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Evaluation of genetic instability of short tandem repeats in hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) ranked as the sixth most common malignancy and the third leading cause of cancer-related mortality with approximately 830, 000 deaths worldwide annually. Genetic instability of short tandem repeats (STRs), which manifested as loss of heterozygosity (LOH) or microsatellite instability (MSI) in the cancerous cells, is a genetic feature of many types of human cancers. The status of STR instablility and its clinical significance in HCC, however, remains to be comprehensively elucidated. In this study, a total of 101 matched DNA samples from HCC individuals were analyzed with 20 "classical STR markers widely used in forensic genetics, our findings demonstrated that 79.21% (80/101) of HCC cases exhibited genetic alterations in at least 1 STR locus, with 16.73% of STR loci altered across all samples. Moreover, our findings also revealed a significant association between an accumulation of STR alterations and the presence of positive hepatitis B surface antigen (HBsAg), as well as moderate-poor/poor differentiation of HCC. Furthermore, LOH at the FGA was found to be significantly correlated with moderate-poor/poor differentiation of HCC ($p = 0.002$), and LOH at the D16S539 was found to be significantly associated with elevated serum levels of AFP ($p = 0.042$) as well as larger tumor sizes ($p = 0.040$). Overall, this study contributes valuable insights into the genetic instability of STRs in HCC and might also enhance insights into the intricate mechanisms underlying hepatocarcinogenesis.

Keywords Forensic genetics, Hepatocellular carcinoma (HCC), Short tandem repeats (STRs), Loss of heterozygosity (LOH), Microsatellite instability (MSI)

In certain particular situations, archival pathologic specimens may become the last source of biological material available for forensic casework, such as when a putative parent was deceased with no other material available, when pathological tissues were suspected to be mixed up in medical disputes, when disaster victims or missing persons are required for individual identification, or other special situations. Most of the archival material, however, is composed of solid tumors known to harbor high rates of genetic alterations randomly and abundantly throughout the tumor genome. Therefore, genetic instability of short tandem repeats (STRs) in cancerous tissues is also possible[1]. STR instability, which manifests as loss of heterozygosity (LOH) or microsatellite instability (MSI), could lead to the genotyping inconsistency between tumor and normal tissues, making forensic identification involving with tumor samples remains challenging in forensic genetics. Although the status of STR instability has been demonstrated in a variety of malignant tissues, including gastric cancer[2]colorectal cancer[3]lung cancer[4]leukemic[5]pancreatic cancer[3]breast cancer[6]papillary thyroid cancer[7]and et al. The status of STR instability and its clinical significance in hepatocellular carcinoma (HCC) remains to be comprehensively elucidated.

Hepatocellular carcinoma (HCC), the predominant subtype (80–85%) of liver cancer[8]continues to be the most formidable malignancies due to the lack of early diagnostic methods and efficient therapeutic strategies. A recent investigation in 2022 reported that HCC ranked the sixth most commonest malignancy and the third in cancer-related mortality with approximately 830,000 deaths worldwide annually[9]. In China, HCC ranked the fourth most prevalent, and the second leading lethal cancer close behind lung cancer[10]. A recent study by Anqi Chen et al. 2024 demonstrated that 63.33% (19/30) HCC cases harbored genetic alterations in at least one STR locus, with an overall STR alteration rate of 10.88%[3]. But this study was limited by a small sample size and no clinical data. To gain a deeper understanding of the patterns and degree of STR instability, as well as its clinical significance in HCC, we carried out a comprehensive investigation with a total of 101 matched HCC DNA from cancerous and adjacent non-cancerous tissues.

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Results

Widespread STR alterations in HCC

In this study, 20 STRs and the Amelogenin gene were all amplified and genotyped successfully in the 101 matched HCC samples. 79.21% (80/101) of HCC cases exhibited STR alternations in least one STR locus. Among these investigated HCC cases, 20.79% of patients (21/101) displayed alterations with 1 ~ 2 STRs, 47.52% of patients (48/101) with 3 ~ 7 STRs, and 10.89% of patients (11/101) with a number of STRs exceeding 7 (Supplementary Materials S1).

