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Screen p53 mutations in hepatocellular carcinoma by FASAY: A novel splicing mutation*

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Abstract: Objective: To establish a routine procedure for the detection of p53 mutations in hepatocellular carcinoma (HCC) surgical resections using the FASAY (functional analysis of separated alleles of p53 on yeast) procedure. Methods: p53 status was analyzed by FASAY and cDNA sequencing in 50 cases of HCC. After the extraction of RNA from the frozen tumor and corresponding normal tissues, reverse transcription RT-PCR was carried out using these samples. The assay can detect mutations of p53 mRNA between codons 67 and 347 by the DNA-binding activity of the protein and reveal them as red colonies. Results: Of the 50 specimens, 29 (58%) were positive (mutant) by FASAY. Sequencing analysis confirmed that all 29 FASAY positive tumors harbored mutations, and that no mutations were detectable in any FASAY negative tumors. In 29 p53 mutations, 22 mutations were point missense mutation, 5 were deletions and 2 were splicing mutations. A novel splice mutation on splice donor of intron 6 was reported, which could produce two different mRNAs, respectively using the nearest upstream and downstream recessive splice donor sites. Conclusion: FASAY is a sensitive method for detecting the various types of p53 mutations in HCC, suggesting that the yeast functional assay for the detection of p53 mutations may be essential for elucidating their clinical significance.

Key words: Hepatocellular carcinoma (HCC), p53 mutation, FASAY, Splicing mutation
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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer in new cases per year (626000) and the third most common cancer resulting in death (598000) (Parkin *et al*., 2005). China is the country most seriously suffering from HCC, accounting for 55% of total cases and deaths worldwide per year. The clinical behavior of HCC is heterogeneous and difficult to predict, and in patients undergoing resection, recurrence rates can be as high as 50% in two years and life expectancy of as short as twelve months can be possible. Great efforts were put to find an ideal prognostic marker, and the tumor suppressor p53 seems to be the most promising biomark of prognosis in HCC.

In an overwhelming majority of clinic studies, the prognostic significance of p53 alterations in HCC relies on the identification of p53 nuclear overexpression by immunohistochemistry (IHC). But IHC would give false-positives from stabilization of wild-type p53 and false-negatives from stop codons, frameshifts and destabilizing mutations (Sjogren *et al*., 1996). Heterogeneity also results from the use of different antibodies, different techniques to prepare sections and different criteria for scoring positives (Chappuis *et al*., 1999). FASAY (functional analysis of separated alleles of p53 on yeast), which was developed by Flaman *et al*.(1995), can evaluate the functional status of p53 proteins expressed in tumor cells using yeast cells yIG397, leading to detection of significant functional mutation of p53 as a transcriptional factor. The

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genotype of yIG397 is *MATa ade2-1 leu2-3*, *112trp1-1his3-11*, *15can1-100ura3-1 URA3* 3×RGC::p*CYC*::*ADE2*, contains an integrated plasmid with the *ADE2* open reading frame under the control of a p53-responsive promoter on *URA3* site.

In this study, we tested the fidelity of FASAY scoring p53 status of analyzed samples and investigated p53 mutations in HCC.

MATERIALS AND METHODS

Sample collection

Fifty patients who underwent surgical resection for primary HCC at the affiliated Eastern Hepatobiliary Surgery Hospital of the Second Military Medical University between 2002 and 2003 entered this study. Cases with preoperative radiation or chemotherapy and liver recurrence were not selected. All patients underwent a complete resection of liver tumor. In each case, fragments of the tumor and corresponding normal tissue were obtained and partly immediately snap frozen in liquid nitrogen and stored at −80 °C until further testing for p53 transcriptional activity to prevent degradation of RNA. The remaining material tumor was formalin fixed and paraffin embedded for pathological evaluation. Anatomopathologic evaluation of the paraffin-embedded tumor specimens was determined stage according to Edmondson classifications.

RNA extraction and cDNA synthesis

Total RNA was extracted using the total RNA isolation kit (Watson, Shanghai, China) according to the manufacture's recommendations of 5~10 mg of tissue ground with a microfuge pestle. The quality of the RNA was visually estimated by the presence of the 18S and 28S rRNA bands upon electrophoresis with ethidium bromide stain.

The RNA was reverse transcribed, using MMLV reverse transcriptase (Promega, Shanghai, China), into single stranded cDNA using an oligop (dT) primer. The reaction was incubated at 65 °C for 5 min, and iced 5 min to allow the primer to anneal and then at 42 °C for 60 min for reverse transcription. To minimize interference with subsequent applications, the MMLV reverse transcriptase was then denatured by heating at 75 °C for 15 min. The reaction products were stored at −80 °C.

