

Nuclear and mitochondrial DNA microsatellite instability in Chinese hepatocellular carcinoma

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Abstract

AIM: To study the nuclear microsatellite instability (nMSI) at BAT26 and mitochondrial microsatellite instability (mtMSI) in the occurrence and development of hepatocellular carcinoma and the relationship between nMSI and mtMSI.

METHODS: nMSI was observed with PCR and mtMSI with PCR-SSCP in 52 cases of hepatocellular carcinoma.

RESULTS: mtMSI was detected in 11 out of the 52 cases of hepatocellular carcinoma (21.2%). Among the 11 cases of hepatocellular carcinoma with mtMSI, 7 occurred in one locus and 4 in 2 loci. The frequency of mtMSI in the 52 cases of hepatocellular carcinoma showed no correlation to sex, age, infection of hepatitis B, liver cirrhosis as well as positive AFP of the patients ($P>0.05$). In addition, nMSI was detected in 3 out of 52 cases of hepatocellular carcinoma (5.8%) and there was no correlation of the incidence of mtMSI to that of nMSI ($P>0.05$).

CONCLUSION: mtMSI may be involved in the occurrence and development of hepatocellular carcinoma and it is independent of nMSI.

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INTRODUCTION

Mitochondria are the energy-transducing organelles of eukaryotic cells in which fuels to drive cellular metabolism are converted into cellular adenosine triphosphate (ATP) through the process of oxidative phosphorylation. Mitochondria are responsible for generating approximately 90% of ATP. The mitochondrion is the only organelle in the cell, aside from the nucleus, which contains its own genome and genetic machinery^[1]. Mitochondrial DNA (mtDNA) is a 16 569 base-pair, double-stranded and closed circular molecule, and encodes 13 polypeptides. All of the polypeptides are components of the respiratory chain/OXPHOS system, plus 24 genes, specifying two ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs), which are required to synthesize the 13 polypeptides. Mitochondrial genome is far more vulnerable to oxidative

damage and undergoes a higher rate of mutation than nuclear genome due to its lack of histone protection, limited repair capacity, and close proximity to the electron transport chain, which constantly generates superoxide radicals^[2-5]. Accumulation of mutations in mtDNA is approximately tenfold greater than that in nuclear DNA^[6].

A high frequency of mtDNA mutations has been identified in cancer of the colon^[7], stomach^[8], liver^[9], pancreas^[10], lung^[11], breast^[12], kidney^[13], prostate^[14], ovary^[15], Barrett's esophagus^[16] and leukemia^[17]. The majority of these somatic mutations were homoplasmic, suggesting that mutant mtDNA becomes dominant in tumor cells. In addition, microsatellite instability has also been shown in mtDNA of colorectal and gastric carcinomas^[18,19]. Further studies demonstrated that mononucleotide could repeat alteration, missense mutation, and small deletion in NADH dehydrogenase genes and alteration in a polycytidine (C)_n tract in the D-loop region of mtDNA could occur in colorectal carcinomas^[20]. These results imply that microsatellite instability in mtDNA (mtMSI) of colorectal carcinoma may be resulted from certain deficiencies in DNA repair. Therefore, it has been proposed that somatic mutations and mtMSI play a role in tumorigenesis and development of cancer^[21].

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer related mortality worldwide. The incidence of HCC shows a considerable geographical variation with a very high incidence in China. Epidemiological studies in high-risk populations have identified chronic hepatitis B virus (HBV) and chronic hepatitis C virus (HCV) infection as well as dietary exposure to aflatoxin B1 (AFB1) as major factors in the etiology of this disease^[22]. It has been reported that the amount of AFB1 combined to hepatocellular mtDNA is 3-4 fold larger than that combined to nuclear DNA (nDNA). This combined product of aflatoxin cannot easily be expelled and stays in mtDNA for a long period^[22]. Since there is a prolonged period between initial HBV and HCV infection and emergence of HCC, multiple genetic events may occur to promote the malignant transformation of hepatocytes. Many chromosomal aberrations have been frequently reported in HCCs including loss of heterozygosity (LOH) at numerous loci^[23,24]. The repeated destruction and regeneration of liver tissue associated with chronic viral hepatitis would lead to accumulation of mtDNA mutations^[25]. Although MSI in nuclear DNA (nDNA) of HCCs has been detected^[26-32], little attention has been paid to MSI in mtDNA (mtMSI) in this tumor. In order to elucidate the role of mtMSI in the hepatocarcinogenesis, we examined mtMSI and nMSI in a set of 52 Chinese HCCs.

