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Association between microsatellite instability status, clinicopathological features and mitochondrial DNA amplification in patients with colorectal cancer

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Abstract. The relationship between BRAF-V600E mutations, mitochondrial DNA amplification and microsatellite instability-high (MSI-H) in colorectal cancer (CRC) has yet to be fully elucidated. The aim of the present study was to assess the association between the MSI status and BRAF-V600E gene mutations/clinicopathological features/mitochondrial DNA amplification in CRC. A non-interventional study analysis was performed using the clinicopathological features of 455 patients with CRC. Immunohistochemistry was used to evaluate four mismatch repair proteins (MutS homolog 2, MutS homolog 6, MutL homolog 1 and postmeiotic segregation increased 2), Ki-67 index, and programmed cell death protein 1 (PD-1) and programmed cell death-ligand 1 (PD-L1) expression. Additionally, PCR coupled with capillary electrophoresis were used to ascertain the MSI status. Moreover, amplification refractory mutation system-PCR was used to detect BRAF-V600E gene mutation and fluorescence *in situ* hybridization analysis was used to assess mitochondrial DNA. A total of 455 patients were divided into the MSI high (MSI-H) group (n=52) and microsatellite stability (MSS) group (n=403) based on their MSI status. Compared with the results of immunohistochemistry of four mismatch repair proteins, the consistency rate between mismatch repair protein deficiency and MSI was 94.23%. There were significant differences in PD-L1, primary tumor site, clinical stage, degree of differentiation, tumor size, lymph node metastasis and the occurrence of multiple primary tumors between the MSI-H group and MSS group ($P<0.05$ or $P<0.001$). However, there were no significant differences for sex, age, PD-1, Ki-67 expression and BRAF-V600E. The 24-60-month survival rate of the patients in the MSI-H group was significantly higher than that of those in the MSS group ($P<0.05$). Furthermore, the number of mitochondrial DNA was significantly amplified in the MSI-H group. In conclusion, the present study demonstrated that the combined detection of PD-L1 and MSI in patients with CRC can provide more accurate and effective guidance for personalized treatment.

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Introduction

Colorectal cancer (CRC) is a key public health and socioeconomic concern. According to GLOBOCAN 2020, CRC ranks third and second in terms of incidence and mortality rates of malignant tumors, respectively (1). Despite an increase in early screening, diagnosis and treatment for CRC, ~20% of patients with CRC are still diagnosed at stage IV of the disease (2). Furthermore, despite advancements in the field of biological knowledge and treatment modalities, CRC continues to pose significant challenges in terms of effective management due to its high incidence of metastasis and the development of drug resistance. The 5-year survival rate of patients with colon cancer at stage I is 90%, which markedly decreases to ~10% in those with stage IV disease (2,3). The ability to identify specific biomarkers and molecular targets related to CRC will provide valuable support for the development of novel targeted therapies and reducing the mortality rate associated with CRC.

Currently, several biomarkers help clinicians make optimal treatment decisions, such as KRAS, NRAS, BRAF, programmed cell death protein 1 (PD-1)/programmed cell death-ligand 1 (PD-L1), human epidermal growth factor receptor 2 and microsatellite instability (MSI). They each serve a critical role in planning the optimal treatment strategy for the patient (4-6). Among these, MSI had attracted widespread attention. Microsatellites are short tandem repeat DNA

sequences with 1-4 base pairs, distributed throughout the coding and noncoding regions of the entire human genome. MSI is recognized as one of the major carcinogenetic pathways of CRC. It represents a molecular hallmark of hereditary non-polyposis CRC, also known as Lynch syndrome, and is usually linked to a germ-line mutation in one of the mismatch repair (MMR) genes. Moreover, it is detected in ~15% of sporadic CRC cases (7,8). MSI occurs due to deficient MMR (dMMR), which accounts for ~15% of cases. The MSI phenotype is characterized by the primary loss of function of proteins involved in repairing DNA base-pair mismatches, namely MutL homolog (MLH) 1, MutS homolog (MSH)2, MSH6 and postmeiotic segregation increased 2 (PMS2) (8).

The mitochondria serve a pivotal role in both physiological homeostasis and pathological processes. Mitochondrial dysfunction is related to metabolic syndrome, neuronal diseases, oncogenesis, cardiovascular ailments, infectious diseases and inflammatory disorders. Moreover, mitochondrial metabolism serves a crucial role in tumor proliferation, survival, metastasis and drug resistance (9,10). However, it is unclear whether there is a difference in mitochondrial metabolism between MSI high (MSI-H) and microsatellite stability (MSS).

The present study aimed to analyze the association between the clinicopathological features of patients with CRC and MSI, and to assess the association between mitochondria and MSI, as well as BRAF gene mutations. Furthermore, the present study aimed to provide a improved understanding of the importance of MSI status in CRC, as well as a theoretical foundation for the molecular classification and clinical treatment of CRC. This may then also provide potential molecular targets for the treatment of CRC.

Materials and methods

Study sample. Data from 455 patients with CRC admitted to Xiangya Second Hospital, Central South University (Changsha, China) from January 2017 to January 2023 were obtained from the laboratory information system (LIS). The inclusion criteria were as follows: i) First diagnosis with CRC; and ii) no prior radiotherapy, chemotherapy or targeted therapy. The exclusion criteria were as follows: Patients who received radiotherapy, chemotherapy or immunotherapy before radical surgery.

The following data were collected from the LIS and Langjia Pathology Information System: Patient age, sex, tumor size, location, degree of differentiation, presence or absence of lymph node metastasis and multiple primary cancer status. The MSI status, BRAF gene mutation status, Ki-67 index and other immunohistochemical data were obtained from the patient specimens by clinical testing in the pathology department, collected from the Langjia Pathology Information System. Telephone follow-up was performed to obtain the survival status of patients post-surgery for >2 years; 25 MSI-H patients and 43 MSS patients accepted telephone follow-up. All samples were obtained with written informed patient consent and the present study was approved by the Second Xiangya Hospital of Central South University Ethics Committee Review Board (Scientific and Research Ethics Committee; approval no. LYEC2022-K0026).

