PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

WJG, 1999 December; 5(6):470-476
World Journal of Gastroenterology
 ISSN 1007-9327

Characterization of six tumor suppressor genes and microsatellite instability in hepatocellular carcinoma in southern African blacks

C. Martins, M.A. Kedda, M.C. Kew*

Subject headings carcinoma, hepatocellular; southern African b lacks; cumulative LOH; tumor suppressor genes; microsatellite genomic instability; liver neoplasms

Abstract

AIM To analyse cumulative loss of heterozygosity (LOH) of ch romosomal regions and tumor suppressor genes in hepatocellular carcinomas (HCC s) from 20 southern African blacks.

METHODS *p53*, RB1, BRCA1, BRCA2, WT1 and E-cadherin- ge nes were analysed for LOH, and *p53* gene was also analysed for the codon 249 mutation, in tumor and adjacent non-tumorous liver tissues using molecular techniques and 10 polymorphic microsatellite markers.

RESULTS *p53* codon 249 mutation was found in 25% of the subjects, as was expected, because many patients were from Mozambique, a country wit h high aflatoxin B_1 exposure. LOH was found at the RB1, BRCA2 and WT1 loci in 20%(4/20) of the HCCs, supporting a possible role of these genes in HCC. No LOH was evident in any of the remaining genes. Reports of mutations of *p53* and RB1 genes in combination, described in other populations, were not confirmed in this study. Change in microsatellite repeat number was noted at 9 /10 microsatellite loci in different HCCs, and changes at two or more loci were detected in 15%(3/20) of subjects.

CONCLUSION We propose that microsatellite/ genomic instability may play a role in the pathogenesis of a subset of HCCs in black Africans.

MRC/CANSA/University Molecular Hepatology Research Unit, Department of Medicine, University of the Witwatersrand Medical School, 7 York Road, Parkto wn 2193, Johannesburg, South Africa.
Supported in part by grants/bursaries from the University of the Witwatersrand, and the Foundation for Research Development, Pretoria, South Africa.
Correspondence to: Professor M.C. Kew, Department of Medicine, Medical School, 7 York Road, Parktown, 2193, Johannesburg, South Africa.
Tel. +27(0)11 488 3626, Fax. +27(0)11 643 4318
Email:014anna@chiron.wits.ac.za
Received 1999-08-10

INTRODUCTION

The evolution of cancer is thought to occur from the stepwise accumulation of ge netic aberrations in the same cell. These include loss of function of tumor supp ressor genes, activation of proto-oncogenes, faulty DNA mismatch repair, and the integration of viral DNA[1,2]. Hepatocellular carcinoma (HCC) is a leading cause of death in both Africa and the Far East, resulting in at least 310000 deaths worldwide each year[3]. HCC is multifactorial in aet iology and its pathogenesis is complex. The major risk factors involved in the development of the tumor are chronic HBV and HCV infections, cirrhosis and aflatoxin B_1 (AFB) exposure[4,5].

Heavy dietary AFB intake is thought to cause a guanine (G) to thymine (T) transversion at the third base of codon 249 of the *p53* gene, and for this reason clustering of this point mutation occurs in HCCs from Africa and China[6-8]. In addition, other mutations in, or deletions of, the *p53* gene (on chromosome 17p13.1) are found with relatively high frequency in human HCCs in other countries. The functional loss of this tumor suppressor gene, as well as its abnormal expression, have been proposed to play a significant role in HCC development[9] or at least in the development of a subset of HCCs. The majority of *p53* alterations reported to date have loss of one allele accompanied by mutations of the second allele[10]. Abnormalities of the *p53* gene, such as gene mutation, deletion, or the nuclear accumulation of mutant *p53* protein have also been found to correlate with increased allelic loss at the Breast Cancer Susceptibility Gene 1 (BRCA1) locus (17q21). This gene is thought to encode a transcription factor which acts as a tumor suppressor[11]. LOH of the BRCA1 gene in HCC was reported in a Korean study[12]. The Breast Cancer Susceptibility Gene 2 (BRCA2) (13q12-13) product is thought to be a tumor suppressor[13] involved in cellular proliferation and differentiation[14], and may be involved in the development of HCC[15]. LOH at the BRCA2 locus has been reported in HCC[15,16] and it has been suggested that mutations of the BRCA2 gene may be involved in hepatocarcinogenesis[15]. The retinoblastoma (RB1) gene (13q14.2) product (pRB) functions as a cell cycle regulator[17], and its