MATERIALS AND METHODS

Fresh tissues were collected from 52 HCC patients undergoing hepatic resection in the Southwest Hospital, Third Military Medical University, Chongqing, China from 1996 to 2002. Neoplastic and nonneoplastic liver tissues were frozen in liquid nitrogen immediately and kept at -70 °C until processing. The 52 patients consisted of 42 males and 10 females, their age ranged from 22 to 71 years with an average of 48.8 years at

Table 1 Sequences of primer for PCR analysis

Repeat sequence	mtDNA region	Position	Annealing (°C)	Primer (5' -3')
(C) _n	270-425	D-loop	58	TCCACACAGACATCAATAACA AAAGTGCATACCGCCAAAAG
(CA) _n	467-556	D-loop	55	CCCATACTACTAATCTCATCAA TTTGGTTGGTTCGGGGTATG
(C) ₆	3529-3617	ND1	55	CCGACCTTAGCTCTCACCAT AATAGGAGGCCTAGGTTGAG
(A) ₇	4555-4644	ND2	55	CCTGAGTAGGCCTAGAAATAAA ACTTGATGGCAGCTTCTGTG
(T) ₇	9431-9526	COIII	55	CCAAAAAGGCCTTCGATACG GCTAGGCTGGAGTGGTAAAA
(C) ₆ and (A) ₈	12360-12465	ND5	55	CACCCTAACCTGACTTCC GGTGGATGCGACAATGGATT
(CCT) ₃ and (AGC) ₃	12940-13032	ND5	55	GCCCTTCTAAACGCTAATCC TCAGGGGTGGAGACCTAATT

diagnosis. Thirty-two patients were positive and 20 were negative for hepatitis B surface antigen (HBsAg). Hepatitis C virus antibody (Anti-HCV) was negative for all cases. Hematoxylin and eosin-stained sections were prepared from the same samples used for mtMSI and nMSI studies and the diagnosis of HCC was confirmed by histology. None of the patients included in the present series had a family history suggestive of HNPCC and none had received previous chemotherapy or radiation therapy. Necrotic tumors were excluded from the study. The tumor samples contained more than 70% malignant cells. Genomic DNA was isolated from tumor and non-tumor liver tissues and blood, using standard proteinase-K digestion and phenol-chloroform extraction protocols.

PCR-single strand conformation polymorphism (PCR-SSCP) was performed to amplify the microsatellite sequence of mtDNA using published primers^[18]. The primer consisted of 2 D-loop regions and 5 coding regions (Table 1). The reaction conditions and procedures were similar to those reported by Hebano *et al*^[18].

Each PCR was digested by appropriate restriction enzymes and electrophoresed at 300V at 22°C for 2 hr on a 7.5% polyacrylamide gel containing 50 mmol/L boric acid, 1 mmol/L EDTA and 2.5% glycerol. After silver staining, PCR products showing mobility shifts were directly sequenced using appropriate internal primer and analyzed using 373A automated DNA sequencer (Perkin Elmer Cetus). All analyses were performed twice to rule out PCR artifact.

MSI at BAT26 microsatellite locus was analyzed using PCR method. The sequence of upper stream primer was 5' -TGACTACTTTTGACTTCAGCC-3' and that of down stream primer was 5' -AACCATTCA ACA TTT TTA ACC C-3'. PCR was performed in 20 µl of reaction mixture containing 10 mmol/L Tris-HCl (pH8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 200 µmol/L each deoxynucleotide triphosphate, 0.5 µmol/L of each primer, 0.5 unit Ampli Taq polymerase (Perkin-Elmer Cetus, Nowak), 100 ng genomic DNA and 0.5 µCi [³³P] dATP. The reaction was carried out in a thermal cycler at 94 °C for 1 min, at 55 °C-62 °C for 1 min, and at 72 °C for 1min, for 35 cycles with an initial denaturation step at 94 °C for 5 min and final extension step at 72 °C for 10 min. The PCR products were then separated on 5% polyacrylamide 7M urea denaturing gel, and visualized by autoradiography. MSI was defined as the presence of a band shift in the tumor DNA not present in the corresponding normal DNA.

χ^2 test was used for statistical analysis and $P < 0.05$ was considered as statistically significant.