Group design. According to the results of the MSI status, determined using PCR and capillary electrophoresis (CE), 455 patients were divided into two groups: MSI-H and MSS.

Processing of paraffin-embedded samples and hematoxylin and eosin staining. All samples were processed according to the standardized sample processing process of the Pathology Department of Xiangya Second Hospital as previously described (11). In brief, the samples were fixed in 10% neutral formalin (Beijing Jiuzhou Berlin Biotechnology Co., Ltd.), for 24-48 h at room temperature, then subjected to dehydrationin an ascending series of ethanol, cleared in xylene and finally embedded in paraffin. Sections were cut at thickness of 4 μm and placed on glass slides. To deparaffinize samples, the slides were placed in xylene and then in ethanol of a decreasing concentration gradient at room temperature. Slides were then stained with hematoxylin for 5 min and with eosin for 10 sec, and finally they were dehydrated in a gradient of alcohol and xylene at room temperature. Then the samples were sealed with neutral balsam on slides and examined by an experienced pathologist under a light microscope (Leica Microsystems GmbH).

Immunohistochemical staining and scores. The EliVision two-step method was used for immunohistochemical staining (12). The selected antibodies were all monoclonal antibodies: Ki-67 (cat. no. RMA-0731), PD-L1 (cat. no. RMA-0854), PD-1 (cat. no. RMA-0734), MSH2 (cat. no. RMA-0836), MSH6 (cat. no. RMA-0831), MLH1 (cat. no. RMA-0789) and PMS2 (cat. no. RMA-0775) (all from Fuzhou Maixin Biotechnology Development Co., Ltd.). The secondary antibody kit [Elivision™ plus Polymer HP (Mouse/Rabbit) IHC Kit, cat. no.KIT-9901], EDTA antigen retrieval solution (cat. no. MVS-0098), DAB chromogenic reagent (cat. no. DAB-0031) and PBS (cat. no. PBS-0060) were also purchased from Fuzhou Maixin Biotechnology Development Co., Ltd. The protocols were in accordance with the instructions provided with the reagents. Immunohistochemical staining was evaluated by qualified pathologists and MSH2, MSH6, MLH1 and PMS2 were defined as no deficiency (+) and deficiency (-). Ki-67, PD-L1 and PD-1 were scored from 0-100.

Detection of MSI status using PCR coupled with CE. The MSI status was detected according to the instructions of the MSI detection kit (cat. nos. C10120230510 and C10120231204; Changzhou Tongshu Biotechnology Co., Ltd.). This kit contained six pairs of fluorescent labeled primers for simultaneous amplification of six microsatellite sites: Two single nucleotide repeat unit sites (BAT25 and BAT26), three dinucleotide repeat unit sites (D2S123, D5S346 and D17S250), and one pentanucleotide repeat unit internal reference site (Penta C). The amplification of microsatellite alleles from normal and tumor tissues of the same individual was detected using multiple fluorescence PCR. Tissue DNA was extracted using the column extraction method (cat. nos. 220812C01X and 221225C02X; Amoy Diagnostics Co., Ltd.). The PCR (cat. nos. C10120230510 and C10120231204; Changzhou Tongshu Biotechnology Co., Ltd.) steps were as follows: Pre-denaturation at 95˚C for 4 min, once; denaturation at 95˚C

for 30 sec; annealing at 60˚C for 90 sec; extension at 72˚C for 30 sec for 30 cycles; and a final extension at 60˚C for 45 min, once. The multiple fluorescence PCR product was used for CE detection; 0.25 μl GeneScan™ 600 LIZ® Size Standard (Applied Biosystems Inc., ABI), 8.75 μl formamide and 1 μl PCR product were mixed and were used to determine the microsatellite status by 3500Dx gene analyzer (Thermo Fisher Scientific, Inc.). For interpretation annotation, ≥2 unstable loci were classified as MSI-H and 1/0 loci were classified as low-frequency MSI/MSS. The primer sequences used for the six microsatellite sites cannot be disclosed due to commercial confidentiality.

BRAF-V600E gene detected using amplification refractory mutation system (ARMS)-PCR. DNA was extracted from paraffin-embedded tissues according to the instructions provided with the Nucleic Acid Extraction kit (cat. nos. 220812C01X and 221225C02X; Amoy Diagnostics Co., Ltd.). The BRAF-V600E gene was tested using the Human BRAF gene V600E mutation detection kit (fluorescence PCR method; cat. nos. 221104C02x and 230308C01x; Amoy Diagnostics Co., Ltd.). The PCR amplification steps were as follows: i) 95˚C for 5 min, one cycle; ii) 95˚C for 25 sec, 64˚C for 20 sec, 72˚C for 20 sec, 15 cycles; and iii) 93˚C for 25 sec, 60˚C for 35 sec, 72˚C for 20 sec, 31 cycles. The internal control HEX (or VIC) signal was a positive curve, and the Cq value was between 13 and 21. The FAM signal amplification curve of the mutation detection tube showed an S-shaped curve and the Cq value was <30, therefore it was considered mutation-positive. The primer used for ARMS-PCR-BRAF-V600E and control cannot been disclosed due to commercial confidentiality.

Fluorescence in situ hybridization (FISH). A total of 5 MSI-H samples and 5 MSS samples were chosen to assess mitochondrial DNA. The human mitochondrial DNA probe labeled with NGT-orange fluorescent dye (cat. no. DF600; Xiamen Longjin Biotechnology Co., Ltd.) was used to detect mitochondrial DNA amplification using FISH technology. The target sequence was *Homo sapiens* mitochondrion, complete genome (NC_012920.1). The following process was used: Slides were dewaxed and washed with water, followed by 100˚C sodium citrate buffer repair for 30 min and pepsin digestion for 37˚C for 20 min. Subsequently, 2X SSC was used at room temperature for 3 min, and the slides were washed and air dried for later use. They were then subjected to probe hybridization (denaturation at 85˚C for 5 min and hybridization at 42˚C for 2-7 h). After removing the rubber cement used for sealing the slide, 2X SSC was used at 37˚C for 10 min, followed by 0.1 NP-40/2X SSC for 5 min at room temperature. The slides were then subjected to ethanol gradient (70, 90 and 100%) dehydration for 3 min each and were then air dried in the dark and immediately sealed using 10 μl anti-quenching/nucleus staining agents (including DAPI) (Xiamen Longjin Biotechnology Co., Ltd) at room temperature. Finally, the glass slides were observed under a fluorescence microscope (Olympus Corporation).

