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RESEARCH ARTICLE

Analysis of genotype polymorphism of tumor-related genes harbored in chromosome arm 1p and 8p in hepatocellular carcinoma patients by cSNP chip

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Abstract The majority of single nucleotide polymorphisms (SNPs) found in the coding region (cSNPs) are single base substitutions that may or may not lead to amino acid substitutions, most of which are related to diseases. Some cSNPs may prove useful for their potential links to functional cSNPs via linkage disequilibrium mapping. We have selected 48 cSNPs located in the coding regions of 25 genes to construct the cSNP chip. These genes are harbored in the high frequency loss regions of the chromosome 1p and 8p and related with apoptosis, cell cycles, signal transduction, oncogene, tumor suppressor genes and so on. All of the cSNPs can lead to amino acid substitutions except TP73 (rs1801174). The PCR products amplified from 31 hepatocellular carcinoma (HCC) specimens were labeled with Dig-dUTP and then hybridized with the cSNP chips. The results showed that there was no hybridization signal when there was more than one site of mutation in the amplification sequence, indicating that the cSNP chip had a high sensitivity. The statistic data of the SNP (MT, homozygous and HT, heterozygous) in the HCC patients with different phenotypes (HBV +/−, differentiation stage, family history positive or negative, tumor size) indicated that the number of MT was distinctly different between patients with positive HBV and negative HBV. The MT and HT numbers of all the 48 cSNPs were significantly different between low differentiation and high differentiation HCC patients. The numbers of MT and HT were not different between positived and negative family history groups and between tumor size >3 cm and $\leqslant 3$ cm groups. The study results provided useful information for understanding the molecular mechanisms of HCC development.

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1 Introduction

Human diversity is generally based on genetic variations, which play an important role in many diseases. Single nucleotide polymorphism (SNP) is the most common source of genomic variations. The majority of SNPs found in the coding region (cSNPs) are single base substitutions that may or may not lead to amino acid substitutions, which are very likely to cause disease (Cargill et al., 1999). Variations in the coding region of important genes in a cell's life may result in changes of the protein function. Some cSNPs alter a functionally important amino acid residue (Wang et al., 1998).

Tumor related genes, especially tumor suppressor genes located in the loss of heterozygosity (LOH) regions in the genome of tumor tissues are believed to play a key role in the carcinogenesis of various cancers (Tamura, 2006; Zhu et al., 2004; Farrand et al., 2002). Allelic loss in the chromosomal regions surrounding known or suspected tumor related genes has been shown to be an important tumor marker of prognosis. Therefore, studies on cSNPs of cancer-related genes harbored in the high frequency loss regions of tumor chromosomes can help reveal genetic and variation mechanisms underlying carcinogenesis and cancer susceptibility.

We chose the tumor related genes located in the high frequency loss regions of chromosomes 1p and 8p of hepatocellular carcinoma (HCC) patients (Shao et al., 1999; Li et al., 2001; Chan et al., 2002; Lu et al., 2007) and collected the cSNP information of these genes to prepare cSNP chips. We performed a polymorphism analysis on 31 HCCs. Our results provide useful information for understanding the carcinogenic mechanism of HCC.

2 Materials and methods

2.1 Materials and main reagents

2.1.1 Materials

The liver carcinoma specimens were surgically resected from 31 patients, including 8 females and 23 males, with ages from 35 to 70 (mean 54.19 ± 9.49) years at the Tianjin Cancer Hospital, Tianjin, China. All of the patients were of Han nationality and were pathologically diagnosed as HCC. Twenty-one patients were accompanied by HBV infection and 4 patients were with HCV infection. Three patients had a family history of cancer. The tumor sizes ranged from 2 to 13 cm in diameter. Fourteen tumor tissues were of high differentiation and 12 tissues of low differentiation. The characteristics of the HCC patients are shown in Table 1.

2.1.2 Enzymes and main reagents

The following enzymes and reagents were used: Taq DNA polymerase and dNTP (TaKaRa Biotechnology Dalian Co., Ltd), nylon membranes (Pall Corp, United States), digoxin and anti-digoxin (Roche Co., Switzerland), DAB condensed liquid (Huamei Biotechnology Co., China).