RESULTS

Fifty-two HCC samples were screened for mtMSI at seven repeat sites using the PCR-RFLP method. Figure 1 exhibits a representative mobility-shift band compared with normal counterpart. mtMSI affecting at least one locus was observed in 11 out of 52 cases (21.2%), in which 7 cases affected 1 locus and 4 cases affected 2 loci. mtMSI occurred in D-loop in 10 cases (19.2%), in which 8 cases occurred in (C)_n region and 2 cases in (CA)_n region. mtMSI occurred in the coding region in 5 cases (9.6%), and concomitant mtMSI locus was found in the D-loop in 4 out of the 5 cases. The frequency of mtMSI in 52 cases of HCC showed no correlation to sex, age, HBV infection, liver cirrhosis and positive AFP of the patients ($P > 0.05$, Table 2).

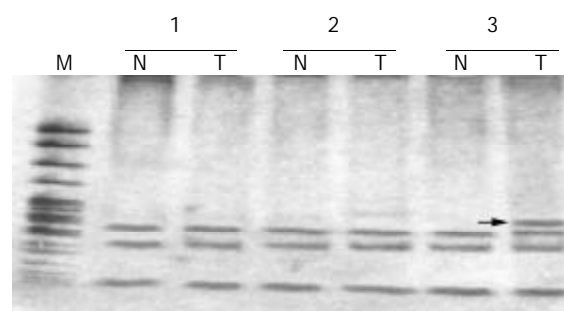


Figure 1 mtMSI in hepatocellular cancer. Arrows indicate conformational variants associated with mtMSI, N: normal DNA, T: tumor DNA.

Table 2 Relationship between MSI and clinical parameters

		<i>n</i>	mtMSI positive	mtMSI negative
Sex	Male	42	10	32
	Female	10	1	9
Age	<30	1	1	0
	30-60	41	8	33
	≥60	10	2	8
HBsAg	Positive	38	8	30
	Negative	14	3	11
Cirrhosis	Positive	37	9	28
	Negative	15	2	13
AFP	Positive	26	6	20
	Negative	26	5	21

The mobility shift in tumor DNA compared to corresponding normal DNA samples representing nMSI is shown in Figure 2. nMSI was found in 3 of 52 cases of HCC (5.8%). In the 3 cases of nMSI, only 1 case showed mtMSI simultaneously. No correlation was found between nMSI and mtMSI in the 52 cases of HCC.



Figure 2 MSI at BAT-26 in hepatocellular cancer. Arrows indicate conformational variants associated with MSI. N: normal DNA, T: tumor DNA.

DISCUSSION

It has been discovered so far that mitochondria are the only organelle to have their own genome and to undergo replication, transcription and translation without dependence on nuclear DNA. They are called as the “25th chromosome of human body”. Many diseases have been found to be related to the structural and functional defects of mitochondria and consequently they are known as mitochondrial diseases^[33]. mtMSI has been found to be a very common phenomenon accompanying gastric carcinoma, colorectal carcinoma and breast carcinoma and may play an important role in the carcinogenesis of these malignant diseases^[12,18-21]. To study the role of mtMSI in liver carcinogenesis, we analyzed 52 cases of HCC using seven microsatellite markers known to be altered in gastrointestinal carcinomas. mtMSI in at least one locus was found in 11 of the 52 cases (21.2%) of HCC, implying that mtMSI might occur not only in gastrointestinal cancers but also in hepatic cancers, and it may play an important role in the occurrence of a certain number of HCC.

Unlike other types of cancer, HCC has been found usually preceded by chronic inflammation due to viral infection^[34-37]. Matsuyama *et al.*^[11] reported that the frequency of mtDNA mutations was markedly increased in both noncancerous and cancerous liver specimens compared with control liver tissue. Accumulation of mtDNA mutations in HCC tissue could reflect its malignant potency. The frequency of mtDNA mutations was significant higher in HBV infection-related HCC than in other tumors, which implies that repeated destruction and regeneration of the liver tissue associated with chronic viral hepatitis would lead to accumulation of mtDNA mutations. In the current study, we did not find any obvious relationship between mtMSI and HBsAg, suggesting that HBV infection might play a limited role in the mtMSI pathway of HCC. In addition, we did not find an obvious relationship between mtMSI and sex, age, cirrhosis as well as positive AFP.