Types and frequencies of STR alterations in HCC

Four types of STR alterations, namely partial loss of heterozygosity (pLOH), complete loss of heterozygosity (cLOH), occurrence of an additional allele (Aadd) and occurrence of a new allele (Anew) were all detected in HCC samples (Fig. 1). A total of 338 STR alternations were observed in 101 HCC tissues. pLOH exhibited as the predominant type of STR alterations with an overall rate of 13.91%, followed by cLOH, Aadd, and Anew with an overall frequency of 1.83%, 0.79%, and 0.2%respectively (Table 1). All investigated STRs exhibited varying degrees of alternation rates ranging from 4.95 to 47.52% (Fig. 2), significantly higher than the average rate of 0.2% in healthy germlines[11]. Notably, FGA and D16S539 exhibited a distinctively high frequency of alterations, reaching up to 47.52% and 42.57% respectively.

Comparison of the average alteration rate of the forensic STRs across different types of malignant tumors

To better understand the degree of STR alterations in HCC, we compared the average rate of STR alterations in HCC with other types of malignant tumors. Interestingly, we found that HCC exhibited the highest level of genetic instability, followed by colorectal cancer (CRC)[3]gastric cancer (GC)[2]lung cancer (LC)[4]renal cell cancer (RCC)[3]pancreatic cancer (PC)[3]breast cancer (BC)[3] and papillary thyroid cancer (PTC)[7] (Fig. 2; Table 2). Our results indicated that HCC individuals are intrinsically much more susceptible to genetic alterations than any other malignant cancers reported to date. Furthermore, although both the incidence and overall frequencies of STR alterations in this study are a little higher than those in previous study for HCC[3]no statistically significant differences existed in the incidence of STR alterations between the two HCC groups.

Association of STR alternations with clinicopathological characteristics

To assess the clinical implication of STR hypermutability in HCC, we analyzed the relationship between STR alterations and various clinicopathological characteristics including gender, age, serum a-fetoprotein (AFP), cirrhosis, HBsAg status, tumor size, and degree of tumor differentiation. However, no statistically significant association was found between STR alternations (grouped by presence or absence of STR alterations) and any clinicopathological features ($p > 0.05$). But when the 101 HCCs were divided into 3 groups based on the cumulative number of altered STR loci, namely the stable group ($n = 0$), the low-alterated group ($n = 1 \sim 2$), and the high-alterated group ($n \geq 3$), the results revealed a significant association between the accumulation of STR alterations and the presence of positive HbsAg ($p = 0.020$) as well as the moderate-poor/poor differentiation ($p = 0.049$) (Table 3). Moreover, LOH at FGA was found to be associated with moderate-poor/poor differentiation ($p = 0.002$) (Table 4). And LOH at D16S539 was found to be significantly associated with an elevated AFP level ($p = 0.042$) as well as a larger tumor size ($p = 0.040$) (Table 5), indicating that LOH at FGA and D16S539 are most common genetic events in HCC.

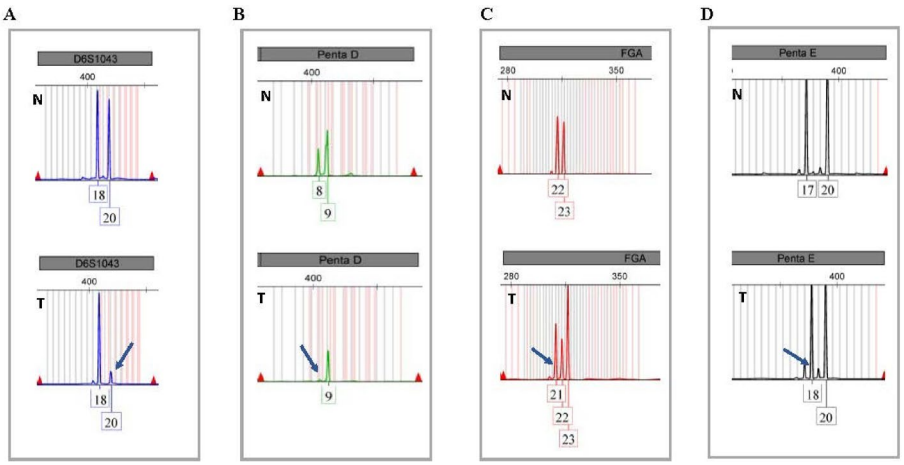

Fig. 1. Four typical types of STR alterations in HCC. (**A**) partial loss of heterozygosity (pLOH), (e.g., allele 18/20 → allele 18/20, but the peak of '20' allele in cancer tissues was significantly reduced); (**B**) complete loss of heterozygosity (cLOH) (e.g., allele 8/9→ allele 9/9); (**C**) an additional allele (Aadd) (e.g., allele 22/23 → allele 21/22/23); (**D**) the occurrence of a new allele (Anew) (e.g., allele 17/20 → allele 18/20).

