A Modified Mutation Detection Method for Large-scale Cloning of the Possible Single Nucleotide Polymorphism Sequences

Ming-Chung Jiang*, Pao-Chu Jiang, Ching-Fong Liao and Ching-Chiu Lee

Institute of Zoology, Academia Sinica, Taipei, Taiwan ROC

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Although the human genome has been nearly completely sequenced, the functions and the roles of the vast majority of the genes, and the influences of single nucleotide polymorphisms (SNPs) in these genes are not entirely known. A modified mutation detection method was developed for large-scale cloning of the possible SNPs between tumor and normal cells for facilitating the identification of genetic factors that associated with cancer formation and progression. The method involves hybridization of restriction enzyme-cut chromosomal DNA, cleavage and modification of the sites of differences by enzymes, and differential cloning of sequence variations with a designed vector. Experimental validations of the presence and location of sequence variations in the isolated clones by PCR and DNA sequencing support the capability of this method in identifying sequence differences between tumor cells and normal cells.

Keywords: Differential cloning, Genome, Normal cells, Single nucleotide polymorphisms, Tumor cells

Introduction

Sequence variations in the human genome, including mutations, translocations, amplifications, insertions and deletions, may alter the functions of genes and predispose an individual to disease formation (Sinden *et al.*, 1992). SNPs are the most frequent type of sequence variation in the human genome. SNPs in the protein-coding regions of genes may alter the functions of the coded proteins. SNPs in the regulatory regions of genes may influence the expression of the coding proteins. SNPs may themselves represent genetic variants that affect disease susceptibility or progression. Most SNPs are found in non-coding regions of the genome, and have no

direct known function in individuals. But these SNPs (also the SNPs in protein-coding regions and regulatory regions) may be used as genetic markers to detect association between a particular genomic region and the disease for discovering genetic factors in diseases (Goodstadt and Ponting, 2000; Becker *et al.*, 2003). The association study can use information from a relatively small set of variants that capture most of the common patterns of variation in the genome, any region or gene can be tested for association with a particular disease (The International HapMap Consortium, 2003).

Identification and genotyping a vast number of genetic polymorphisms in large populations are increasingly important for disease gene identification, pharmacogenetics and population-based studies (Cantor, 1999; Elahi *et al.*, 2004; Nelson *et al.*, 2004). Most of the current employed SNP detection methods use allele-specific probes or polymerase chain reaction (PCR) and designed primers to detect SNPs (sequence variations) of specified sequences (Mir and Southern, 2000; Chen *et al.*, 2003; Kwok, 2003). The differential cloning method described herein is aimed to clone the possible SNPs between tumor cells and normal cells at a large-scale to facilitate the discovery of genetic factors that associated with tumor formation and progression. The strategy of this method is described clearly in the text.

Materials and Methods

Cells and enzymes Human cell lines derived from hepatoma, the Hep G2 cells, and from normal liver, the Chang liver cells, were used in the experiments. Both cells were from the American Type Culture Collection (Manassas, Virginia, USA) and were grown in DMEM medium as described previously (Jiang and Liao, 2004). Nuclease S1 was from Invitrogen (Carlsbad, California, USA) and other enzymes were from New England Biolabs (Beverly, Massachusetts, USA).

DNA precipitation Ethanol precipitation of DNA described in this section was performed by adding 0.1 volume of 3 M sodium acetate (pH 7.0) and 1.5 volumes of cold (−20°C) absolute ethanol

*To whom correspondence should be addressed.
Tel: 886-2-27899533; Fax: 886-2-27858059
E-mail: jiangmcedu@yahoo.com.tw

to a 200- μ l DNA solution in a 0.5-ml microcentrifuge tube, mixing this completely, incubating the tube at -70°C for 30 min, liquefying the frozen sample with fingers, centrifuging the tube at 14,000 rpm at 4°C for 20 min, briefly rinsing the DNA pellet with cold (-20°C) 75% ethanol, centrifuging the tube at 14,000 rpm at 4°C for 1 min, removing the ethanol, centrifuging the tube at 14,000 rpm at 4°C for 0.5 min to spin down the ethanol in the wall, removing the ethanol with a mini-tip, drying the DNA pellet at 50°C for 5 min, and dissolving the DNA pellet in H_2O at 4°C overnight. The DNA pellet may become transparent; avoid DNA loss when removing the ethanol.