Amplification of p53 cDNA by PCR

The p53 cDNA was amplified by PCR using the following primers (Invitrogen, Shanghai, China). Human specific primers P3: 5'-ATT-TGA-TGC-TGT-CCC-CGG-ACG-ATA-TTG-AA(S)C-3', P4: 5'-ACC-CTT-TTT-GGA-CTT-CAG-GTG-GCT-GGA-GT(S)G-3', where (S) represents a phosphorothioate linkage.

PCR was performed for 35 cycles of 94 °C for 30 s, 65 °C for 60 s and 72 °C for 60 s using a thermal cycler that had been preheated to 95 °C. The PCR mix was allowed a final extension at 72 °C. Two microlitres of the cDNA reaction product was amplified in 20 μl of Pfu buffer (Strategene, Shanghai, China) plus 1.25 units of Pfu DNA polymerase, 100 ng of primers, 10% dimethyl sulphoxide, and 50 mol/L dNTPs. PCR controls included (1) negative control incubation without template DNA and (2) positive controls included plasmids pLS76 known to contain the p53 cDNA sequence.

PCR reaction product (5 μl) was then run on a 1.5% agarose gel, alongside a molecular weight marker, to check the PCR product.

Vector preparation for transformation of yeast

The plasmid pRDi22 (Waridel *et al*., 1997) was obtained from Dr. R. Iggo (I.S.R.E.C. Switzerland). One microgram of plasmid was cut by restriction digest using *Stu*I and *Hin*dIII. The linearised vector was then dephosphorylated using calf intestinal alkaline phosphatase (Takara, Shanghai, China) at 37 °C for 30 min. The linearised vector was gel purified using the gel DNA isolation kit (Vitagene, Shanghai, China) and redissolved in TE buffer (pH 8.3). Yeast transformation and growth. The yeast strain yIG397 was obtained from Dr. R. Iggo (I.S.R.E.C. Switzerland) and was routinely cultured on complete medium (yeast extract, peptone, dextrose: YEPD), supplemented with adenine at 10×excess concentration (200 g/ml), to avoid selection of spontaneous suppressors of the endogenous *ade2* locus. The resulting colonies were all white. Any red colonies would have been discarded.

Transformation of competent cells

The TE/LiAc/PEG method (Gietz *et al*., 1992) was used to make the yeast competent, and was also used for the transformation. Linearised vector DNA (50 ng), 5 μl of PCR product, 5 μl ssDNA (10 mg/ml)

(Sangon, Shanghai, China) were used for the transformation. The components were mixed by vortexing vigorously. The cells were then incubated for 30 min at 30 °C, and again vortexed at 15 min intervals during the incubation, then heat-shocked for 15 min at 40 °C. Cells (100 μl) were plated on synthetic minimal media minus leucine, left to grow for 2~4 d at 30 °C, and the number and colour of the yeast colonies were noted. Control transfections included transfection with linear vector only, pLS76 whole vector only, and PCR product only. Untransfected cells were also grown on leucine plus and leucine minus media. PCR products from plasmids with known p53 status i.e. wild-type and known mutants were also transfected.

Sequence analysis

Sequencing analysis was carried out using automated ABI 3730 apparatus (Applied Biosystems, Shanghai, China). cDNA, synthesized by the polymerase chain reaction protocol described above, was directly sequenced using the following oligonucleotide primers: 5'-ATT-TGA-TGC-TGT-CCC-CGG-ACG-ATA-TTG-AAC-3', 5'-TAC-TCC-CCT-GCC-CTC-AAC-AAG-ATG-3', 5'-TTG-CGT-GTG-GAG-TAT-TTG-ATG-AC-3', and 5'-ACC-TTT-TTG-ACT-TCA-GGT-GCT-GGA-GTG-3' in both directions. The cycle sequencing reactions were carried out using Taq Dye Deoxy Terminator Cycle Sequencing kit according to the manufacturer's protocol. Plasmid containing p53 cDNA, recovered from single red yeast colonies, was similarly sequenced following this protocol. For each patient sample, 5 colonies were sequenced.

RESULTS

Determination of the production of RT-PCR for p53 gene

The production of PCR using P3, P4 as primers and vector pLS76 (Flaman *et al*., 1995) as template, which had the wild-type p53 gene CDS, was about 1 kb fragment. The result of RT-PCR coming from the tumor and corresponding normal tissues is shown in Fig.1.