2.2 Methods

2.2.1 Extraction of DNA

Extraction of DNA was carried out using protein K, hydroxybenzene and chloroform (Sambrook and Russell, 2003; Niu and Shen, 2000). Clotted blood (0.5 mL) was mixed with 1 mL hemolytic reagent (0.32 mol/L sucrose, 1.0 mmol/L Tris-HCl, pH 7.5, 5 mmol/L $MgCl_2$, 1% Tritone X-100) and centrifuged at 4000 rotation/min for 10 min. The supernatant was discarded. The deposits were treated to trituration at −20°C, then mixed with 0.8 mL digest buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0, 100 mM NaCl, 1% SDS) and 10 μL protein K (0.1 mg/mL final concentration), and incubated at 60°C for 30 min. Following that, 0.3 mL hydroxybenzene and chloroform/isoamyl alcohol (24:1) were added to each sample and centrifuged at 12 000 rotation/min for 10 min. The supernatant was transferred to fresh tubes. An equal volume of isopropanol was added to each sample, and mixed well. The samples were

Table 1 Data of the HCC patients

No.	gender	age/yr	HBV infection	HCV infection	family history of cancer	tumor size/cm	differentiation degree	other information
1	male	67	−	+	+	11.0 × 9.0 × 6.0	high	
2	male	64	−	−	−	2.0 × 2.0 × 2.0	high	
3	male	37	−	−	−	2.5 × 2.0 × 2.0	low	
4	female	59	−	−	−	5.5 × 4.0 × 3.0	low	diffuse type
5	female	48	+	−	−	7.5 × 8.0 × 7.0	high	with clear margin
6	male	64	+	+	−	9.5 × 6.2 × 3.5	low	
7	male	70	+	−	−	3.0 × 1.5 × 1.0	Low	multifocal, diffuse type
8	male	62	+	−	−	5.5 × 5.5 × 6.0	high	with clear margin
9	female	57	+	−	−	3.5 × 5.5 × 3.0	high	
10	male	52	+	−	−	6.0 × 4.0 × 3.0	high	multifocal
11	female	40	−	−	−	3.0 × 2.0 × 2.0	high	
12	male	56	+	−	−	7.0 × 6.0 × 3.5	high	with clear margin
13	male	50	+	−	−	13.0 × 8.0 × 6.0	low	
14	female	67	−	−	−	9.0 × 5.0 × 7.0	low	
15	male	46	−	−	+	12.0 × 11.0 × 6.0	low	
16	male	45	+	−	−	9.0 × 9.5 × 5.0	low	multifocal
17	male	55	+	−	−	6.0 × 3.5 × 3.0	low	
18	male	61	+	+	−	5.0 × 5.0 × 6.0	low	
19	male	57	+	−	+	8.0 × 6.0 × 3.5	high	
20	male	44	+	−	−	6.0 × 4.5 × 3.5	low	
21	female	55	+	−	−	5.0 × 4.0 × 3.5	high	with clear margin
22	female	60	−	−	−	3.0 × 4.0 × 3.0	high	
23	male	66	+	−	−	9.0 × 5.5 × 3.0	high	
24	male	49	−	−	−	7.0 × 5.2 × 4.0	low	
25	female	58	−	+	−	11.0 × 8.5 × 5.0	low	diffuse type
26	male	52	+	−	−	3.0 × 2.5 × 1.0	high	
27	male	65	+	−	−	6.0 × 4.5 × 3.0	high	
28	male	35	+	−	−	no record	no record	
29	male	40	+	−	−	no record	no record	
30	male	57	+	−	−	no record	no record	
31	male	42	+	−	−	no record	no record	

incubated at −20°C for 20 min. The samples were then centrifuged at 4°C, 12 000 rotation/min for 10 min. The pellet was washed with 70% ethanol, dried and finally resuspended in 300–500 μL sterile dH_2O. DNA samples were measured for concentration at 260 nm and then stored at −20°C for further use.