absence leads to unrestricted cell growth. Although there is no definite evidence that mutations of the RB gene are involved in HCC, LOH of the RB1 gene has been documented in human HCCs[18,12]. LOH of WT1 and 11p13 have been reported in human HCCs[12,16]. WT1 appears to be involved in proliferation, differentiation and apoptosis[19,20]. The product of the E-cadherin (Uvomorulin) gene (16q221) is the primary adhesion molecule in epithelium[21]. Loss of function of E-cadherin may lead to decreased cell-cell adhesion[22], cellular phenotypic changes, and the development of invasive properties[23]. In HCC, multicentric development and the formation of intrahepatic metastases is common[24]. LOH on chromosome 16q has been previously reported to be important in the initiation or progression of HCC[25,26].

Polymerase chain reaction (PCR) amplification of microsatellites (sequences uniformly distributed throughout the human genome) provides a simple and effective method of rapidly detecting loss of heterozygosity/microsatellite instability (LOH/MI)[27]. Microsatellite instability is defined as the loss or gain of microsatellite repeats at 2 or more loci and is detected by the presence of extra bands or band shifts between tumor and non-tumorous tissue DNA.

In this study, we examined the G-T transversion at codon 249 of the *p53* gene, LOH of the *p53*, RB1, BRCA1, BRCA2, WT1 and E-cadherin genes, and micr osatellite instability at 10 loci flanking these genes, in HCC and adjacent non -tumorous liver, from 20 southern African blacks.

MATERIALS AND METHODS

Subjects

The subjects included 20 southern African black men, aged between 20 and 40 years. HCC tissue and matched non-tumorous liver were obtained at necropsy or during surgical resection. DNA was extracted from the tissues using a modified "salting-out" procedure[28].

HBV markers

The HBV status of the subjects was determined previously using commercially available kits to detect HBV markers in serum (Abbott Labs, Chicago, IL, USA).

LOH and microsatellite instability

Microsatellite instability (MI) and loss of heterozygosity (LOH) studies were carried out by PCR and gel electrophoresis using polymorphic repeat markers (Table 1).

PCR products of the polymorphic loci *p53*, D17S846 and RB1.20 were resolved on 4% composite agarose gels, while radioactively labeled PCR products of the remaining loci were resolved on polyacrylamide gels, and viewed by autoradiography. Band mobility shifts between tumor and matched non-tumorous liver DNA were scored as a change in allele repeat number. LOH was characterized by the disappearance of one band or a considerable (£¾80%) decrease in band intensity in heterozygotes, whilst microsatellite instability was determined by expansion and/or contraction of microsatellite sequences.

PCR for LOH and MI

A standard PCR protocol (primers, Table 1) was followed for the *p53*, WT1, D1 3S137, RB(1.20), D13S120, D13S127, D17S855, and D17S846 loci. Each PCR reaction consisted, at final volume, of 100ng DNA, 1U *Taq* DNA polymerase (Promega, Madison, USA), 1×buffer, 1 mM each dATP, dTTP, dGTP, 0.1 mM dCTP, 0.025 μCi α^{32} P dCTP, and 50 pmol of each pr imer; in a total volume of 50 μL, amplification for 30 cycles of denaturation at 94 ℃ for 30 s, annealing 55 ℃ for 30 s, extension at 72 ℃ for 1min, and a final cycle of 72 ℃ for 10 minutes.

The PCR reaction for the D16S301 and D16S260 loci (primers, Table 1) consisted, at final volume, of 100 ng DNA, 1U Taq- DNA Polymerase, 1×buffer, 0.1 % gelatin, 1 mM each dGTP, dATP, dTTP, 0.1 mM dCTP, and 0.025 μCi α ^{32}P dCTP, 50 pmol of each primer; in a total volume of 25 μL, amplification for 25 cycles of denaturation at 94 ℃ for 1 min, annealing at 55 ℃ for 2 min, extension at 72 ℃ for 2.5 min, and a final cycle of 72 ℃ for 10 minutes.