Identification of p53 statues in tumors and corresponding normal tissue

Ade2^{-} cells grown on medium contained limiting adenine turned red because of the accumulation of an intermediate in adenine metabolism. The strain used for assessing p53 status of yIG397 carrying a reporter with a p53-binding site ribosomal gene cluster (RGC) upstream of the *ADE2* gene. When the yeast was transfected with the cDNA from a wild-type p53 gene, the yeast cells express *ADE2*, and grow normally as white colonies on minimal synthetic defined agar. Cells transfected with the cDNA from a mutant tumour, failed to transactivate the *ADE2* gene and grow to form red colonies. The p53 status can be easily determined by the color of transfected yeast cells (Fig.2). But the percentage of yeasts transfected with wild-type cDNA was not 100% white clones, and there were generally ≤10% red colonies as background due to PCR-induced point mutations, alternative splicing of intron 9 affecting the p53 carboxy-terminus. p53 was considered as wild type when <10% of red or pink colonies were detected (negative FASAY). Above this cutoff value, the FASAY was considered positive. The activity of the p53 mutant was determined by the color of at least 300 colonies per strain. Using this criterion, 50 of the tumors tested contained 29 mutant p53 (58%).

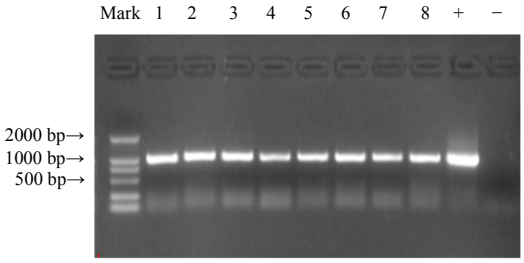


Fig.1 RT-PCR products of p53 gene
Mark: DL2000 (TaKaRa); 1~8: Different tissue samples respectively; +: pLS76 as positive control; −: ddH_2O as negative control

The FASAY results of the corresponding normal tissue varied from 5% to 20% (the data of each sample were not shown). Considering that the tumors tissue may be mixed into the neighboring normal tissue, another cutoff value (25%) was used in the tumor corresponding normal tissues. But according to this cutoff value, no positive FASAY was screened. Though p53 249^{Ser} mutation induced by aflatoxin B1 (AFB1) (Bressac *et al*., 1991) could be detected in the normal hepatic tissue surrounding tumor or even the patients without HCC (Kirk *et al*., 2000; Huang *et al*., 2003), in these tissues FASAY positive samples were not examined.

Sequencing the mutations in p53

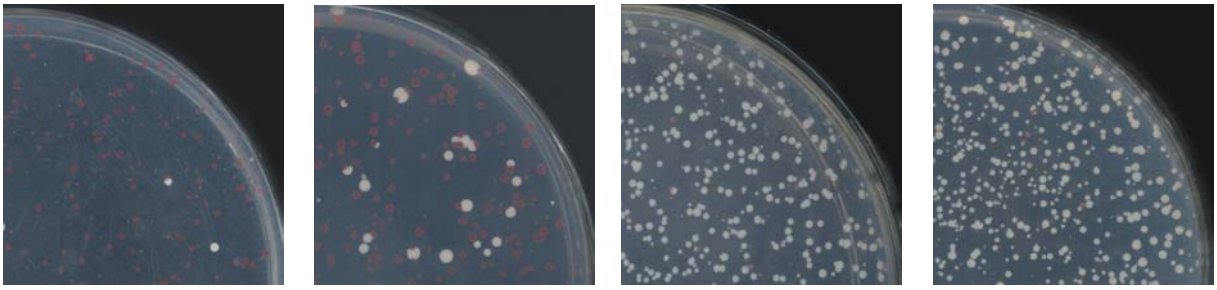
Sequencing of plasmids rescued from red yeast colonies showed that 22 mutations were point missense mutation, 2 were frameshift deletion mutations, 3 was in-frame deletion and 2 were splicing mutations (Table 1). The hotspot mutation 249^{Ser} that occurred 11 times in this study, accounted for 37.9% in total mutations.

In the 5 deletion mutations, the sample T2 (deletion of T in 124 codon) and T8 (deletion of A in 62 codon) both caused frameshifts and premature truncation of p53 translation at codon 168 and 121 respectively; the mutations of the sample T5, T26, T45 all caused in-frame deletion, missing several amino acids in final proteins.