Locus	Types of alterations (*n*, %)				Total alterations (*n*, %)
	pLOH	LOH	Aadd	Anew	
D2S1338	7 (6.93%)	0	0	0	7 (6.93%)
TPOX	3 (2.97%)	0	2 (1.98%)	0	5 (4.95%)
D2S441	7 (6.93%)	0	0	0	7 (6.93%)
D3S1358	7 (6.93%)	1 (0.99%)	0	0	8 (7.92%)
FGA	37 (36.63%)	4 (3.96%)	5 (4.95%)	2 (1.98%)	48 (47.52%)
D5S818	11 (10.89%)	3 (2.97%)	1 (0.99)	0	15 (14.85%)
CSF1PO	10 (9.90%)	1 (0.99%)	0	0	11 (10.89%)
D6S1043	20 (19.80%)	2 (1.98%)	0	0	22 (21.78%)
D7S820	8 (7.92%)	0	0	0	8 (7.92%)
D8S1179	20 (19.80%)	1 (0.99%)	2 (1.98%)	0	23 (22.77%)
TH01	9 (8.91%)	1 (0.99%)	0	0	10 (9.90%)
D12S391	15 (14.85%)	2 (1.98%)	3 (2.97%)	1 (0.99%)	21 (20.79%)
vWA	13 (12.87%)	1 (0.99%)	1 (0.99%)	0	15 (14.85%)
D13S317	20 (19.80%)	4 (3.96%)	0	0	24 (23.76%)
Penta E	11 (10.89%)	2 (1.98%)	0	1 (0.99%)	14 (13.86%)
D16S539	38 (37.62%)	5 (4.95%)	0	0	43 (42.57%)
D18S51	13 (12.87%)	4 (3.96%)	1 (0.99%)	0	18 (17.82%)
D19S433	5 (4.95%)	1 (0.99%)	0	0	6 (5.94%)
Penta D	15 (14.85%)	2 (1.98%)	0	0	17 (16.83%)
D21S11	12 (11.88%)	3 (2.97%)	1 (0.99%)	0	16 (15.84%)
Average	281 (13.91%)	37 (1.83%)	16 (0.79%)	4 (0.20%)	338 (16.73%)

Table 1. Types and frequencies of the alterations at the 20 STR loci in HCC.

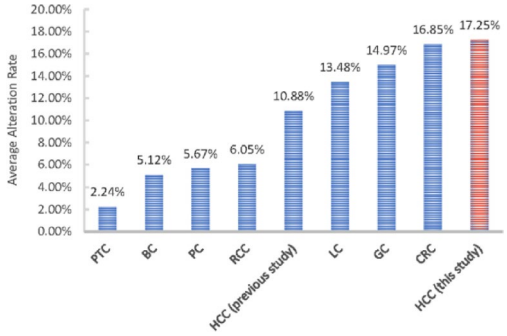

Fig. 2. Average alteration frequency of the 19 STRs across different tumor types: PTC: papillary thyroid cancer[7]BC: Breast cancer[3]PC: pancreatic cancer[3]RCC: renal cell cancer[3]HCC: hepatocellular cancer[3]LC: lung cancer[4]GC: gastric cancer, CRC[2]: colorectal cancer[2].

Discussion

In current study, we performed a genome-wide investigation of the status of STR alterations in 101 paired HCCs using "classical" forensic STRs widely used in forensic genetics. Our results revealed that 79.21% (80/101) of HCCs exhibited STR alterations in at least one locus, with 16.73% of STR loci altered across all samples. Although these frequencies were slightly higher than those in the previous study for HCC[3] no statistically significant differences in the incidence of STR alterations was observed between the two HCC groups. And the slight differences might partly due to the differences in sample size, geographical region, inclusion criteria for HCC volunteers, or various clinical characteristics. By reviewing existing literature, we compared the average frequencies of the STRs alterations in different types of malignancies with sufficiently powered samples ($n \geq 20$) for statistical validation. The results revealed that the average frequencies of the STR alterations varied in different tumors, and HCC exhibited a significantly higher alteration frequency compared to any other malignancy reported to date, indicating a remarkable high degree of genetic instability in HCC. Furthermore, our investigation also revealed that different tumors exhibited varied alteration tendencies across the STR loci (Table 2), and no significant regularity could be observed. Intriguingly, both wide ranges and high frequency of STR alterations were detected in CRC, GC and HCC, while the STRs were selectively altered in RCC, PC, BC, LC, and PTC, indicating the inclined alteration in STRs. Although four types of STR alterations were all detected in HCC, LOH (including pLOH and cLOH) was revealed as the predominant type of STR alterations (Supplementary Fig. 1), in agreement