p53 codon 249 mutation

The *p53* codon 249 mutation was detected by PCR -RFLP using primer sequences F3 and R3 (Table 1), and confirmed by sequence analysis. The PCR reaction consisted of, at final volume[6], 100 ng DNA, 2.5U of Taq-DNA polymerase (Promega), 1×buffer, 1mM $MgCl_2$, 0.8 mM each of dCTP, dATP, dGTP, dTTP, and 50 pmol of each primer; in a total volume of 50 μL, amplification for 30 cycles of denaturation at 94 ℃ for 15 s, annealing at 56 ℃ for 15 s, and extension at 72 ℃ for 30 s. The 110 bp PCR product was sized on ethidium bromide stained agarose gels against a 100 bp DNA ladder (Promega). AG to T transversion at the third base of codon 249 was detected by the presence or absence of a *Hae*III restriction site[6]. All samples shown by digestion to have the codon 249 mutation were sequenced in both directions both upstream and downstream in separate reactions, to confirm the presence of the mutation.

Table 1 PCR primers

Gene / Locus	Primer	Primer sequence	Amplicon	Amplicon length
p53	*p53*F3 *p53*R3	5'GTTGGCTCTGACTGT-ACCAC 5'CTGGAGTCTTCCAGT-GTGAT	exon 7 spanning codon 249[6]	110bp
p53	*p53*ivs1a *p53*ivs1b	5'GCACTTTCCTCAACTCTACA 5'AACAGCTCCTTTAATGGCAG	ALU sequence within intron 1 of *p53* gene[43]	200bp-300bp
D13S120 (BRCA2)	1353L 1353R	5'ATGACCTAGAAATGATACTGGC 5'CAGACACCACAACACACATT	$(AC)_{73}$repeat at D13S120[44]	112bp-136bp
D17S846 (BRCA1)	FF RF	5'TGCATACCTGTACTACTTCAG 5'TCCTTTGTTGCAGATTTCTTC	$(GGAA)_{25}$ repeat a t D17S846[45]	250bp-300bp
D17S855 (BRCA1)	FS RS	5'GGATGGCCTTTTAGAAAGTGG 5'ACACAGACTTGTCCTACTGCC	AC repeat at D17S855[46]	145bp
WT1	400 401	5'AATGAGACTTACTGGGTGAGG 5'TTACACAGTAATTTCAAGCAACGG	AC repeat within 3' untranslated sequence of WT33[47]	100bp-200bp
RB1	B57 B103	5'TGTATCGGCTAGCCTATCTC 5'AATTAACAAGGTGTGGTGGT	$[CTTT(T)]_n$ (n=14-26) repeat within intron 20 of RB gene[48,49]	400bp-600bp
D13S127 (BRCA2 / RB1)	1341L 1341R	5'CAGATATGTACTCATGCACATG 5'AAACAAATGAGTTTGGCTGT	(AC)35 repea t at D13S127[44]	130bp-142bp
D13S137 (RB1)	F R	5'TTTCCTCATTCTTTCCCAATTG 5'CAGGAGGGATGGACTCACTTC	$(GT)_{22}$ repeat at D13S137[50]	±135bp
E-cadherin	E-cadF1 E-cadF1	5'GATCCTAAGGACAAATGTAGATGCTCT 5'AGCCACTTCCCAGAACTTGGCTTCC	D16S301 locus polymorphic AC region[51]	146bp
E-cadherin	E-cadF2 E-cadR2	5'GGTTGAGATGCTGACATGC 5'CAGGGTGGCTGTTATAATG	D16S260 locus polymorphic AC repeat region[52]	±234bp

Note: WT1: Wilm's tumor gene; RB1: Retinoblastoma gene; BRCA1: Brea st cancer susceptibility gene 1; BRCA2: Breast cancer susceptibility gene 2; bp: base pairs.

Sequencing

All sequencing was carried out using the Sequenase PCR Product Sequencing Kit (United States Biochemical Corp., Cleveland, Ohio), according to the manufacturer's instructions.

RESULTS

HBV status

Seven patients were currently infected with HBV (5 of these were HBsAg-positive ; HBeAg-negative; the HBeAg status of the remaining 2 was unknown), and 6 were previously infected (anti-HBc and anti-HBs-positive). The HBV status of the remaining patients was not known (Table 2).

LOH/MI analyses

LOH was noted for the WT1 (1/13 subjects), RB (1.20) (1/10 subjects), D13S120 (1/20 subjects) and D13S127 (2/14 subjects) loci (Table 2).