For the two splicing mutations, in the sample T9, as the splice donor site of intron 6 AG mutated into TG (Fig.3a), there existed two different splicing products: one that used the nearest upstream AG as splice donor had been inserted 49 bp sequence of 6 intron into the final mRNA (Fig.3a); the other that used the nearest downstream AG as splice donor therefore deleted a 47 bp sequence of 7 exon in mRNA (Fig.3b); both of them caused premature truncation of proteins, stopping at codon 243 and 246. In 6 clones randomly chosen for sequencing, 5 of them were the first splicing forms and only one was the second splicing form, suggesting that the upstream one may be more preferable for splicing than the downstream one. In another sample T4, the entire exon 8 was skipped, which was reported before (Holmila *et al*., 2003).

DISCUSSION

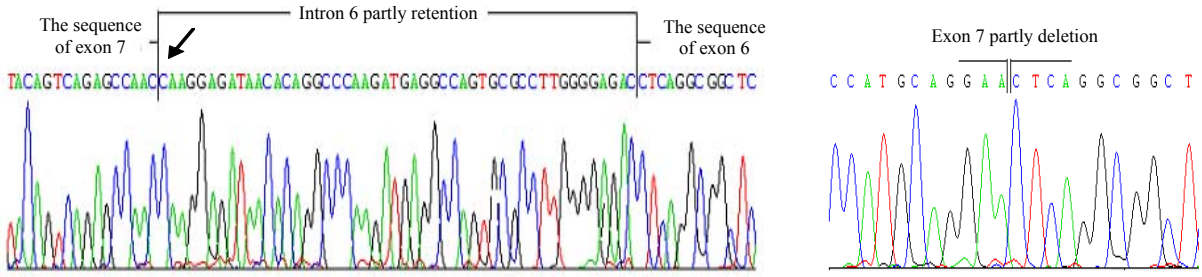
The p53 gene mutation is one of the commonest genetic alterations found in human cancers. The majority of these mutations are missense point mutations that are scattered along the entire p53 gene (MIM191170). The high degree of heterogeneity of p53 mutations and their heterogeneous distribution



(a) (b) (c) (d)

Fig.2 FASAY analysis of HCC tissues. (a) T7 cancer tissue (99%); (b) T16 cancer tissue (87%); (c) T3 cancer tissue (5%); (d) T7 corresponding normal tissue (6%)

The percent in the brackets is ratio of the red clones to total clones on the plate



(a) (b)

Fig.3 The electropherograms of different splicing products detected in the tumor T9. (a) The first splicing product in T9 which used upstream recessive splice donor AG in 6 intro in T9, the mutant splice donor site is shown by the arrow; (b) The secondary splicing products in T9 which used downstream recessive splice donor AG in 7 exon

Table 1 Clinical pathologic characteristics and p53 status in 50 patients with primary HCC

No.	Age/sex	Stage[a]	HBV	FASAY (%)[b]	p53 statue[c]	DNA sequence	Consequence
Wild type							
T3	49/M	III	+	5	WT	\	\
T6	58/M	III	+	9	WT	\	\
T11	55/M	III	+	10	WT	\	\
T12	56/M	III	+	10	WT	\	\
T14	58/M	III	+	9	WT	\	\
T15	59/M	III	+	10	WT	\	\
T18	62/M	III	+	7	WT	\	\
T19	63/M	III	+	10	WT	\	\
T20	54/M	II	−	6	WT	\	\
T21	57/F	II	+	8	WT	\	\
T24	80/M	I	−	3	WT	\	\
T25	36/M	I	+	4	WT	\	\
T30	47/F	II	−	4	WT	\	\
T33	69/M	II	−	9	WT	\	\
T34	57/M	II	+	10	WT	\	\
T37	84/M	II	−	3	WT	\	\
T38	42/M	II	+	4	WT	\	\
T41	47/M	I	+	3	WT	\	\
T44	65/M	II	+	7	WT	\	\
T48	75/M	I	+	2	WT	\	\
T49	56/M	I	+	9	WT	\	\
T50	40/M	I	+	7	WT	\	\
Missense mutation							
T1	50/M	III~IV	−	93	M	747 G→T	249 R→S
T7	50/M	III	+	99	M	747 G→T	249 R→S
T10	65/F	III	+	97	M	736 A→G	246 M→V
T13	57/M	III	+	92	M	157 G→T	157 V→F
T16	60/M	III	+	87	M	747 G→T	249 R→S
T17	61/M	III	+	68	M	747 G→T	249 R→S
T22	37/M	I	+	97	M	747 G→T	249 R→S
T23	63/M	I	−	58	M	584 T→A	195 I→N
T27	55/M	II	+	65	M	747 G→T	249 R→S
T28	50/M	I	+	79	M	747 G→T	249 R→S
T29	40/M	II	+	79	M	747 G→T	249 R→S
T31	43/M	II	+	81	M	329 G→T	110 R→L
T32	62/M	I	+	69	M	747 G→T	249 R→S
T35	47/M	I	+	31	M	844 C→T	282 R→W
T36	59/M	II	−	67	M	817 C→A	273 R→S
T39	53/F	II	+	80	M	747 G→T	249 R→S
T40	61/M	I	+	79	M	535 C→A	179 H→N
T42	47/M	II	+	95	M	747 G→T	249 R→S
T43	73/M	II	−	40	M	438 G→T	146 W→STOP
T46	35/M	II	+	81	M	422 G→A	141 C→Y
T47	52/F	III	+	75	M	484 A→T	162 I→F
Frameshift mutation							
T2	65/M	III	+	28	M	373 Δ1 bp	Truncated 168
T8	46/M	III	+	80	M	186 Δ1 bp	Truncated 121
In-frameshift mutation							
T5	43/M	III	+	91	M	791~793 Δ3 bp	ΔL
T26	45/M	II	+	74	M	313~375 Δ62 bp	ΔGS~CT
T45	51/M	II	+	64	M	720~734 Δ15 bp	ΔSCMGG
Splicing mutation							
T4	42/M	III	+	29	M	I7 MD gDNA?	Δ8 exon
T9	42/M	III	+	60	M	I6 MD AG→TG	Multiple[d]

