. Furthermore, their association with clinicopathological characteristics in CRC was also explored. The present results revealed that MSI alterations in TP53 introns are a valuable predictive marker for the tumor-nodes-metastasis (TNM) stage of CRC and LOH alteration may be a useful marker for the TP53 exon mutation status.

Materials and methods

Patients. CRC samples were collected from the Clinical Data and Biobank Resource of Beijing Friendship Hospital (a specimen bank; Beijing, China) between November 2016 and November 2018. The establishment of the specimen bank requires informed consent from the patients, and therefore, ethical approval was obtained from the ethics committee prior to the start of the study. According to the TNM system classification of the American Joint Committee on Cancer (12), specimens of CRC at stage II/III were selected and the principal inclusion criteria were as follows: Histologically proven papillary/tubular adenocarcinoma, signet ring carcinoma and mucinous carcinoma of the colon or rectum. Specimens were collected from fresh tumors and matched normal tissues for the genetic analysis of MSI and LOH in a specific MS (TP53ALU). A total of 512 specimens from 256 CRC patients were stored at -80˚C and analyzed. Relevant clinical data were collected from the patients' medical charts. Vital status and cause of death were obtained from medical records, tumor registry correspondence or death certificate. The present study was approved by the institutional review board of the Beijing Friendship Hospital (Beijing, China).

Genomic DNA extraction. Genomic DNA was extracted from 256 pairs of CRC and their matched normal tissues using a standard phenol-chloroform extraction and ethanol precipitation method, as previously described (13). DNA was quantified using the absorbance ratio at 260/280 nm measured with a microplate absorbance reader (Bio-Rad 680; Bio-Rad Laboratories, Inc.) and then further analyzed by agarose gel electrophoresis. DNA samples were diluted to a concentration of 50 ng/μl and stored at -80˚C.

MS analysis. An MS locus in intron 1 of the tumor suppressor gene TP53 (referred to as TP53ALU) with the repeat unit $(AAAAT)_8$ was assessed. The sequences of the primers designed were as follows: 5'-GGCAATAAGAGCTGAGAC TCC-3' (sense) and 5'-GACAAAACATCCCCTACCAAA-3' (anti-sense). The forward primer of the locus was labeled at the 5' end with a fluorescent marker (labeled using 6-carboxyfluorescein) for later use in short tandem repeat (STR) scanning. The PCR amplification system contained 2 μl 10X buffer, 125 μmol/l dNTP (4X), 0.5 μmol/l of each primer, 1.0 units of Taq DNA polymerase, 1.5 mmol/l $MgCl_2$ and 100 ng template DNA. PCR was performed under the following conditions: Denaturation at 94˚C for 5 min; 35 cycles of denaturation at 94˚C for 30 sec, annealing at 62˚C for 30 sec and extension at 72˚C for 30 sec. This was followed by an extension step at 72˚C for 7 min. The PCR products were visualized on 2% agarose gels stained with ethidium bromide and assessed with an ultraviolet transilluminator (Gel Doc™ XR+; Bio-Rad Laboratories Inc.). Products amplified successfully and correctly by PCR were stored at 4˚C for subsequent STR scanning. Fluorescently tagged PCR products were examined with an ABI3730XL DNA Analyzer system (Perkin Elmer Biosystems). GeneMarker version 1.75 (Tianyi Juiyuan Company) was used to quantify each fluorescent PCR product. In order to confirm whether the new alleles exist, PCR products were purified using an ABI BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), cloned into the PMD18-T vector (Takara Bio Inc.) and then sequenced using an ABI 3730XL DNA sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Mutation analysis of exons of TP53. According to the MSI and LOH status of TP53 intron 1, 31 CRC and their paired normal tissues were selected for further analysis (Table I). A total of

Table I. Clinical features of patients with colorectal cancer (n=31).

Clinical feature	Value
Mean age (years)	68.26±9.2
Sex	
Male	20 (64.52)
Female	11 (35.48)
Drinking	
Yes	11 (35.48)
No	20 (64.52)
Smoking	
Yes	14 (45.16)
No	17 (54.84)
TNM stage	
II	12 (42.86)
III	16 (57.14)
Histologic grade	
Well/moderate	12 (41.38)
Poor	17 (58.62)
Adjuvant therapy	
Yes	13 (41.94)
No	18 (58.06)
Survival time (months)	
<12	3 (15.79)
12-36	8 (42.11)
>36	8 (42.11)

Values are expressed as n (%) unless otherwise specified. TNM, tumor-nodes-metastasis.