2.2.2 Preparation of cSNP chips

Preparation of the cSNPs was done according to a previous method (Wang et al., 2005). Forty-eight SNPs situated in 25 genes harbored in the high frequency loss regions 1 p and 8 p and related to HCC were included in the chips. At the same time, eight different concentrations of the house-keeping gene G_3PDH amplified by PCR were used to detect the hybridization efficiency in the chip. Primers and oligonucleotide probes were designed using primer premier 5.0 based on information about SNP sequences from the SNP database. Each SNP site corresponded to two probes, differing only in the middle base (perfect-match probes and mismatch probes). Probes with the same concentration were arranged on the nylon membrane using the NKG-Microarray III instrument. Each chip was irradiated for 3 min using a CL-1000M ultraviolet instrument to strengthen the stability of the combination between the probes and the membrane. The chips were then stored in a refrigerator at 4°C.

2.2.3 Multiple-primer PCR amplification

Multiple-primer PCR amplification was performed with reference to the literature (De BK and Srinivasan, 1989; Durigon et al., 1993). As the first step of multiple-primer PCR procedure, multiple PCR primers were analyzed with Vector NTI 7.0. In the second step, primers having different lengths of PCR products and no dimmers between them were compounded in the same PCR reaction system.

The PCRs were carried out on a Perkin-Elmer thermocycler in a total volume of 25 μL including ddH_2O 18.7 μL, 10 × PCR buffer 2.5 μL, primers 1 and 2 (10 μmol/L) 1 μL each, dNTP 0.5 μL (10 mmol/L) (the proportion of dTTP and Dig-dUTP was 10:1), DNA 0.8 μL (100 ng/μL), and Taq polymerase 0.5 μL (2 U/μL). The touch-down PCR cycling profile was as follows: denaturation at 94°C for 5 min; five cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 30 s; five cycles of 94°C for 30 s, 61°C for 30 s, 72°C for 30 s; five cycles of 94°C for 30 s, 58°C for 30 s; 72°C for 30 s; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 10 min. The length of the PCR amplified products was between 80 to 390 bp. The primer sequences are shown in Table 2.

2.2.4 Hybridization, membrane washing and coloring

The three hundred μL hybridization solution was added to a hybridization bag with a cSNP chip. Pre-hybridization was done at 45°C for 30 min. The PCR products were incubated in 100°C water for 5 min and then in ice for 5 min. The probe was added to the hybridization bag and incubated at 45°C for 12 h. The membrane was then placed in a container and washed with 2 mL/cm^2 of 2 × SSC and 0.1% SDS at room temperature twice for 2 min. Washing was done again with 0.2 × SSC and 0.1% SDS at 42°C twice for 2 min. The membranes were washed with TNT solution (10 mmol/L Tris-Cl, pH 8.0, 150 mmol/L NaCl, and 0.05% Tween-20) for 5 min at room temperature and was then placed in a fresh bag and blocked with 10 × blocking solution (0.2% polysaccharide, 0.2% PVP, and 0.2% BSA) and 180 μL TN (10 mmol/L Tris-Cl, pH 8.0 and 150 mmol/L NaCl). These were incubated at 37°C for 30 min. The liquid was removed and the following agents were added: fresh 10 × blocking solution, 180 μL TN and 0.3 μL digoxigenin antibody. Incubation was done at 37°C for 30 min. The membrane was washed twice with TNT solution for 3 min. Incubation of the membrane in DAB solution was done until the color was satisfactory. Finally, the reaction was terminated with 1 × PBS. The procedure was performed according to a previous method (Gao et al., 2003).

2.2.5 Chip analysis

The hybridized chips were input into a computer by using a scanner and analyzed with the software chip 3.0 designed by Nankai Chromosome Lab, Tianjin, China. The A1/A1 (wild-type, WT), A2/A2 (homozygous mutation, MT) and A1/A2 (heterozygous mutation, HT) genotypes were simply distinguished as follows: 3-fold and above, wild-type (A1/A1 genotype); 0.33-fold and below, mutant (A2/A2 genotype); lower than 1.5-fold but higher than 0.67-fold, heterozygous (A1/A2 genotype) (Huber et al., 2002).

2.2.6 Statistical analysis

The hybridization process of each sample was repeated for 4 times. The data were expressed as the mean ± standard deviation (SD). The data between different groups were analyzed by χ^2 test.