[a]Edmonson classification; [b]The ratio of red colons in total colons on SD-L plate; [c]The status of p53 is determined by the red clones ratio with 10% cutoff; WT: Wild type; M: Mutant; [d]Two different splicing products: 6I+ and Δ7E (6 intron partly retention and 7 exon partly deletion). Δ: Delete; MD: Mutant splice donor; I7: Intron 7; I6: Intron 6; ?: Exact mutant sites need sequencing gDNA

have led to the development of various screening procedures in order to increase the speed, sensitivity and specificity of p53 mutation detection. Compared with IHC, genomic sequencing, which usually detect exon 5~8 and a single from normal tissue can easily mask the mutant peak, and single-strand conformation polymorphism (SSCP), FASAY was proved to reach the highest sensitivity (Meinhold-Heerlein *et al*., 2001; Watanabe *et al*., 2004), due to the fact that a larger region exon 4~10 of p53 gene was tested, mutations can be detected in the presence of large amounts of normal tissue because hundreds of clones are examined from each sample and the simple red vs white read-out means that mutations are not easily overlooked (Chappuis *et al*., 1999).

The FASAY detected 29 mutations in the 50 tumors investigated (58%), and the sequencing analysis confirmed that all 29 FASAY positive tumors harbored mutations, with no mutations detected in any FASAY negative tumors. In these mutations, the non-missense mutations including frameshift, in-frame deletion and splice mutation took place 7 times, accounting for 24% in total mutations detected by FASAY in HCC. In contrast, less than 20% of p53 mutations in the UMD database (http://www.umd.be:2072/) were non-missense mutations, with splice mutations in the p53 gene described as rare events that occur at a frequency of less than 1% (Holmila *et al*., 2003). Using RNA as starting material for a test, relatively rare mutant sequences can be detected by FASAY (Chappuis *et al*., 1999), partly due to the reason mentioned above. Recently, several reports about novel splicing mutations of p53 in other cancers were all detected by FASAY assay (Chappuis *et al*., 1999; Verselis *et al*., 2000; Holmila *et al*., 2003).

Geographic and ecologic studies of HCC are limited by the fact that regions with a high incidence of HCC frequently have a population that is simultaneously exposed to AFB1 and HBV (Kirk *et al*., 2000). China has a population with a high incidence of HCC, chronic HBV infection, and a high exposure to AFB1 (Hsu *et al*., 1991; Parkin *et al*., 2005). Among patients with HCC, we did not observe any differences in chronic infection with HBV (based on HBsAg status) between those patients with HCC who were positive and negative for p53 mutation. In this study, the patients with HBV positive (based on HBsAg status) were 41, in which 16 were p53 wild type (16/21) and 25 p53 were mutants (25/29), $P>0.05$. On the other hand 249^{Ser}, as a "hotspot" mutation for HCC, in our study the frequency percent was 37.9% (11/29), and no statistically significant correlation was found between this mutation and various clinical pathologic characteristics.

We have established a routine procedure for the detection of p53 mutations in HCC samples using the FASAY procedure. The results of the present study demonstrated that FASAY is a sensitive method for detecting various p53 mutations. Additional large-scale studies are needed to determine whether p53 status may be associated with tumor progression and chemosensitivity, and whether p53 mutation have independent prognosis value in China.

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