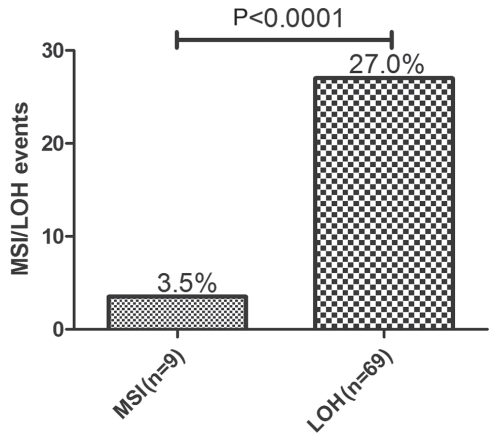

Figure 1. MSI and LOH alteration events of TP53ALU detected in colorectal cancers. The LOH rate of TP53ALU was significantly higher than that of MSI (P<0.0001). MSI, microsatellite instability; LOH, loss of heterogeneity; TP53, tumor protein 53.

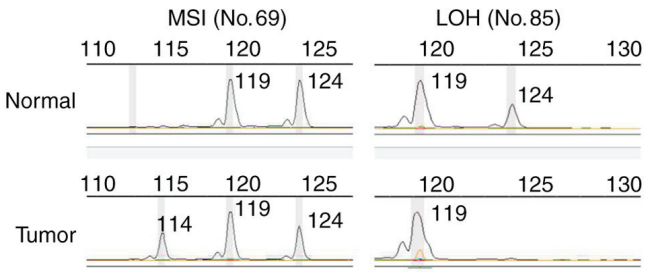

Figure 2. Alteration patterns of tumor protein 53ALU detected in colorectal cancers. The left row indicates the MSI mutation. The allele size in the tumor tissues was changed from 119/124 to 114/119/124 bp. The right row indicates the LOH mutation, with the allele size changed from 119/124 to 119/119 bp. MSI, microsatellite instability; LOH, loss of heterogeneity.

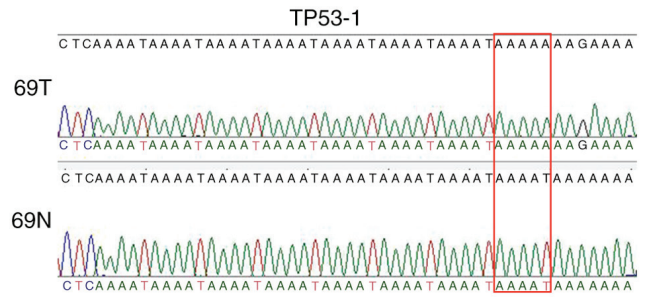

Figure 3. Sequencing result of microsatellite instability in TP53ALU. Compared with normal tissue, TP53ALU indicated deletion of one (AAAAT) repeat unit. 69T, tumor tissue of case no. 69; 69N, normal tissue of case no. 69; TP53, tumor protein 53.

11 primers were designed to screen for nucleotide alterations in exon 1-11 of the TP53 gene (Table SI). PCR-sequencing was performed using an ABI 3730XL DNA Sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Statistical analysis. Pearson's Chi-squared and Fisher's exact test were performed to explore the associations of MSI, LOH and MSS within the intron and nucleotide alterations of TP53 exons with clinicopathological characteristics.