Statistical analysis. Statistical analysis was performed using the SPSS 22.0 statistical software package (IBM Corp.). Continuous data are presented as the mean ± standard deviation and comparisons between two groups was performed using independent sample t-tests. Categorical data are presented as n (%) and group comparisons were made using the χ^2 test or Fisher's Exact Test. Kaplan-Meier curves and the log-rank test were used to analyze the survival rates. Cox proportional-hazards model analyses were performed to evaluate the associations between microsatellite status and mortality. P<0.05 was considered to indicate a statistically significant difference. Due to the incomplete information of some patients in this study, the data were considered unknown and were not included in statistical analysis.

Results

Microsatellite status and dMMR. According to the results of PCR and CE, 455 patients were divided into two groups as follows: The MSI-H group (52/455; 11.43%) and the MSS group (403/455; 88.57%). Among the 52 MSI-H cases, 98% exhibited instability in BAT25, 94% in BAT26, 46% in D2S123, 34% in D17S250 and 26% in D5S346. Immunohistochemical analysis was used to detect the expression of MMR proteins (MSH2, MSH6, MLH1 and PMS2). If all four proteins were positively expressed, the MMR function was considered normal (proficient mismatch repair, pMMR); if any protein expression was lost (negative expression), the MMR function was considered deficient. According to the results of immunohistochemistry, 94.23% (49/52) of the MSI-H cases were dMMR, and the consistency rate between dMMR and MSI-H was 94.23%. Among these, the highest deficiency was MLH1(-) + PMS2(-), accounting for 44.23% (23/52). Secondly, the deficiency rate of MSH2(-) + MSH6(-) was 17.31% (9/52). The deficiency rates of MSH2(-), MSH6(-), PMS2(-), MSH6(-) + PMS2(-), MLH1(-) + PMS2(-) + MSH6(-), and MLH1(-) + MSH2(-) + MSH6(-) were 9.62% (5/52), 7.69% (4/52), 5.77% (3/52), 3.85% (2/52), 3.85% (2/52) and 1.92% (1/52) respectively. No loss (pMMR) was 5.77% (Fig. 1).

Association between BRAF-V600E mutation and MSI. The mutation of BRAF-V600E in the 39 MSI-H and 71 MSS cases was assessed using ARMS-PCR. Among the MSI-H cases, 10.26% (4/39) had BRAF-V600E mutations, whilst the remaining 89.74% (35/39) had wild-type BRAF-V600E. In contrast, only 1.41% (1/71) of the MSS cases exhibited a BRAF-V600E mutation, with 98.59% (70/71) had the wild-type. However, statistical analysis revealed there was no significant statistical difference between these two groups (P>0.05; Table I and Fig. 2). Notably, all four BRAF-V600E mutation cases in the MSI-H group were also demonstrated to have deficiencies in MLH1 and PMS2 proteins. The distribution of the BRAF-V600E mutation results are presented in Fig. 1.

Association between microsatellite status and patient clinicopathological characteristics. Among the 455 cases of CRC, the ages ranged between 16-89 years, with a mean of 59.87±0.5530 years (n=455). The average age was 60.80±0.7398 years and 58.55±0.8216 years for the male (n=267) and female (n=188) patients, respectively. The average age of the male patients was significantly higher than that of the female patients (P<0.05). The average age