MtDNA contains several mono- and dinucleotide repeats. The most frequently used mtDNA in the test of mtMSI is a (CA)_n microsatellite starting at 514 bp position of the D-loop^[38] and a homopolymeric C tract extending from 16 184 to 16 193 bp of the D-loop, which could be interrupted by a T at 16 189 bp position^[39]. Alonso *et al.*^[40] studied mutations in the mtDNA D-loop region and found three mutations in eight gastric tumors. Richard *et al.*^[41] studied 40 pairs of normal/cancer breast specimens for the presence of mtMSI and found a 216-fold increase in the D-loop point mutations of cancer cells with regard to the spontaneous rate detected in female

gametes. Maximo *et al.*^[19] utilized PCR-SSCP to examine mtDNA large deletions and mutations in 32 gastric carcinomas and found that most of the mutations corresponded to insertions/deletions in the D-loop region or transitions in ND1, ND5, and COXI. Earlier studies revealed the presence of mutations in the D-loop of both non-malignant and malignant gastric tumors^[42,43]. Analysis of HCCs indicated that mutations in the D-loop were a frequent event and could be used as a molecular tool for the determination of clonality^[9,44]. Two recent studies reported the frequency of D-loop mutations in esophageal cancer. One group focused on adenocarcinomas of Barrett’s esophagus. In that study, D-loop alterations were identified in 40% of the patients examined^[16]. The other study showed that D-loop mutations were much less frequent in esophageal cancer, occurring in only 5% of the specimens analyzed^[45]. Clearly, analysis of mtDNA from more esophageal tumor samples is needed in order to determine the frequency of D-loop mutations and their relevance in this type of cancer. In our series of 52 cases of HCC, mtMSI was found in 11 (21.2%). MtMSI occurred in the D-loop region of 10 cases and in the coding region of 5 cases. Among the 10 cases, mtMSI occurred in (C)_n region of 8 cases and in (CA)_n region of 2 cases. So (C)_n region of D-loop is the site at which mtMSI occurs more frequently than in other regions. Our findings are consistent to those reported by Habano *et al.* and Maximo *et al.*^[18-20].

Microsatellite markers might provide evidences of faulty DNA mismatch repair (MMR) via the detection of MSI^[46-49]. The choice of microsatellite markers may impact on the MSI detection rate. BAT-26, a repeat of 26 deoxyadenosine localized in intron 5 of hMSH2 gene, has been reported as a reliable indicator of replication error phenotype in colorectal cancers, enabling analysis of tumour DNA in the absence of paired normal DNA^[50]. The frequency of nMSI in hepatic cancer varied in different reports^[51,52]. Karachristos *et al.*^[52] studied 27 cases of HCC and found none of the tumors examined showed alterations in BAT-26. In our series of 52 cases, 3 cases were found to have nMSI at BAT26 (5.8%). Our finding indicates that nMSI at BAT26 is not common in cases of HCC and support the hypothesis that HCC is a “low” MSI tumor in China. Carcinogenesis of HCC may undergo a different molecular route other than that of nMSI.

Mutation of mtDNA may result in the occurrence of tumor but its mechanism remains unknown. Further studies are required to determine if mtDNA mutations are correlated with malignant transformation. Recently, scholars have shifted their attention to the interactions between mtDNA and nDNA. Fragments of mtDNA are sometimes found in nuclear genes, and the insertion of mtDNA has been suggested as a mechanism by which oncogenes are activated^[53]. For example, sequences representing subunits ND4 (Complex I) and subunits cytochrome C oxidases I, II and III (complex IV) have been found in the nuclear DNA of various tissues^[53]. In yeast cells, migration of DNA from the mitochondria to the nucleus occurred 100 000 times more frequently than in the opposite direction^[54]. In our series of 52 cases of HCC, nMSI was detected in 5.8% and coexistence of nMSI and mtMSI in only 1 out of 3 cases. We failed to confirm there was a correlation of mtMSI to nMSI in our cases of HCC. This finding is in agreement with the recently published data on gastrointestinal cancer^[55].

In conclusion, mtMSI could play an important role at multiple stages in the process of carcinogenesis. The mitochondrial production of ROS might be involved in the initiation and promotion of carcinogenesis, in part due to ROS-triggered mutagenesis of both mtDNA and nDNA^[56]. Also, other evidences exists for a mechanism of nDNA mutagenesis involving the integration of mtDNA fragments. Many primary tumors revealed a high frequency of mtDNA mutations and

the majority of these somatic mutations were homoplasmic in nature, indicating that the mutant mtDNA has become dominant in tumor cells. The mutated mtDNA was readily detectable in paired bodily fluids from each type of cancer and was 19 to 220 times as abundant as mutated nuclear p53 DNA. By virtue of their clonal nature and high copy number, mitochondrial mutations might provide a powerful molecular marker for noninvasive detection of cancer^[57]. Important areas for future research should include intergenomic signaling pathways in carcinogenesis and the potential role of mitochondria and mtDNA mutations in immunological surveillance of tumor cells. Finally, the role of mitochondria in stimulating apoptosis could be exploited in cancer therapeutics^[58].

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