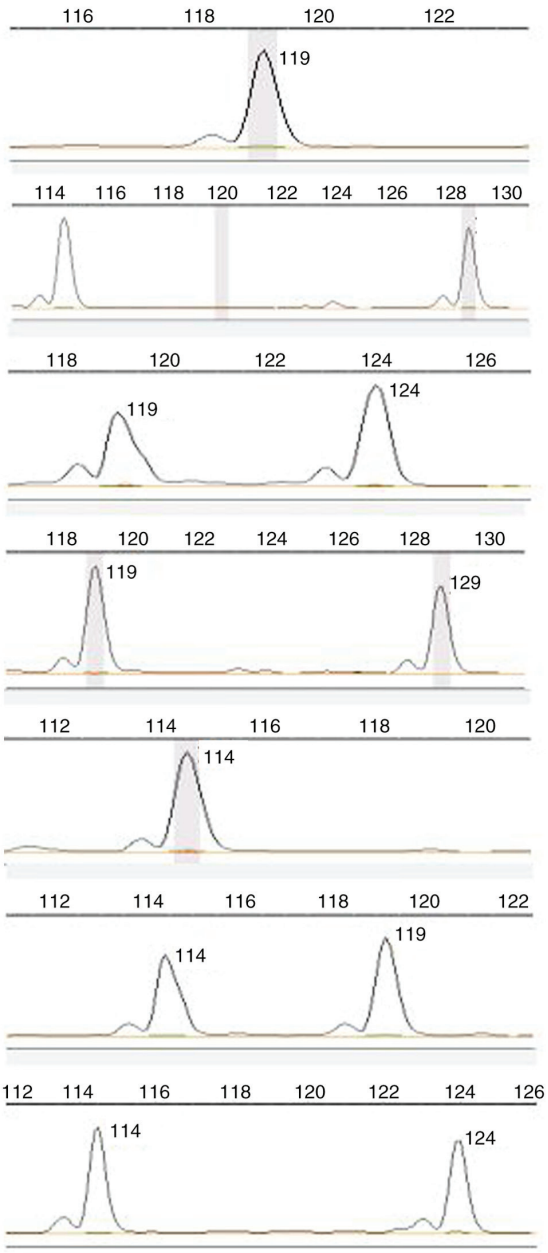

Figure 4. The 7 genotypes of tumor protein ALU in normal tissues. The genotypes from top to bottom were 119/119, 114/129, 119/124, 119/129, 114/114, 114/119 and 114/124.

Survival analysis was performed for overall survival (OS) and disease-free survival (DFS) using the Kaplan-Meier method (log-rank test). Statistical analysis was performed using SPSS 16.0 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

MS analysis of TP53 in CRCs. MSI and LOH of TP53 intron 1 were analyzed in tumors and their matched normal tissue samples of 256 patients. Of these, 9 tumors (3.5%) were identified as having MSI and 69 (27.0%) as having LOH in TP53 intron 1 (TP53ALU) by STR scanning. The frequency of LOH was significantly higher than that of MSI (P<0.0001; Fig. 1). MSI refers to the presence of new fragments, whereas LOH refers to a

Table II. Association of TP53ALU alterations with clinical characteristics of 256 cases.

Clinical feature	Patients (n)	Value			P-value			
		MSI	LOH	MSS	MSI vs. MSS	LOH vs. MSS	MSI+LOH vs. MSS	MSI vs. LOH
Mean age (years)	256	72±8.9	67.3±8.8	67.4±8.2	0.791[a]	0.322[a]	0.316[a]	0.879[a]
Sex					0.736	0.305	0.252	1
Male	146	6 (4.1%)	43 (29.5%)	97 (66.4%)				
Female	108	3 (2.8%)	26 (24.1%)	79 (73.1%)				
Smoking					0.742	0.392	0.552	0.535
Yes	88	2 (2.3%)	27 (30.7%)	59 (67.0%)				
No	167	7 (4.2%)	42 (25.1%)	118 (70.7%)				
Drinking					1.000	0.592	0.610	1.000
Yes	57	2 (3.5%)	17 (29.8%)	38 (66.7%)				
No	198	7 (3.5%)	52 (26.3%)	139 (70.2%)				
TNM stage					0.048	0.421	0.965	0.027
II	127	7 (5.5%)	36 (28.3%)	84 (66.1%)				
III	93	1 (1.07%)	23 (24.7%)	69 (74.1%)				
Depth of tumor invasion					0.443	0.868	0.719	0.667
pT2	20	1 (5%)	6 (30%)	13 (65%)				
pT3	190	8 (4.2%)	51 (26.8%)	131 (68.9%)				
pT4	32	0 (0.0%)	8 (25.0%)	24 (75.0%)				
Lymph node involvement					0.122	0.288	0.105	0.281
pN0	144	8 (5.6%)	42 (29.2%)	94 (65.3%)				
pN1	73	1 (1.4%)	19 (26.0%)	53 (72.6%)				
pN2	22	0 (0.0%)	4 (18.2%)	18 (81.8%)				
Metastasis					1.000	1.000	1.000	1.000
M0	252	9 (3.6%)	68 (26.9%)	175 (69.4%)				
M1	3	0 (0.0%)	1 (33.3%)	2 (66.7%)				
Pathological type					1.000	0.514	0.449	0.412
Adenocarcinoma	216	8 (3.7%)	62 (28.7%)	146 (67.6%)				
Mucinous carcinoma	17	1 (5.9%)	3 (17.6%)	13 (76.5%)				
Histologic grade					0.682	0.245	0.185	1.000
Well	50	1 (2.0%)	11 (22.0%)	38 (76.0)				
Moderate/poor	168	7 (4.2%)	50 (29.8%)	111 (66.1%)				
Survival time (months)								
OS	210	37.09	27.9	30	0.307[b]	0.760[b]	0.560[b]	0.326[b]
PFS	218	36.23	25.68	28.22	0.315[b]	0.415[b]	0.290[b]	0.310[b]