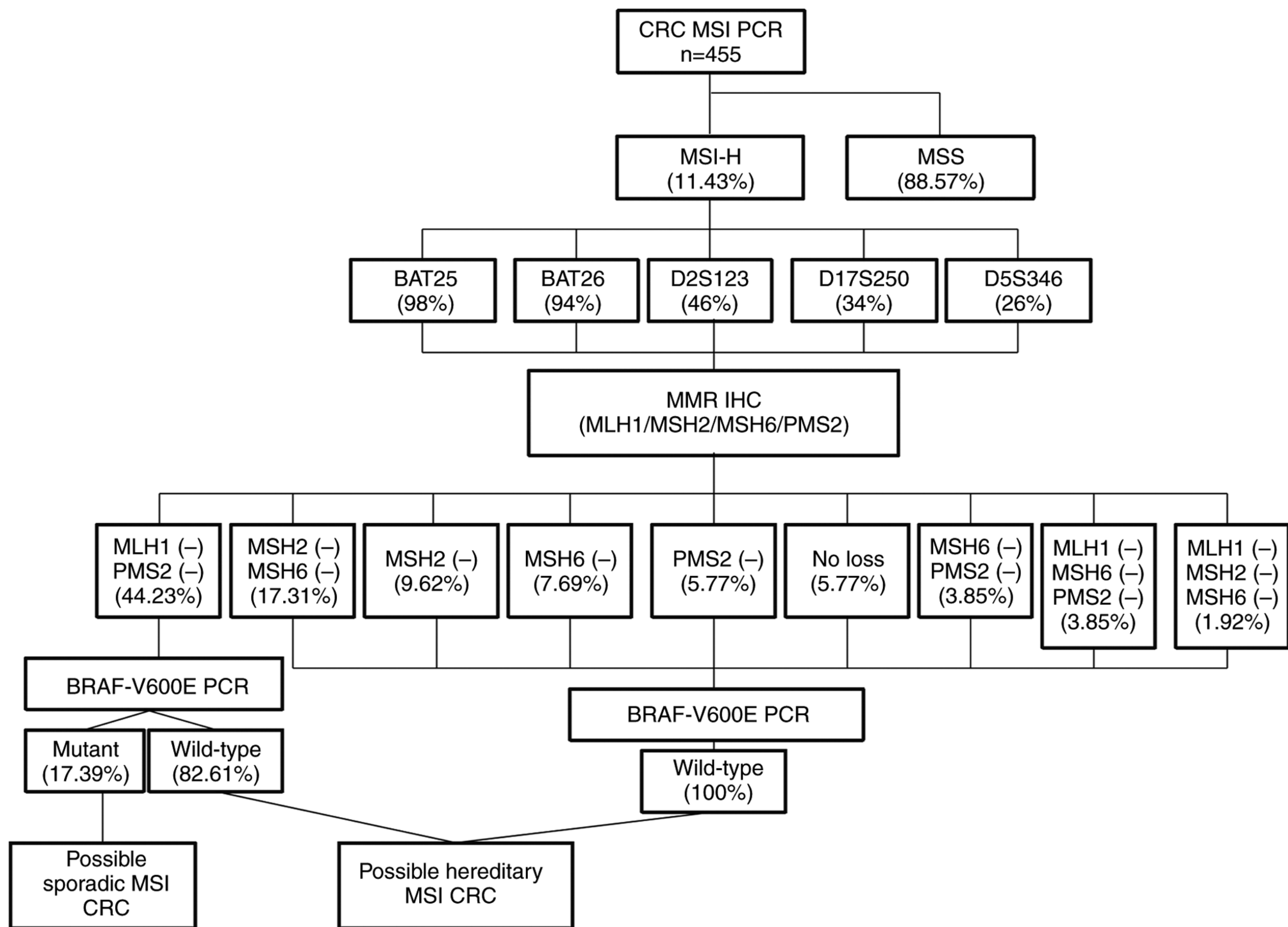

Figure 1. Microsatellite status and MMR protein deficiency status in 455 patients with CRC. CRC, colorectal cancer; MSI, microsatellite instability; MSI-H, high-frequency microsatellite instability; MSS, microsatellite stability; MMR, mismatch repair; IHC, immunohistochemistry; MSH, MutS homolog; MLH1, MutL homolog 1; PMS2, postmeiotic segregation increased 2; (-), deficiency detected by IHC.

of the patients in the MSI-H group was 57.15±1.7570 years (n=52), compared with 60.22±0.5802 years (n=403) in the MSS group was, with no significant difference demonstrated between the two groups. Clinical staging [tumor-node-metastasis (TNM)] analysis revealed that the proportion of patients with I-II stage (early stage) disease in the MSI-H group was significantly higher than that in the MSS group (P<0.05). The incidence of lymph node metastasis in the MSI-H group was significantly lower than that in the MSS group (P<0.05). According to the degree of tumor cell differentiation, there were 96 cases of low differentiation (21.38%), 287 cases of medium differentiation (63.92%) and 66 cases of high differentiation (14.70%). The incidence of low differentiation in the MSI-H group was significantly higher than that in the MSS group (P<0.001). The maximum diameter of tumors in the MSI-H group was 0.5-10 cm, with an average maximum diameter of 5.21±0.2817 cm (n=48). For the MSS group, the tumor size ranged between 0.5-11.8 cm, with an average maximum diameter of 4.02±0.0963 cm (n=316). The average maximum diameter of the tumors in the MSI-H group was significantly higher than that in the MSS group (P<0.001). In the MSI-H group, there were 27 cases (51.92%) with tumors in the right colon, 20 cases (38.46%) with tumors in the left colon, 4 cases (7.69%) with tumors in the rectum, and 1 case (1.92%) with a tumor at the straight sigmoid junction. In the MSS group, 73 patients (18.25%) had tumors in the right colon, 120 patients (30%) had tumors in the left colon, 195 patients (48.75%) had tumors in the rectum and 12 patients (3%) had tumors in the rectum sigmoid junction. Statistical analysis revealed that the incidence of tumors in the right colon in the MSI-H group was significantly higher than that in the MSS group (P<0.001; Table I).

Association between microsatellite status and the expression of Ki-67, PD-1 and PD-L1. The expression of PD-L1, Ki-67 and PD-1 in the MSI-H and MSS groups was detected using immunohistochemical analysis. The results revealed that the average score of PD-L1 in the MSS group was 6.02±0.4529 (n=329), whilst in the MSI-H group it was 16.74±3.9980 (n=36). There was a statistically significant difference between the two groups (P=0.0117; Fig. 3). The average score of Ki-67 in the MSS group was 69.12±0.9697 (n=355), whilst in the MSI-H group it was 70.59±3.9400 (n=41); however, there was no significant difference between the two groups. The average score of PD-1 in the MSS group was 0.39±0.1260 (n=198), whilst in the MSI-H group it was 4.70±2.5390 (n=30); however, there was no statistically significant difference between the two groups. Representative immunohistochemical images are presented in Fig. 3.

Table I. Analysis of the association between microsatellite instability and clinicopathological features of patients with colorectal cancer.

	MSI		
Clinicopathological feature	MSS	MSI-H	P-value
Age			
≤60 years (n=244)	211 (86.5)	33 (13.5)	0.131
>60 years (n=211)	192 (91.0)	19 (9.0)	
Sex			
Female (n=188)	166 (88.3)	22 (11.7)	0.911
Male (n=267)	237 (88.76)	30 (11.24)	
Clinical stage			
I-II (n=208)	173 (83.2)	35 (16.8)	0.003[c]
III-IV (n=216)	200 (92.6)	16 (7.4)	
LNM			
No (n=212)	177 (83.5)	35 (16.5)	0.007[c]
Yes (n=176)	163 (92.6)	13 (7.4)	
Histological grade			
Low (n=96)	74 (77.1)	22 (22.9)	<0.001[c]
Moderate (n=287)	263 (91.6)	24 (8.4)	
High (n=66)	61 (92.4)	5 (7.6)	
Diameter, cm	4.02±0.0963[a]	5.21±0.2817[b]	<0.001[c]
Location			
Right (n=100)	73 (73.0)	27 (27.0)	<0.001[c]
Left (n=140)	120 (85.7)	20 (14.3)	
Rectosigmoid junction (n=13)	12 (92.3)	1 (7.7)	
Rectum (n=199)	195 (98.0)	4 (2.0)	
Primary carcinoma			
Single primary carcinoma (n=429)	385 (89.7)	44 (10.3)	0.003[d]
SMPC (n=13)	11 (84.6)	2 (15.4)	
HMPC (n=13)	7 (53.8)	6 (46.2)	
Survival status			
Alive (n=53)	30 (56.6)	23 (43.4)	0.029[c]
Dead (n=15)	13 (86.7)	2 (13.3)	
BRAF V600E			
Wild-type (n=105)	70 (66.7)	35 (33.3)	0.052
Mutated-type (n=5)	1 (20.0)	4 (80.0)	
Mitochondrial DNA amplification			0.048[d]
Un-clustered amplification	5	1	
Clustered amplification	0	4	