Locus	HCC (this study) ($n=101$)	HCC (previous study)[3] ($n=30$)	CRC[2] ($n=129$)	GC[2] ($n=121$)	RCC[3] ($n=22$)	PC[3] ($n=35$)	BC[3] ($n=36$)	LC[4] ($n=75$)	PTC[7] ($n=68$)
D2S1338	6.93%	6.67%	13.18%	15.70%	4.55%	2.86%	2.78%	5.33%	5.88%
TPOX	4.95%	3.33%	7.75%	5.79%	0.00%	0.00%	2.78%	4.00%	2.94%
D3S1358	7.92%	10.00%	9.30%	12.40%	13.64%	5.71%	5.56%	6.67%	2.94%
FGA	47.52%	30.00%	24.03%	19.33%	4.55%	6.45%	2.78%	5.33%	2.94%
D5S818	14.85%	0.00%	24.81%	12.40%	0.00%	8.57%	0.00%	9.33%	1.47%
CSF1PO	10.89%	3.33%	23.26%	21.49%	0.00%	6.25%	2.78%	2.67%	4.41%
D6S1043	21.78%	26.67%	13.18%	17.50%	15.00%	7.14%	2.78%	1.33%	1.47%
D7S820	7.92%	0.00%	10.08%	14.05%	4.55%	2.94%	2.78%	2.67%	1.47%
D8S1179	22.77%	16.67%	16.28%	15.70%	9.09%	5.71%	11.11%	1.33%	2.94%
TH01	9.90%	3.33%	10.08%	9.09%	9.09%	2.86%	8.33%	5.33%	1.47%
D12S391	20.79%	13.33%	13.95%	16.53%	4.55%	9.68%	8.33%	6.67%	2.94%
vWA	14.85%	13.33%	16.67%	16.81%	4.55%	11.43%	8.33%	2.67%	0.00%
D13S317	23.76%	36.67%	13.28%	9.92%	4.55%	2.94%	11.11%	4.00%	2.94%
Penta E	13.86%	6.67%	26.56%	19.33%	9.09%	7.14%	11.11%	0.00%	2.94%
D16S539	42.57%	23.33%	9.30%	14.88%	0.00%	2.86%	11.11%	2.67%	0.00%
D18S51	17.82%	6.67%	43.41%	21.85%	9.09%	9.38%	5.56%	4.00%	1.47%
D19S433	5.94%	0.00%	14.73%	14.05%	0.00%	2.86%	0.00%	1.33%	1.47%
Penta D	16.83%	3.33%	15.50%	16.81%	13.64%	6.45%	0.00%	5.33%	0.00%
D21S11	15.84%	3.33%	14.73%	10.74%	9.09%	6.45%	0.00%	1.33%	2.94%
Average	17.25%	10.88%	16.85%	14.97%	6.05%	5.67%	5.12%	3.79%	2.24%

Table 2. Comparison of the alteration frequency of STRs in different malignant cancers (%).

Characteristics	n	No. of the accumulation of STR alterations			p-value
		The stable group ($n=0$)	The low-alterated group ($n=1\sim2$)	The hihg-alterated group ($n\geq3$)	
Age					
≥ 60	50	8	15	27	0.064
< 60	51	13	6	32	
Sex					
Female	17	2	5	10	0.438
Male	84	19	16	49	
Tumor size					
≥ 5 cm	58	8	13	37	0.154
< 5 cm	43	13	8	22	
Tumor number					
Single	71	14	18	39	0.224
Multiple	30	7	3	20	
AFP (ng/ml)					
≥ 20 ng/ml	58	12	9	37	0.287
< 20 ng/ml	43	9	12	22	
Hepato-cirrhosis					
Positive	13	2	5	6	0.291
Negative	88	19	16	53	
HBsAg					
Positive	83	16	14	53	0.049
Negative	18	5	7	6	
Degree of differentiation					
Well /well-moderate	35	8	12	15	0.020
Moderate-poor /poor	66	13	9	45	

Table 3. Association of the accumulation of STR alterations with clinicopathological characteristics of HCC.

Characteristic	Alterations at FGA (n)			p-value
	Positive			
	LOH (pLOH and cLOH)	MSI (Aadd and Anew)	Negative	
Degree of differentiation				0.002
Moderate-poor /poor	33	1	33	
Well/well-moderate	8	6	20	

Table 4. Association of STR alterations at FGA with clinicopathological characteristics of HCCs.