Values are expressed as n (%) unless otherwise specified. P-values were obtained using the χ^2-test, [a]t-test or [b]log-rank analysis. MSI, microsatellite instability; LOH, loss of heterozygosity; MSS, microsatellite-stable status; OS, overall survival; PFS, progression-free survival.

complete or partial loss of one of the two alleles. The two alteration patterns of TP53ALU are provided in Fig. 2. Next, clone sequencing was performed to detect the PCR product of sample no. 69 and verify a new allele of 114 bp (Fig. 3). In addition, the results suggested that MS TP53ALU, which has seven genotypes, is a polymorphic locus in normal human tissues (Fig. 4).

The 256 patients with CRC were further stratified based on their TP53ALU status, including MSI, LOH and MSS, and its association with clinicopathological characteristics was determined (Table II). No significant differences in age, sex, smoking, drinking, depth of tumor invasion, lymph node involvement, metastasis, pathological type, histologic grade and survival time were observed. However, compared to the LOH (P=0.027) and MSS (P=0.048) groups, MSI tumors exhibited a greater association with TNM stage II.

Mutation analysis of TP53 exons in the CRCs. DNA sequencing of the exons of the TP53 gene in 31 CRC samples (MSI, n=4; LOH, n=7; MSS, n=20) revealed a total of 6 mutations, which were mainly distributed in 4 exons, in 4 samples (4/31, 12.9%).

Table III. Mutations of tumor protein 53 exons detected in colorectal cancer samples (n=31).

Case	MS status	Exon2	Exon3	Exon5	Exon7
T7	LOH	-	12:G>A	-	71:G>G+A
		-	rs1800370	-	rs28934576
T8	LOH	-	-	25:T>C	-
		-	-	rs760043106	-
T11	LOH	66:C>T	108:G>A	-	-
		rs878854070	rs746814615	-	-
T24	MSS	-	-	-	36:G>G+A
		-	-	-	rs112431538

LOH, loss of heterozygosity; MSS, microsatellite-stable status.

Of all of the mutations, 66 (C>T) were in exon 2 (codon 13), 12 (G>A) in exon 3 (codon 36), 108 (G>A) in exon 3 (codon 68), 25 (T>C) in exon 5 (codon 195), 71 (G>G+A) in exon 7 (codon 273) and 36 (G>G+A) in exon 7 (codon 285). All of these mutation sites have been defined as single nucleotide polymorphism (SNP) loci and previously registered in the PubMed database. The tumor samples T7 and T11 were regarded as the most unstable cases, owning to two mutations. Exon 3 and exon 7 were prone to mutations, as they were harboring 2 mutation loci (Table III).

Furthermore, the mutation patterns of TP53 exons were divided into 2 types. Among the six mutations detected, the genotype of three mutations changed from homozygous to homozygous and the remaining mutations ranged from homozygous to heterozygous. For instance, compared to matched normal tissues, the genotype of tumor sample 7 changed from G/G to A/A. These genotypes ranging from homozygous to homozygous were classified as Type 1 mutations, whereas the genotype of tumor sample T8 changed from T/T to T/C, which ranged from homozygous to heterozygous, and was classified as a Type 2 mutation (Fig. 5).

Unexpectedly, no correlation was identified between the TP53 exon mutation and any of the clinicopathological characteristics (Table IV). While there was a slight trend towards a worse prognosis, no significant association between TP53 exon mutation and survival (OS and DFS) was detected (P_{OS}=0.093, P_{DFS}=0.095).

Association between TP53 exon mutation and TP53ALU alterations. A total of 6 mutations were identified in 3 LOH samples (3/7, 42.9%) and in 1 MSS sample (1/20, 5.0%). None of the exon mutations in TP53 was detected in the MSI samples (0/4, 0%). The mutation rate in the LOH group was significantly higher than that in the MSS group (P=0.0419; Fig. 6A). In addition, regarding the 6 mutation positions in 31 tumor tissues, the mutation rate in the MSI group was 0% [0 mutations/(6 positions x 4 MSI-tumor cases)]; however, it was 11.9% [5 mutations/(6 positions x 7 LOH-tumor cases)] in the LOH group, which was significantly higher than that in the MSS group [1 mutation/(6 positions x 20 MSS-tumor cases), 0.8%; P=0.0047; Fig. 6B].