Data are presented as n (%) or mean ± standard deviation. [a]n=316; [b]n=48; [c]$P<0.05$, χ^2 test; [d]$P<0.05$, Fisher's Exact Test. MSI, microsatellite instability; MSS, microsatellite stability; MSI-H, MSI-high; LNM, lymph node metastasis; SMPC, simultaneous multiple primary carcinoma; HMPC, heterochronous multiple primary carcinoma.

Association between MSI status and mitochondrial DNA amplification. The amplification of mitochondrial DNA in the colon cancer tissues and adjacent tissues from patients in the MSI-H and MSS was assessed using FISH analysis. There was no significant change in mitochondrial DNA in the two groups of adjacent cancer cells. However, when comparing the tumor cells from patients in the MSI-H group with those of patients from the MSS group, it was revealed that the number of mitochondrial DNA in the MSI-H group was significantly higher than that in the MSS group (P=0.048; Table I). Orange fluorescence signal points were clustered in the cells, indicating clustered amplification of mitochondrial DNA in the MSI-H group of tumor cells (Fig. 4).

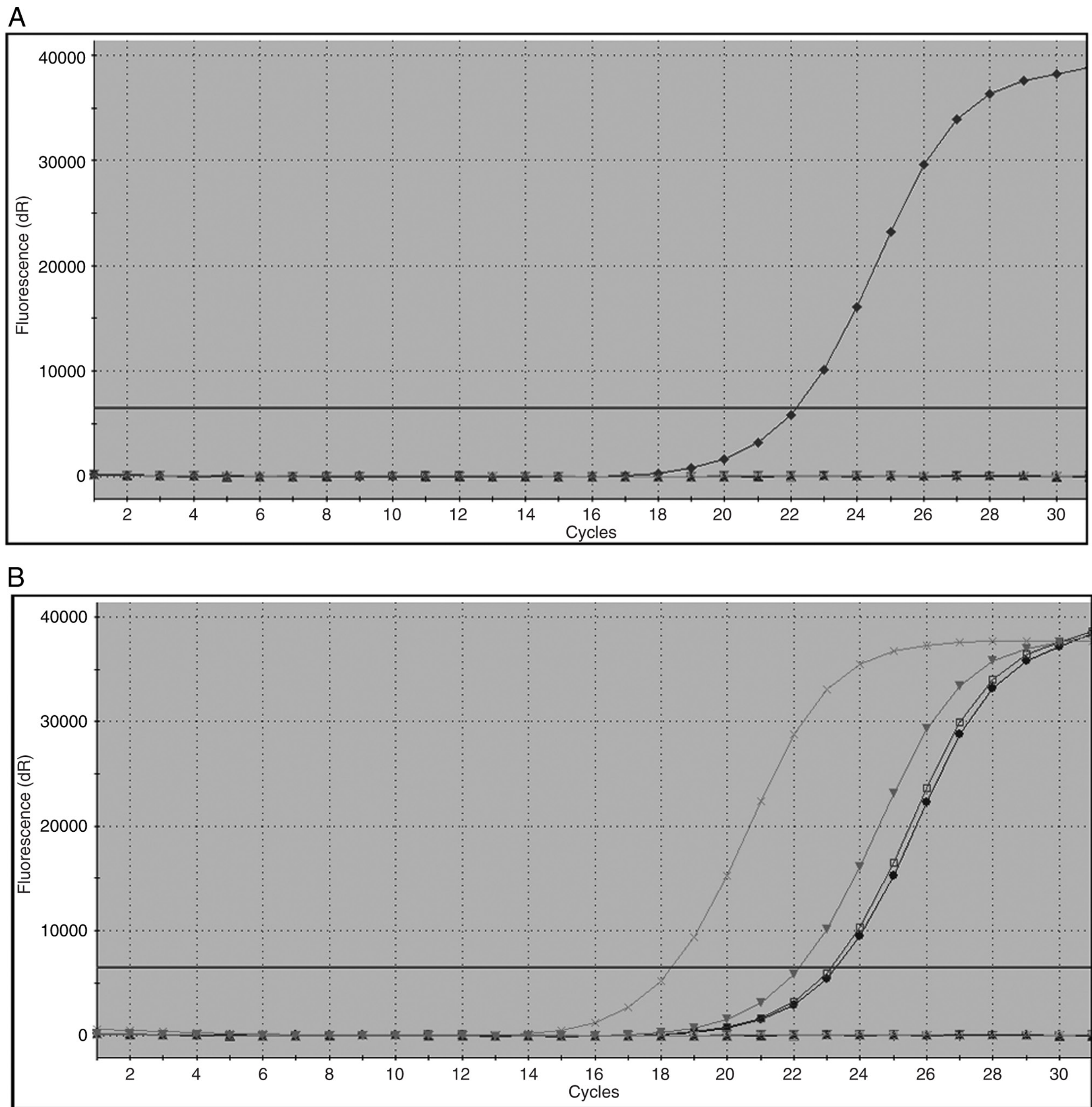

Figure 2. BRAF-V600E mutations were assessed using amplification refractory mutation system-PCR. Amplification curves of the (A) microsatellite stability group (n=71), in which one case had a BRAF-V600E mutation; and (B) high-frequency microsatellite instability group (n=39), in which 4 cases had BRAF-V600E mutations (P=0.052). A Cq value of <30 and an S-shaped PCR amplification curve was defined as a BRAF-V600E mutation.