3 Results

3.1 Results of multi-primer PCR amplification

The touch-down and multiple-primer PCR amplification procedures were used. In the multiple PCR procedure,

Table 2 Thirty five pairs of primer sequences

gene name	sense primer	anti-sense primer	length of product/bp
EGFL3	5′AGACAAGGAGGACGTGGAG3′	5′ATGGTGGGACCTGCGACT3′	161
EGFL3	5′CATGAAAGGAAAGGGTGCC3′	5′AGGACACCGCTTCCCACA3′	288
TP73	5′AAGACATGCCCCATCCAGAT3′	5′ACGTGCTCCGCTTTCTTGT3′	92
ENO1	5′GGGCGTCATGGTGTCTCA3′	5′CCAGGTCAGCGATGAAGGTAT3′	95
TNFRSF1B	5′TTCCTGACCAAGCCTCCTC3′	5′CCATTGGGAGCAGGAAGG3′	213
MASP2	5′GACATTGACGAGTGCCAGGT3′	5′CGGCAGGAGCAGTAGAAACC3′	92
CASP9	5′TGGAAGAGCTGCAGGTGGA3′	5′GTCCTCGATCATATGGGGC3′	83
CASP9	5′GCGAACTAACAGGCAAGCAG3′	5′TCTGGGTGTTTCCGGTCTG3′	115
CASP9	5′GCCACTGCCTCATTATCAACA3′	5′GCCCTTCACCTCCACCAT3′	141
FGR	5′GCCCACCTGTGACTCTACTTC3′	5′CTGATCTCCCAGGCGTCC3′	130
SEPN1	5′GCTTCTGGTGGGACGAGTTC3′	5′GGCATCAGGAGTGTGCAAC3′	61
E2F2	5′GAATGTTTGAAGACCCCACCA3′	5′ACCCAGGAAGGAAGAAACAGAT3′	271
PINK1	5′CATCTAAGCCTCTGGGGTGAA3′	5′CAGCCAACCATCTTGTCTAACTT3′	68
STMN1	5′GAAGTGCGGAAGAACAAAGAAT3′	5′GGGATGGGGAGAAAGTCAGT3′	92
STMN1	5′CAGAAGGCAATAGAAGAGAACAAC3′	5′CGGTTCTCTTTATTAGCTTCCATT3′	86
MUTYH	5′GCCTCCCTCCTTCCATTTT3′	5′TGTCACTGGGCTGCACTGAA3′	160
MUTYH	5′GCCCTCACCTCCCTGTCTT3′	5′CAAGTGGGTCTGTAAGCAATCAA3′	157
MUTYH	5′GATCTCCGTTCCCAGCTCC3′	5′GAATGGAGGGAATCGGCAG3′	270
MUTYH	5′TTCCGAGGGAGCCTGCTAA3′	5′GAAGACGGGCAGAAGAGGT3′	80
MUTYH	5′ATGAGGAAGCCACGAGCAG3′	5′ACCGACGGACGAAGGACA3′	152
JUN	5′ATCGCTGCCTCCAAGTGC3′	5′GCTGTGCCACCTGTTCCCT3′	134
THEA	5′CTGGTAGAGAAGCCCAAACACT3′	5′CCTTGTGCTATTTCAGGAGACTT3′	390
THEA	5′GAGGCAGGACCAGCTCCACA3′	5′CGTTCCCTCCCTTATCCCAT3′	107
THEA	5′GCCTCGGAAGTGGAACA3′	5′GGCATTCCTTCTGTCTC3′	94
GSTM1	5′CCTTATTTGTCCTCTTTCCTTGC3′	5′CGTGAACTCGGGTGTACCT3′	334
PHGDH	5′CACTGGTGTCAGATGGGGAG3′	5′AGGTTAGAAGTGGAACTGGAAGG3′	113
DEFA1	5′CCTGTCCCAGGCTCAAGG3′	5′TTCCATAGCGACGTTCTCC3′	79
DEFB1	5′CAATCATGGGCCAATTTCTT3′	5′CTGCGTCATTTCTTCTGGTCA3′	309
MTMR9	5′AGCAACTGCTGTTGAATTTTCA3′	5′GCACCTCCTCCCGTTTGT3′	91
FDFT1	5′TGGACCAGGACTCGCTCAG3′	5′CAGCGCCTGGATAACAGC3′	92
NAT1	5′ACATTGTCGATGCTGGGTTT3′	5′CTGTTCCCTTCTGATTTGGTCTA3′	150
DOK2	5′GCCTCCAGCATGTCCAGC3′	5′GTACTTTGGGCCTTTCTTTAG3′	102
EPHX2	5′ CACCGGGAGGAGCAGATG3′	5′GAGGGAAGCAAAGAGGGAGTAT3′	154
ADRB3	5′CTTTCCGCCCGAGGAGTCT3′	5′TTCCCGGAGAGGCAGGAG3′	127
BNIP3L	5′TACCATCCTCATCCTCCATCC3′	5′CTCTGTCCTGATTCATGTTGTGC3′	75