The D13S137 and D13S127 loci flank the RB1 gene, while the RB (1.20) repeat sequence is within intron 20 of the same gene. LOH at the D13S127 locus suggests loss of at least a portion of the RB1 gene as shown in 2/14 informative subjects. LOH at RB (1.20) indicated loss of the RB1 gene in a further 1/ 10 informative subjects. The RB1 gene was thus lost in 3/18 informative subjects (Table 2). LOH at the D13S120 and D13S127 loci flanking the BRC A2- gene was shown in 2/20 informative subjects (Table 2). No LOH was found for any of the remaining loci (Table 2).

Microsatellite/genomic instability (or a gain/ loss of microsatellite repeats) was found in 15% (3 /20) of subjects.

p53 gene codon 249 analysis

The *p53* codon 249 mutation was detected in 25% of the subjects using PCR-RFLP analysis, and confirmed by sequencing. The *p53* codon 249 mutation was det ected in the tumor tissue of 3 subjects, in the non-tumorous liver of 1 subject, and in both the tumor and non-tumorous liver tissue of 1 subject (Table 2).

Sequencing gel electrophoresis of the *p53* gene product revealed a gel artifact, in all subjects with wild-type chromosomes, previously described by Kapelner *et al* (1994).

All tumors were at an advanced stage. No attempt was made to correlate the presence of LOH or microsatellite instability with clinical or other features.

Table 2 LOH, SSCP and sequence analysis

Subject number	VNTRs										*p53* codon 249		HBV status
	p53 (ALU)	WT1 (AC)	RB1 D13S137 $(GT)_{22}$	RB1.20 $[CTTT(T)]_n$	BRCA2 D13S120 $(AC)_{73}$	BRCA2 D13S127 $(AC)_{35}$	BRCA1 D17S855 (AC)	BRCA1 D17S846 $(GGAA)_{25}$	E-*cadherin* D16S301 (AC)	E-*cadherin* D16S260 (AC)	T	NT	
1	NI	NI	-	-	-	NI	-	-	-	NI	-/-	-/-	HBsAg+; HBeAg-
2	-	-	-	-	-	NI	-	-	-	NI	-/-	-/-	HBsAg+
3	-	-	-	-	-	-	-	-	NI	-	+/-	-/-	HBsAg+; HBeAg-
4	-	-	-	NI	-	-	?	NI	-	-	-/-	-/-	anti-HBc+; anti-HBs+
6	-	?	?	?	-	NI	?	-	?	-	+/-	-/-	HBsAg+; HBeAg-
7	?	?	?	?	-	-	?	?	-	-	-/-	-/-	anti-HBs+; anti-HBc+
8	↑	△	NI	+	↓	↓	△	△	↑	-	+/-	-/-	anti-HBs+; anti-HBc+
14	-	NI	?	NI	-	+	-	?	?	NI	-/-	-/-	anti-HBs+; anti-HBc+
16	?	?	△	?	+	+	?	?	△	↑	-/-	-/-	HBsAg+
18	↑	+	?	?	↑	-	?	?	?	↑	-/-	-/-	HBsAg+; HBeAg-
24	NI	-	NI	-	-	-	-	-	NI	-	-/-	+/-	anti-HBs+; anti-HBc+
39	-	NI	-	?	-	-	-	NI	?	-	-/-	-/-	HBsAg+; HBeAg-
40	NI	-	?	-	-	NI	-	-	?	-	-/-	-/-	anti-HBs+; anti-HBc+
48	NI	-	-	-	-	-	-	-	?	?	-/-	-/-	?
50	NI	-	-	NI	-	-	-	NI	-	NI	-/-	-/-	?
51	NI	-	?	NI	-	NI	-	-	-	-	-/-	-/-	?
52	NI	-	NI	-	-	NI	-	-	-	-	+/-	-/-	?
53	-	-	-	-	-	-	-	-	NI	-	-/-	-/-	?
54	-	?	-	-	-	-	-	-	-	NI	-/-	-/-	?
56	NI	-	-	NI	-	-	-	NI	NI	NI	-/-	-/-	?