Association between MSI status with patient survival rate and multiple primary tumors. The survival status was analyzed in the 25 patients in the MSI-H group and in the 43 patients with CRC in the MSS group from January 2017 to September 2021. It was demonstrated that there was a significant difference in the survival rate between the two groups (P<0.05; Fig. 5A). A Cox proportional-hazards model was then used to evaluate the association between microsatellite status and mortality. A favorable association was revealed between MSI-H and a lower risk of mortality in the unadjusted model [model I; hazard ratio (HR), 0.22; 95% confidence interval (CI), 0.05-0.98; P=0.046; Table II]. In the adjusted models, model II (adjustment for age and sex) and model III (adjustment for age, sex, TNM stage and site), the HR values were 0.24 (95% CI, 0.05-1.08) and 0.32 (95% CI, 0.06-1.69), respectively. Furthermore, analysis of the association between MSI and clinicopathological features of CRC (68 follow-up cases data; Table SI) demonstrated a significant association between MSI-H and TNM stage, site, status and time (P<0.001, P<0.001, P=0.033 and P=0.039, respectively), but no significant association between MSI status and age and sex (Table SII). Following an analysis of 52 cases of MSI-H and 403 cases of MSS with multiple primary tumors (including simultaneous multiple primary carcinoma and heterochronous multiple primary carcinoma), it was revealed that the incidence of multiple primary tumors in the MSI-H group was 15.38%

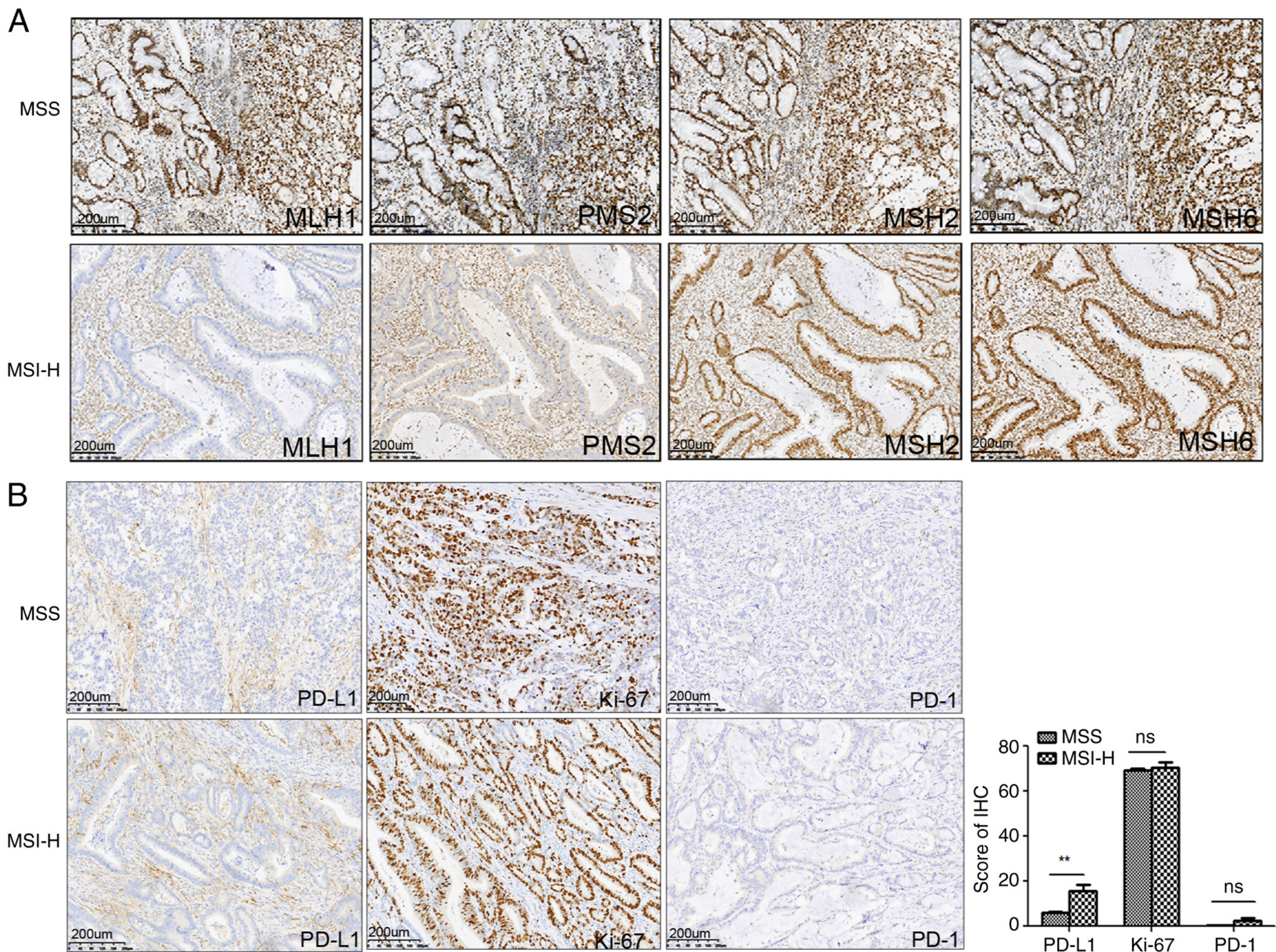

Figure 3. Representative immunohistochemical images of MLH1, PSM2, MSH6, MSH2, PD-L1, Ki-67 and PD-1. (A) MLH1 and PMS2 proteins exhibited deficiency in the tumor cells from the patients in the MSI-H group. (B) PD-L1 expression was significantly increased in the MSI-H group. Scale bars 200 μm. **P<0.01. MLH1, MutL homolog 1; PSM2, postmeiotic segregation increased 2; MSH, MutS homolog; MSI-H, high-frequency microsatellite instability; MSS, microsatellite stability; IHC, immunohistochemistry; PD-1, programmed cell death protein 1; PD-L1, programmed cell death-ligand 1; ns, not significant.

(8/52), of which 5 cases were other digestive system tumors, and 3 cases were endometrial cancer, bladder cancer and nasal cancer. The incidence of multiple primary tumors in the MSS group was only 4.47% (18/403). There was a significant difference between the two groups (P<0.01; Table I and Fig. 5B).