Characteristics	*n*	LOH at D16S539 (*n*)		*p*-value
		Positive	Negative	
Tumor size				
≥ 5 cm	58	29	29	0.040
< 5 cm	43	14	29	
AFP (ng/ml)				
≥ 20	58	30	28	0.042
< 20	43	13	30	

Table 5. Association of LOH at D16S539 with clinicopathological characteristics of HCCs.

with previous literature in which LOH was demonstrated as a major event in almost all malignant tumors[12]. In actual forensic practice, only the types of cLOH, Anew and Aadd could lead to STR genotype alteration (STR_{GA}), complicating the forensic evaluation. Whereas in this study STR_{GA} occurred in 31 of 101(30.69%) HCC cases, and STR_{GA} was detected at almost all locus except D2S1338, D2S441 and D7S820 in this study. Obviously, the "Gold standard" method of STR-genotyping in forensic application remains challenging when involving with malignant tissues. Alternative approaches such microhaplotypes (MHs) strategy, genome-wide sequencing or high-throughput genotyping of single nucleotide polymorphism (SNPs) might be powerful options.

Increasing evidence has demonstrated that there may be associations between STR alterations and medical conditions[13–15]. In recent years, it has been proposed that STR alteration in cancerous tissue can be used as a biomarker for certain cancers or as a predictor of certain susceptibility disease[3,6,16,17]. Although in our study no significant associations were found between the presense or absence of STR alterations and age, sex, tumor size, hepato-cirrhosis or AFP level, a significant association was observed between the accumulation of STR alterations and HbsAg as well as degree of differentiation, indicating that HCC patients with hepatitis B virus or poor differentiation are more susceptible to STR alterations.

LOH, indicative of tumor suppressor gene (TSG) pathway, has been established as an important mechanism in the carcinogenesis and/or subsequent progression of human cancers. LOH at 4q has been documented in several human cancers including cervical carcinoma[18]oesophageal carcinoma[19]squamous cancer in head and neck[20]small-cell lung cancer[21]HCC[22–24] and et al. However, LOH at 4q occurred more frequently in HCC than in all other cancers. Among which LOH at 4q21-22, 4q28, 4q34, 4q35, 4q24-26, 4q34.3-35 have been frequently observed in HCC. And several HCC-associated tumor suppressor genes (TSGs) including *PLAC8* (4q21.22), *PRDM5* (4q26), *PCDH10* (4q28.3), *FBXW7* (4q31.3), *SORBS2* (4q35.1) have been identified from these regions[25–31]. Consistent with previous reports on high frequency of LOH at 4q31 in HCC[22,23]our data not only revealed a distinctively high frequency of LOH at 4q31.3 (FGA), but also a significant association with moderate-poor/poor differentiation of HCC, indicating an important TSG associated with HCC may reside at the region of 4q31.3. Interestingly, the STR marker of FGA is exactly located in the third intron of the fibrinogen α (*FGA*)(4q31.3) gene, which encodes the alpha subunit of the coagulation factor fibrinogen. Mutations in *FGA* have been identified in several diseases including hypofibrinogenemia, afibrinogenemia, dysfibrinogenemia as well as renal amyloidosis[32–35]. A recent study has established *FGA* as a "drive" gene regulating the progression and metastasis of HCC[36]. And *Xi Han* et al. 2024 further validated that *FGA* acted as a TSG suppressing invasion and metastasis in HCC via the PI3K/AKT signaling pathway[37]. Whereas our findings provide novel clinical evidence for the critical role of *FGA* in HCC.

LOH at 16q occurs frequently in various human malignancies including breast cancer[38]ovarian cancer[39]prostate cancer[40] and HCC[23,41,42]suggesting that one or more tumor suppressor genes (TSGs) may lie within the region of 16q. Although thus far no definite TSGs associated with HCC at chromosome 16q have yet been identified, LOH on 16q is yet a most frequent genetic events in HCC and occur more frequently with increased tumor size, poor differentiation and metastasis in HCC[1,23,43,44]. Intriguingly, in this study our data not only demonstrated a markedly high frequency of LOH at 16q24(D16S539), but also revealed a significant association with increased tumor size as well as elevated AFP level. To our knowledge, it is the first to report that LOH on chromosome 16q is associated with an elevated AFP in HCC. These findings indicated that an important TSG, which could not only regulate hepatocyte growth but also involve in postnatal re-expression of *AFP*, may reside within the region of 16q24.1. But further study is required to clarify this issue.