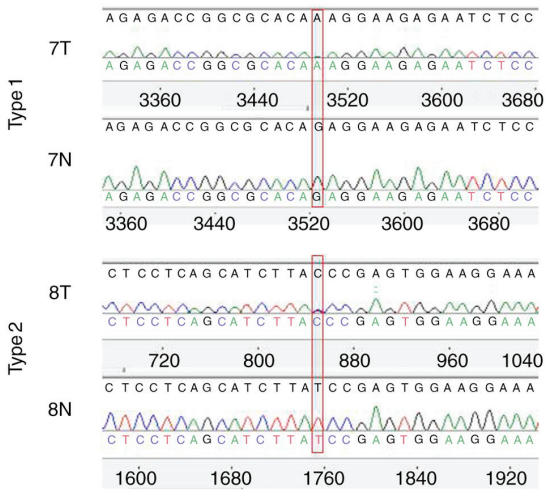

Figure 5. Mutation patterns of tumor protein 53 exons detected in colorectal cancers. T, tumor tissues; N, matched normal tissues; Type 1 mutation, the genotype changed from G/G to A/A; type 2 mutation, the genotype changed from T/T to T/C.

Discussion

CRC is the second most common cancer type with 1.2 million novel cases per year worldwide (14). Chromosomal and MS alterations constitute the major genetic instability events in CRC (15,16). However, few studies have explored the correlation between the MS status of a single gene and the exon mutations in the same gene. In the present study, the MSI and LOH status of the MS in an intron of the TP53 gene was detected and the association between MSI/LOH alterations and the mutations in TP53 exons was analyzed. The results suggested a positive association between LOH alterations in the MS of the TP53 intron and mutations in TP53 exons in CRCs. These results indicated that MS analysis is not sufficient to predict the mutation status of TP53 exons, but was higher than the probability for MSS or MSI when LOH was present.

MS are widely abundant in the genome and certain MS are highly polymorphic in the normal population. The MS TP53ALU was initially identified to be a polymorphic locus and has been frequently used as a marker in previous studies (17,18). In the present study, 7 genotypes of TP53 ALU were detected in the matched normal tissues, and it was confirmed that it is a polymorphic MS.

CRCs with MSI have unique clinicopathological features. Studies have indicated that patients with MSI in their tumors frequently exhibit localization in the right colon, female sex,

Table IV. Association of tumor protein 53-exon mutations with clinical characteristics of the patients (n=31).

Item	Patients (n)	Mut	Non-Mut	P-value
Mean age (years)	31	67	68.32	0.815
Sex				0.115
Male	20	1	19	
Female	11	3	8	
Drinking				1
Yes	11	1	10	
No	20	3	17	
Smoking				0.304
Yes	14	3	11	
No	17	1	16	
Histologic grade				1
Well	12	2	10	
Moderate/poor	17	2	15	
TNM stage				1
II	12	2	10	
III	16	2	14	
Survival time (months)				
OS	31	10.65	37.53	0.093
PFS	31	10.64	36.24	0.095

P-values were obtained using χ^2-tests and log rank test. Mut, mutation; Non-Mut, no mutation; OS, overall survival; DFS, disease-free survival; TNM, tumor-nodes-metastasis.