Discussion

CRC is one of the most common malignant tumors of the digestive system, which is also the second cause of cancer-related mortality worldwide (13). Whilst targeted therapy and chemotherapy have been widely used, ICIs have exhibited improved efficacy, particularly in patients with metastatic CRC with tumors with dMMR or MSI. Moreover, the microsatellite status can serve as a predictive biomarker for ICI therapy in CRC. Among the 455 patients with CRC included in the present study, MSI-H was present in 52/455 patients (11.4%). Notably, the present study demonstrated that an early TNM stage (I-II stage), no lymph node metastasis, a low degree of differentiation, a large tumor diameterand tumors in the right colon were more common in patients with MSI-H. Furthermore, patients with CRC with MSI-H had a higher overall survival rate than those with MSS. This result is also in accordance with previously reported data on patients with CRC (14). In addition, no significant association was demonstrated between the microsatellite status and other features, including age, sex, BRAF-V600E gene mutation and the expression of PD-1 and Ki-67 (Table I). Recently, the PD-1/PD-L1 axis was identified as a target for cancer immunotherapy (3). The present study indicated that the expression of PD-L1 was significantly higher in patients with MSI-H compared with those with MSS, which illustrated that patients with CRC with MSI-H had a prolonged overall survival due to the clinical response of ICIs.

dMMR/MSI-H is a key subtype of CRC (15). To accurately detect the microsatellite status, PCR combined with CE is generally used, and immunohistochemistry is used to evaluate the expression of MMR proteins (16). Previous studies have demonstrated heterogeneity in PCR combined with CE and immunohistochemistry owing to the low quantity and poor quality of tumor cells in the formalin-fixed paraffin-embedded samples (17,18). In the present study, when comparing the results of the two assays, the consistency rate of MSI (detected using PCR) and dMMR (detected using immunohistochemistry) was 94.23%. Moreover, in the present study, the instability rates of the single nucleotide sites, BAT25 and BAT26, were the highest in the MSI-H group, reaching 98 and 94%, respectively. In the

Table II. Association between microsatellite status and mortality.

			Model I[a]		Model II[b]		Model III[c]	
Variable	Total, n	Event, n (%)	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
MSS	43	13 (30.2)	1 (Reference)		1 (Reference)		1 (Reference)	
MSI-H	25	2 (8)	0.22 (0.05-0.98)	0.046	0.24 (0.05-1.08)	0.063	0.32 (0.06-1.69)	0.182

[a]Unadjusted; [b]adjustment for age + sex; [c]adjustment for age + sex + tumor-node-metastasis grade + site. MSS, microsatellite stability; MSI-H, microsatellite instability-high; HR, hazard ratio; CI, confidence interval.

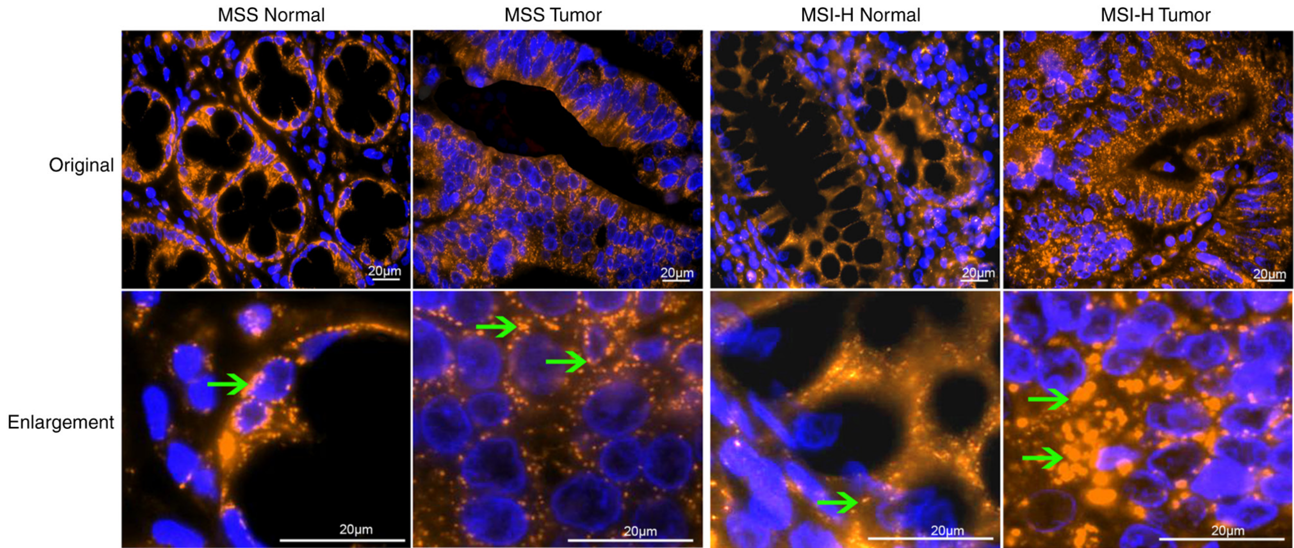

Figure 4. Mitochondrial DNA was assessed using fluorescence *in situ* hybridization in the colorectal cancer and adjacent tissues of patients in the MSI-H and MSS groups. Green arrows represent mitochondrial DNA (orange fluorescence). The enlarged area is a partially enlarged image of the above image; scale bars, 20 μm. MSI-H, high-frequency microsatellite instability; MSS, microsatellite stability.