Conclusions

Taken together, our findings advanced our understanding of the genetic basis of HCC with a robust sample size and clinically relevant correlations. More importantly, our findings holds considerable potential for advancing both forensic genetics and oncology. Future research will focus on the potential roles and underlying mechanisms of FGA as well as D16S539 in hepatocellular carcinoma.

Materials and methods

Participants and samples

101 HCC individuals were recruited from the Hunan Provincial People's Hospital (the First Affiliated Hospital of Hunan Normal University), Changsha, PR China during 2022–2024. Fresh cancerous and corresponding control tissues were collected with histopathologically verified HCC. Informed consent was obtained from each HCC volunteer.

Patients who had received any treatment including chemotherapy, radiotherapy, or other cancer-related treatments prior to surgery were excluded from the current research cohort. This study included 84 male and 17 female with ages ranging from 30 to 82. This study was approved by the Ethics Committee of Academy of the Hunan Provincial People's Hospital, China.

DNA preparation

DNA was extracted utilizing the DNeasy®Blood & Tissue Kit (Qiagen, Venlo, The Netherlands) according to the instructions. The DNA concentration was acquired using a NanoDrop One (Thermofisher Scientific, Waltham, MA, Germany).

STR genotyping

PCR was amplified using the Microreader™21ID kit (MR21, MicroReader, Beijing, China) comprising 20 autosome loci (13 CODIS STRs, D2S441, Penta E, Penta D, D2S1338, D19S433, D12S391, and D6S1043) and the Amelogenin gene (Amel). The primer sequences were those detailed in supplementary S3, and the PCR reaction conditions were as follows: 37 °C for 5 min, then 96 °C for 4 min, followed by 28 cycles at 94 °C for 5 s and 60 °C for 70 s, then 60 °C for 30 min and hold at 4 °C. Genotyping was conducted using the ABI 3500 Genetic Analyzer (Applied Biosystems, USA), and STR genotyping data were validated with an allelic ladder and positive and negative controls using GeneMapper™ ID-X Software (Applied Biosystems, USA). All data from paired tissues were analyzed in a double-blind manner.

To distinguish genuine alternations from genotyping errors, 101 paired samples with inconsistent genotypes or null alleles were re-analyzed to confirm the genotypes using the PowerPlex 21 kit (Promega, Madison WI, USA), which includes 20 autosomal loci (13 CODIS STRs, D1S1656, Penta E, Penta D, D2S1338, D19S433, D12S391, and D6S1043) and the Amel gene. The PCR reaction conditions were as follows: 96 °C for 1 min, followed by 25 cycles at 94 °C for 10 s and 72 °C for 30 s, then 60 °C for 20 min and hold at 4 °C. Four types of STR alterations in HCC cases (HCCs) were observed as previously described, i.e. occurrence of an additional allele (Aadd) (e.g., allele 22, 23 →allele 21, 22, 23); Occurrence of a new allele (Anew) (e.g., allele 17,20→ allele 18,20); and complete loss of heterozygosity (cLOH), and specimens with the ratio of peak intensity in normal/tumor pair < 0.5 or > 2.0 were defined as partial loss of heterozygosity(pLOH)[2], both cLOH and pLOH were classified as LOH. In addition, considering that Aadd (occurance of an additional allele) and Anew (occurrence of a new allele) were characterisized by length alterations of simple repeats and caused by DNA mismatch repair deficiency, both Aadd and Anew are also generally referred to as microsatellite instability (MSI).

Statistical analysis

The statistics were executed using SPSS software (version 27.0). the count data are submitted as proportions (%) or frequencies (n), the χ^2 test or Fisher's exact probability method was employed to analyze and compare the data between different cohorts. The p-value < 0.05 was considered statistically significant.

Data availability

The patient data and genotyping data were collected and provided by the Hunan Provincial People's Hospital. All data presented in the study is included within the manuscript or supplementary table S1 files. Further inquiries can be directed to the corresponding author.

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Author contributions

YH.S performed statistical analyses and revised the manuscript; M.X and XL.Z performed the experiments and prepared all figures and tables; YF.L collect the samples and clinical data; W.X designed the study and wrote the main manuscript text. All authors reviewed and approved the final version.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

This study was approved by the Ethics Committee of Academy of the Hunan Provincial People's Hospital, China. (approval No. 2022–156). All HCC patients provided informed consent to participate in the study, and all experiments were performed in accordance with relevant guidelines and regulations.

Additional information

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