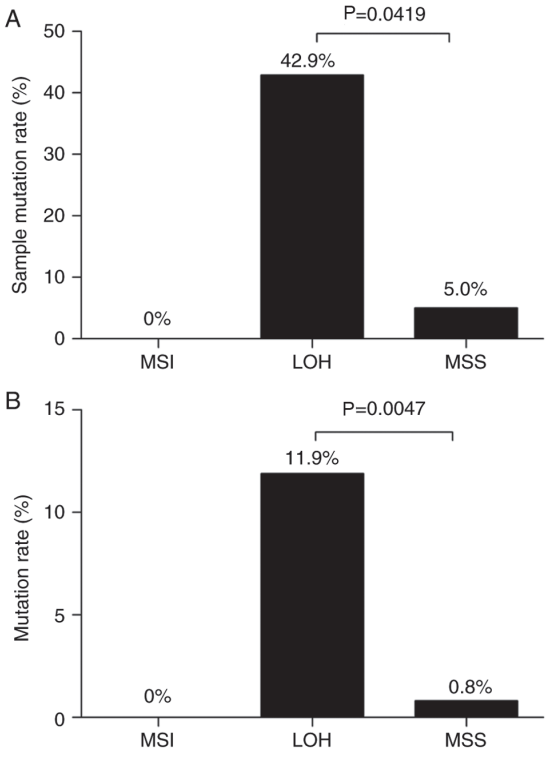

Figure 6. Mutation profiles in TP53ALU MSI, LOH and MSS group tumors from the 6 unstable positions. (A) The sample mutation rate of TP53ALU LOH tumors was significantly higher than that of MSS tumors (P=0.0419); (B) the mutation rate of TP53ALU LOH tumors was significantly higher than that of MSS tumors (P=0.0047). MSI, microsatellite instability; LOH, loss of heterogeneity; MSS, microsatellite-stable status; TP53, tumor protein 53.

mucinous histology, larger tumor size and less advanced disease stage (19-22). In the present study, the TP53ALU MSI phenotype was significantly associated with the TNM stage, as TP53ALU MSI tumors were more frequently stage II than LOH (P=0.027) and MSS tumors (P=0.048). These results indicated that the status of TP53ALU may be of prognostic value regarding the TNM stage of CRC.

A total of 6 different mutations in the TP53 exons were identified in 4 unrelated CRC patients by comparing the sequence of the tumor's TP53 with those of the matched normal tissues. Although all of the mutations (6/6) are known as SNP loci, position 71 in exon 7 (codon 273) is a hotspot that has been previously reported (23). All of the mutations were in coding sequences and only three missense mutations were detected: Codon 13 (p. Pro13Leu), codon 195 (p. Ile195Thr) and codon 273 (p. Arg273His).

The role of SNPs in the regulation of different functions of proteins is an important genetic research field aimed at understanding the molecular basis of disease (24). Among the SNPs, non-synonymous coding SNPs in the coding regions result in an amino acid variation in the protein products of genes, which are thought to have an impact on the phenotype (25). The TP53 rs28934576 variation (p.Arg273His) is a known pathological mutation that is important in the initiation and progression of CRC, which has been previously described in a public database. The rs1800370 (CCG to CCA, both encoding proline) is a synonymous SNP, which does not change the amino acid sequence, but this silent mutation has been indicated to reduce the ability of p53 to activate apoptosis by lowering its synthesis through a reduction

in the affinity of its mRNA to MDM2 (26). The rs112431538 and rs760043106 variations of TP53 are likely pathogenic according to the records in the PubMed database (https://www.ncbi.nlm.nih.gov/clinvar/variation/420133/#clinical-assertions; https://www.ncbi.nlm.nih.gov/clinvar/?linkname=snp_clinvar&from_uid=760043106).

However, no correlation was identified between TP53 exon mutations and any of the clinicopathological characteristics; this may be due to the limited number of cases examined. However, patients with TP53 exon mutation exhibited a slight but insignificant tendency to have shorter OS (P=0.093) and DFS (P=0.095). This is in accordance with the mutations in tumor suppressor genes that are associated with highly malignant tumors in a previous study (27). Driver mutations in CRC include intracellular KRAS, B-Raf proto-oncogene, serine/threonine kinase, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, TP53, F-box and WD repeat domain-containing 7 and NRAS gene mutations, and the first four of these mutations have been proposed as indicators of CRC prognosis. Therefore, the correlation between TP53 gene mutation and chemotherapy requires further exploration.

The present study had several limitations that require further discussion. The number of samples tested was small due to the exclusion of the effects of mutations at other sites. The small sample size may have affected the accuracy of the results, and therefore, the present results require to be verified using a larger sample size. Still, the results of the present study provided a basis for further in-depth research. Due to the limited number of specimens, no histological control of the samples used for DNA extraction was performed. Although exon mutations were not detected, it cannot be ruled out that these mutations may affect protein sequence/transcription.

In conclusion, the present study indicated that the prevalence of TP53 exon mutations was significantly higher in CRC tumors with LOH of TP53 than in CRC tumors with MSI and MSS of TP53. Exonmutations of TP53 were associated with TP53ALULOH. Furthermore, an MSI status was closely associated with stage II CRCs.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

ZC, XL, XH and DF contributed to the conception and design of the present study. XL and DF performed the experiments. XL, DF, XH and XX analyzed the data. XL, DF and XH drafted and revised the manuscript. All authors critically revised the manuscript and approved the final version.

Ethics approval and consent to participate

The study was reviewed and approved by China National center for Biotechnology development (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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