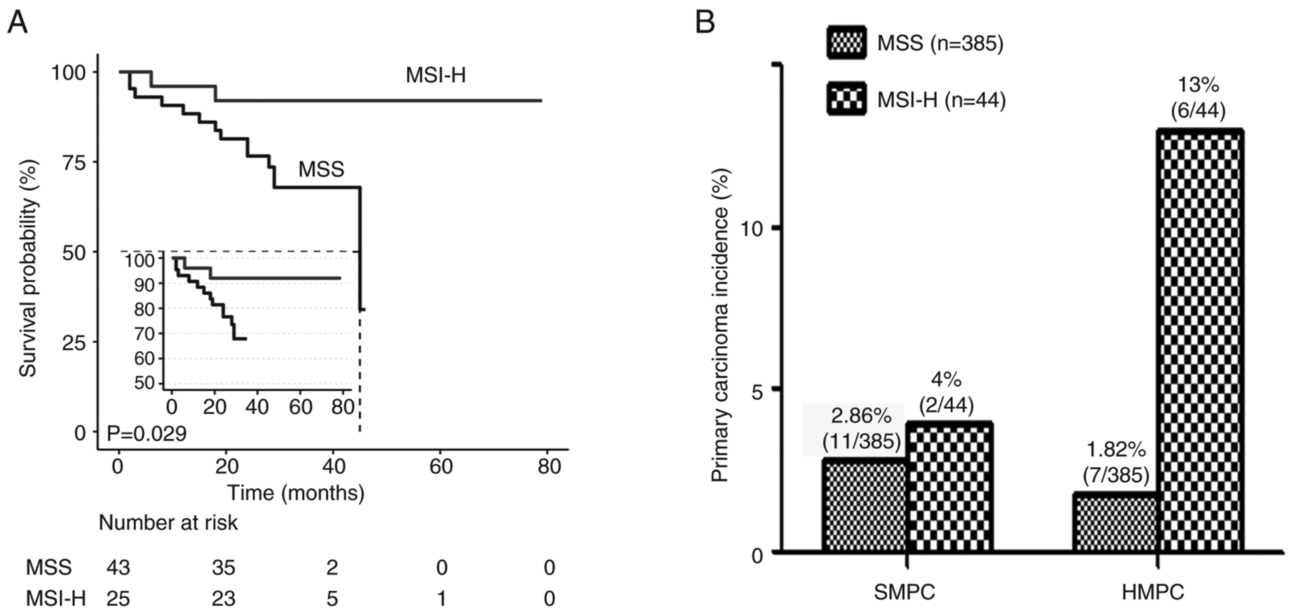

Figure 5. MSI status is associated with the overall survival rate and categories of multiple primary cancers in patients with CRC. (A) Overall survival rates were significantly lower in MSS patients, compared with the overall survival rates of MSI-H patients. (B) Compared with the patients with MSS, those with MSI-H were markedly more susceptible to multiple primary cancers (including SMPC and HMPC); the difference between the MSS and MSI-H groups was statistically significant (P<0.01). CRC, colorectal cancer; MSI, microsatellite instability; MSI-H, high-frequency MSI; MSS, microsatellite stability; SMPC, simultaneous multiple primary carcinoma; HMPC, heterochronous multiple primary carcinoma.

dMMR group, the MLH1 combined with PMS2 deficiency rate was the highest, accounting for 44.23%. Notably, patients with the BRAF-V600E mutation in the MSI-H group all had MLH1 combined with PMS2 deficiency. This suggests that in patients with MSI-H, detecting BRAF mutations could help distinguish between hereditary CRC and sporadic CRC. In the MSI-H cases, CRC may be considered hereditary in patients when no BRAF gene mutations were present.

The mitochondria serve a key role in cancer. A previous study reported an elevation in mitochondrial DNA in patients with gastric cancer compared with patients without gastric cancer, and it was also elevated in patients with grade II-IV inflammation and atrophy compared with patients with grade 0-I inflammation and atrophy (19). The present study demonstrated a marked amplification of mitochondrial DNA in the tumor cells of patients in the MSI-H group compared with those of patients in the MSS group. Therefore, we hypothesize that mitochondrial DNA is a novel biomarker and therapeutic target for patients with CRC with MSI-H. However, the role and underlying mechanisms of mitochondrial amplification in MSI-H remain unclear and require further research.

As 80-90% of patients with CRC who undergo surgical treatment experience recurrence within 2-3 years after surgery, the present study performed a survival follow-up for patients who underwent surgery >2 years prior (25 MSI-H patients and 43 MSS patients were included). Through survival analysis, it was demonstrated that MSI-H was a favorable prognostic factor for patients with CRC. Moreover, compared with the MSS group, the survival rate was significantly increased in the MSI-H group. MSI-H was revealed to be associated with a lower risk of mortality, as revealed by Cox proportional-hazards model. Although P>0.05 was obtained, the HR value was <1 in models II and III, indicating that MSI-H may serve as a biomarker for predicting a good prognosis in patients with CRC.

Multiple primary cancers are defined as >1 synchronous or metachronous cancer in the same individual. Underlying causes for multiple primary cancers may include host and lifestyle-related factors, environmental and genetic factors and treatment-related factors (20). In the present study, 15.38% (8/52) of patients in the MSI-H group had multiple primary cancers (including simultaneous multiple primary carcinoma and heterochronous multiple primary carcinoma), whilst only 4.47% (18/403) of patients in the MSS group had multiple primary cancers. This suggests that MSI may be one of the reasons for the occurrence of multiple primary tumors.

However, the present study has limitations. As certain patients went to other hospitals for treatment and follow-up examinations after surgery, the present study was unable to obtain the true drug efficacy and disease progression of these patients, so these data were not analyzed. In addition, the study was unable to further explore the mechanism of mitochondrial DNA amplification in the MSI-H group. We aim to conduct further research in the future to explore whether mitochondrial DNA amplification combined with MSI-H is involved in CRC cell proliferation and migration, which may provide new targets for the treatment of CRC.

In conclusion, the results of the present study demonstrate that the combined detection of PD-L1 and MSI in patients with CRC may provide accurate and effective guidance for selecting more personalized treatment strategies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ZT and HT contributed to the conception and design. ZT and HT acquired the funding. HT, JL and XC performed the experiments. ZT and TG analyzed the data and drafted the original manuscript. ZT and JL contributed to the manuscript writing and revision. ZT and JL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Second Xiangya Hospital of Central South University Ethics Review Board (Scientific and Research Ethics Committee; Changsha, China; approval no. LYEC2022-K0026). The methods were performed in accordance with the approved guidelines. All samples were obtained with written informed patient consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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