primers having different lengths of the PCR products and no dimers between them were compounded in the same PCR reaction system, which could decrease the usage of Dig-dUTP. By this procedure, we obtained clear PCR bands. The 35 PCR products ranged from 61 to 390 bp (Fig. 1).

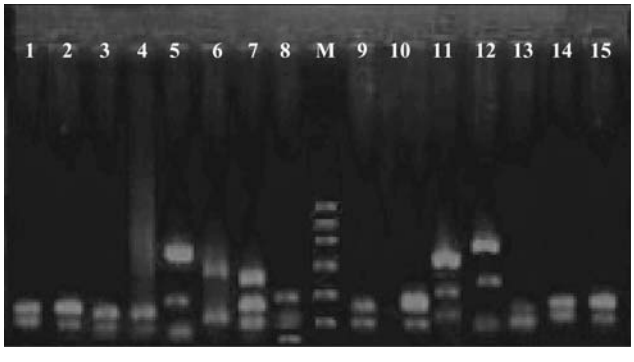

Fig. 1 Results of touch-down and multiple-primer PCR amplification. M: 100 bp marker (from 100 to 600 bp); lanes 1–15: multi-primer PCR products

The PCR products amplified from the 1st patient were purified, respectively, and then sequenced by the Shanghai Sangon Bio-Technology Ltd Co.(Shanghai, China) on an ABI 377 automated DNA sequencer. The result of sequence analysis shows that all of the 35 PCR products were amplified correctly.

3.2 Analysis of genotype polymorphism in HCC patients by cSNP chip

DNAs extracted from the 31 HCC patients respectively were served as the temple of PCR reaction. The PCR products were labeled with Dig-dUTP in the amplification procedure and then hybridized with the cSNP chip. The genotype polymorphisms in all the HCC patients were analyzed.

3.2.1 Exceptional results of polymorphism in several patients

The hybridization results of four patients were exceptional (Fig. 2–5). Two probes of the MTMR9

(rs3021506) in the 20th patient did not have any hybridization signals (Fig. 2, indicated by black arrows), while the PCR product of the MTMR9 (rs3021506) could be detected by 2% agarose gel electrophoresis. The PCR product was sequenced and the result showed that there were another two mutation sites in the 25 bp probe fragment (Fig. 6). Thus, the sequence could not entirely match with the designed probes, which indicates that there was no hybridization signal when more than one mutation site were present in the probe sequence and also that the cSNP chip had a high sensitivity.

Fig. 2 Hybridization result of the 20th patient

Fig. 3 Hybridization result of the 4th patient

Fig. 4 Hybridization result of the 8th patient

Fig. 5 Hybridization result of the 5th patient

There was no signal of the TNFRSF1B (rs106161622) in the 4th patient (Fig. 3) and the ADRB3 (rs4995) in the 8th patient (Fig. 4). Moreover, the PCR products of the TNFRSF1B (rs1061622) and the ADRB3 (rs4995) could not be detected by the agarose gel electrophoresis. We deduced that there were homozygous deletions of the TNFRSF1B and ADRB3 in the 4th and 8th patients, respectively.

3.2.2 Analysis of genotype polymorphism in HCC patients with different phenotypes

Analysis of the SNP (MT, homozygous and HT, heterozygous) in the HCC patients with different phenotypes (HBV +/−,differentiation degree, family history positive or negative, tumor size) showed that the number of MT was distinctly different between HBV + and HBV − patients (Table 3). The number of MT and HT was significantly different between low differentiation and high differentiation patients, while they were not significantly different between family history positive and negative groups or tumor size >3 cm and $\leqslant 3$ cm groups (Tables 4–6).