Note: LOH: loss of heterozygosity; -: HBV status-negative for particular antigen / antibody, mutation studies-mutation absent; LOH studies: no LOH; →*p53* codon 249 mutation analysis: G→T transversion absent; +: HBV status-positive for particular antigen / antibody, mutation studies-mutation present, →LOH studies: LOH, →*p53* codon 249 mutation analysis: G→T transversion present; ?: results not obtained because of unsuccessful PCR or HBV status unknown; △: a change in repeat number between tumor (T) and non-tumorous liver (NT) in both chromosomes; ↑ / ↓: an increase / decrease in repeat number between tumor (T) and non-tumorou s liver (NT) in one chromosome; NI: not informative; VNTRs: Variable number of tandem repeat sequences; HBV: Hepatitis B virus; WT1: Wilm's tumor gene; RB1: Retinoblastoma gene; BRCA1: Breast cancer susceptibility gene 1; BRCA2: Breast cancer susceptibility gene 2; HBsAg: hepatitis B virus S antigen; HBeAg: hepatitis B virus E antigen; anti-HBs: antibody to hepatitis B virus S antigen; anti-HBc: antibody to hepatitis B virus C antigen

DISCUSSION

LOH of the *p53* gene has been reported with relatively high frequency in HCCs from Japan (29%-69%)[29,30], and also from southern Africa (60%), an d Taiwan (39.3%)[6,31]. No LOH was detected for *p53* in this study, although inactivation/ reduction of *p53* gene expression or of its product by means other than LOH may have occurred in our population. In a study by Walker *et al* (1991), *p53* allele loss occurred only in HBV-negative tumors. It thus appeared as if a mechanism other than loss of one *p53* allele and mutati on of the second allele was operating in HBV-positive tumors, thereby eliminati ng fully functional *p53* protein. The obvious mechanism would be the formation of complexes between wild-type *p53* protein and viral protein/?s leading to the loss of function of wild-type *p53* protein. Such associations have been well docume nted in the literature[32]. In our study most samples were HBV positive and a mechanism such as that mentioned above, rather than *p53* gene inactivation by physical mutation and LOH, may have been operating in our tumors to eliminate the function of the *p53* protein. Alternatively, should both alleles of t he *p53* gene be mutated in ways other than LOH in our samples, such as point mutations and small deletions (<50bp)[18], these would not have been de tected by the techniques employed in this study.

The *p53* codon 249 mutation was detected in 25% (5/ 20) subjects. This was expected as the subjects were southern African blacks, some of whom came from Mozambique and other areas where aflatoxin exposure is prevalent. The *p53* codon 249 mutation was found in both tumor and non-tumorous liver tissues of one subject. This could have been caused by contamination of the non-tumorous liver with tumor tissue. In another subject the mutation was detected in the non-tumorous liver only. The presence of this mutation has been documented in non-tumorous liver and not in the corresponding tumor tissues[33], where it was proposed that normal liver subjected to prolonged aflatoxin exposure could gradually accumulate high levels of AGT mutations, whereas the mutation would not necess a rily arise in neoplastic populations that were cloned from single progenitor cells resistant to aflatoxin. Unfortunately, insufficient

tissue was available in these two patients for histopathological examination, so we cannot exclude microscopic contamination as a cause of this finding in the two subjects. Two patients with a codon 249 G¡úT transversion were HBsAg positive, 2 subjects were anti-H Bs/anti-HBc positive and the HBV status of 1 subject was unknown. This concur red with previous studies where mutations at codon 249 were not found in non-HB V-related HCCs[34], and is in agreement with previous work which suggests that both aflatoxin exposure and HBV infection are required for this mutation to occur[29,33].

A gel artefact generated by formation of a mini hairpin secondary structure in the codon 249 region of the *p53* gene in 34 wild type chromosomes, lead to a "missing" G at the third base of codon 249 in the sequence of the sense strand[35]. In a study by Kapelner *et al* (1994), as with our samples, Hae-III digest confirmed the presence of the recognition sequence GGCC. However, since there has been no other report of this "G deletion" in such a commonly sequenced region, Kapelner *et al* (1994) suggested that this artifact may not occur often.

LOH appears to have occurred in 4 subjects at the RB1 (3/18 ro 17%), BRCA2 (2/20 or 10%) and WT1 (1/13 or 8%) loci (2 of these subjects had LO H at both the RB1 and BRCA2 genes). Although reduction to homozygosity h as been apparent in certain individuals, and has consistently been scored as LOH, 'band disappearance' may also be caused by a gain/loss in microsatellite repeats. We think, however, that this is unlikely to be the case in so many indiv iduals.

LOH of the retinoblastoma gene has been documented in 33% of HCCs from Korea[12], 16%-73% of HCCs from Japan[18,30]and 27% of HCCs from Austra lia[36]. One copy of the RB1 gene was lost in 17% (3/18) of HCCs in this study. Although our sample is small, this frequency differs from the higher percentages found thus far. This may reflect population differences, LOH of t he RB1 gene may play a role in a small subset of southern African HCCs. Coin cident mutation of the *p53* and RB1 genes has been observed in 25%[18], and 12.9%[37] of advanced HCCs in Japan and Australia respectively[36]. Mutations and LOH in these genes is most frequently observed in advanced stage HCCs, like those investigated here. However, no coincident mutati on of these genes was detected in this study.

LOH of the BRCA2 gene has been reported in 3% of Japanese HCCs[15], and in 40% of HCCs from the USA[16]. In this study one copy of the BRCA2 gene was lost in 10% (2/20) HCCs. This finding supports the notion that BRCA2 may function as a tumor suppressor gene in the liver[15], and that it may in some way be involved in the progression of a small number of HCCs. To our knowledge, LOH of the BRCA1 gene has been reported only once, in a study in which 11.5% (3/6) of HCCs showed LOH at this locus[12]. No LOH was found for this gene in our sample population. LOH at 11p13, the region containing the WT1 gene, as well as LOH of the gene itself has been reported in 4%-7% HCCs[12,16]. Our result of 8% (1/13) LOH agrees with these findings. LOH of the region where the E-cadherin gene is located (16q22) has been reported in 64%-91% of Chinese and Japanese HCCs[25,26]. No LOH w as found for this gene in our study. Although all the HCCs used in this study were in advanced stages, it was not established whether they were highly undifferentiated. There may be retention of *E-cadherin* expression in these samples and no loss of intercellular adhesiveness.

We cannot say whether HBV played any role in the chromosome losses reported here[37]. Cumulative LOH is thought to reflect the sequential development of HCC progression.

LOH of a number of tumor suppressor genes may be important in the advancement of HCC[37]. Frequent loss of tumor suppressor genes has been reported in Korean HCCs, where 86% HCCs had LOH of 1 gene, and 59% had LOH of 2 - 4 genes[12]. Piao *et al* (1997), investigated 10 tumor suppressor genes (-VHL, APC, EXT1, WT1, RB1, *p53*, BRCA1, nm23, DPC4, DCC). The genes most often lost were *p53* (66%), RB1 (33%), EXT1 (33%), and APC (20%). The genes found to be lost most often in our study were RB1 (17%) and BRCA2 (10%). However, as the total LOH of the *p53*, RB1, BRCA1, BRCA2, WT1 and *E-cadherin* genes in this study was 20% (4/20), we conclude that LOH of tumor suppre ssor genes is infrequent in our HCCs.

Microsatellite/genomic instability is reflected in the expansion/contraction of microsatellite sequences, and is thought to be a product of replication errors[38]. Microsatellite instability has been considered to be insignificant in HCC development by some authors, while others believe that it may be significant[39]. To our knowledge, this is the first time microsatellite instability has been looked at in HCCs from a southern African black population. It is important to note that there are differences in allele frequency between our so uthern African Negroid population and the Asian, European and Australian populat i ons, characterized previously at the WT1, D13S137, D13S120,

D13S127, D17S855 , D16S301, and D16S260 loci (paper in preparation). Microsatellite instability has been documented in 40% of HCCs from the USA[39], and in 41% of HCCs at two or more loci in a French study[40]. In a Korean study microsatellite instability was detected in 4/10 (40%) HCCs, where each subject showed inst ability at two or more loci[39]. Of the 9 markers used in their study, 3 showed genetic instability in one or more subjects. In our study 3/20 (15%) H CCs showed instability at two or more loci. Of the 10 loci investigated, 9 showed genetic instability in one or more subjects. Cumulative microsatellite in stability indicates advanced HCC. One of these subjects also had the codon 249 mutation in the *p53* gene. We were not able to determine whether joint changes at the loci were necessary for tumor development, or whether they represented independent events in tumor initiation and/or progression. Microsatellite/ genomic instability is believed to occur at random and may reflect alteration of the entire genome of the cancer cell[41]. The order of these changes is most likely insignificant. Their cumulative effect however, may be important [42]. We propose that microsatellite/genomic instability may play a role only in a small subset of HCC in our population.

In conclusion, our observations support a possible role of *p53*, WT1 and BRCA2 genes in the pathogenesis of HCC, and that microsatellite instability appears to be an important factor contributing to HCC development in a subset of our HCCs.

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Edited by MA Jing-Yun