Table 3 Data of genotype polymorphism of the HCC patients with HBV(+/−) phenotypes

type	HBV +	HBV −	*P* value
MT number	3.48 ± 2.52	5.60 ± 2.65	0.029*
HT number	7.33 ± 3.18	10.90 ± 6.01	0.069

Note: MT, homozygous mutation; HT, heterozygous. *: $P \leqslant 0.05$.

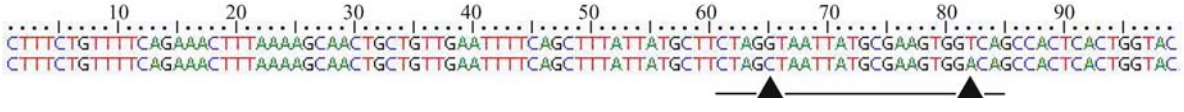

Fig. 6 Comparison results of the MTMR gene sequence between GenBank and the 20th patient. 1: MTMR gene sequence in GenBank; 2: MTMR gene sequence in the 20th patient. The sequence underlined is the probe. Base changes of the MTMR gene sequence in the 20th patient are indicated by the black arrow.

Table 4 Data of genotype polymorphism of the HCC patients with different differentiation degrees

type	low differentiation	High differentiation	*P* value
MT number	6.08 ± 2.72	3.57 ± 1.65	0.005**
HT number	10.62 ± 3.37	6.29 ± 2.01	0.0039**

**: $P \leqslant 0.01$.

Table 5 Data of genotype polymorphism of the HCC patients with different heredity phenotypes

type	family history +	family history –	*P* value
MT number	5.50 ± 2.29	4.62 ± 2.78	0.29
HT number	14.25 ± 6.41	8.62 ± 3.53	0.11

Table 6 Data of genotype polymorphism of the HCC patients with different tumor size

type	tumor size (>3 cm)	tumor size ($\leqslant 3$ cm)	*P* value
MT number	4.85 ± 2.77	4.60 ± 3.05	0.47
HT number	8.5 ± 4.32	11.60 ± 5.77	0.14

4 Discussion

We chose the genes located in the high frequency loss regions of the HCC chromosomes to construct the chips by collecting cSNP sequences. We set dots to control the efficiency of the hybridization, designed the oligo-nucleotide probes (including perfect match probes and mismatch probes) according to the conservative area of household gene G_3PDH to examine the precision of the chips, set a blank site to detect the uniformity of the hybridization backgrounds, and avoided the non-specific fragments by using touch-down PCR cycling conditions. Thus, the reliability of the hybridization was increased. Moreover, the multiple-primer PCR amplification procedure could decrease the usage of Dig-dUTP and experimentation cost.

In addition, the hybridization results of the MTMR9 in the 20th patient could further prove the accuracy of the cSNP chips. The results of the TNFRSF1B in the 4th patient and that of the ADRB3 in the 20th patient indicated that there were many kinds of variations in the HCC patients.

Tumor suppressor genes located in the LOH regions in the genomes of tumor tissues are believed to play a key role in carcinogenesis. Most of previous studies elucidated the chromosome gains and losses by SNP arrays (Monzon et al., 2008; Iwamoto et al., 2007; Pandya et al., 2007). Recently, increasing numbers of research shows that inactivation of the suppressor genes located in the LOH regions is critical in the pathogenesis of human cancer (Midorikawa et al., 2006). In the present study, we constructed the cSNP chip of tumor-related genes located in the LOH regions and evaluated the genotype polymorphism according to the cSNP chips analysis results (the MT and HT numbers of individual patients). The statistic data showed that the number of MT was distinctly different between the HBV + and HBV- patient groups. The significant difference in MT and HT number between patients with low and high differentiation indicated that the differentiation degree of HCC is very important. The genotype was different between patients with different phenotypes. The frequency of gene mutation is different between various populations and geographic regions. Therefore, the factors of population and natural environment should be taken into consideration in the investigation of susceptibility and carcinogenesis. Furthermore, the number of MT and HT was not different in the positive or negative family history groups and in the tumor size >3 cm or $\leqslant 3$ cm groups, which may be related with population or due to our small group size.

The analysis of the single nucleotide polymorphism in HCC patients can provide useful information for the early diagnosis of HCC and the understanding of carcinogenesis in HCC. Our methods are applicable to many cancers. In future studies, we will increase the scanning density of genes in chromosomes and enlarge the scanning area to the whole genome.

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