Preparation of chromosomal DNA Chromosomal DNA was extracted as described (Sambrook *et al.*, 1989). The chromosomal DNA was incubated in *Tsp509I* buffer (10 mM Bis Tris Propane-HCl, pH 7.0, 10 mM MgCl_2 , 1 mM DTT) containing 1.5 U/ μ g *Tsp509I* at 65°C for 4 h. During incubation, the sample was pipetted with a 1-ml tip and centrifuged briefly every 30 min. The DNA fragments were extracted twice with phenol/chloroform and once with chloroform/isoamyl alcohol, and were then precipitated with ethanol.

Dephosphorylation of *Tsp509I*-generated DNA fragments *Tsp509I*-cleaved DNA was incubated in dephosphorylation buffer ((10 mM Tris-HCl, pH 8.3, 0.1 mM ZnCl_2 , 1 mM MgCl_2 , and 0.5 U/ μ g calf intestinal alkaline phosphatase (CIP)) at 37°C for 70 min. During incubation, the tube was vortexed and centrifuged briefly every 25 min. Dephosphorylation was stopped by incubating the reaction mixture in proteinase K buffer (0.3% SDS, 5 mM EDTA, pH 8.0, and 100 μ g/ml proteinase K) at 50°C for 30 min. DNA was extracted 3 times with phenol/chloroform and once with chloroform/isoamyl alcohol, and was then precipitated with ethanol.

Ligation with *EcoRI* adaptors *EcoRI* adaptors were prepared by mixing equal molar quantities of the oligonucleotides, 5'-AAGGGGTTTCG-3', and 5'-P-AATTCGAACCCCTT-3'. The oligonucleotide mixture was heated at 95°C for 5 min and cooled at room temperature for 30 min and was then precipitated with ethanol. Dephosphorylated DNA fragments (20 μ g) were incubated with 6-fold excess moles of *EcoRI* adaptors in T4 DNA ligation buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , 10 mM DTT, 1 mM ATP, 25 μ g/ml BSA, and 5,000 U T4 DNA ligase) at 4°C for 12 h in a total volume of 100 μ l. Another 5,000 U T4 DNA ligase was added to the ligation buffer, and the tube was incubated at 16°C for another 8 h. During incubation, the tube was vortexed and centrifuged briefly every hour. DNA was extracted with phenol/chloroform and chloroform/isoamyl alcohol and was then precipitated with ethanol.

Repair of nicks Nicks in the DNA fragments were repaired by incubating the DNA in *E. coli* DNA polymerase I buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl_2 , 7.5 mM DTT, 5 mM dNTP, and 50 U *E. coli* DNA polymerase I) at 16°C for 70 min in a total volume of 50 μ l. During incubation, the tube was vortexed and centrifuged briefly every 10 min. DNA fragments were extracted with phenol/chloroform and chloroform/isoamyl alcohol, followed by ethanol precipitation. DNA was then incubated in T4 DNA ligation buffer containing 4,000 U T4 DNA ligase at 4°C for 12 h in a total volume

of 50 μ l. Another 4,000 U T4 DNA ligase was added to the ligation buffer and the tube was incubated at 16°C for another 6 h. During incubation, the tube was vortexed and centrifuged briefly every hour. DNA fragments were extracted with phenol/chloroform and chloroform/isoamyl alcohol, followed by ethanol precipitation.

Preparation of heteroduplex and homoduplex fragments Each Hep G2 and Chang liver DNA fragment was divided into 4 equal fractions. Heteroduplex fragments were prepared by mixing 1 fraction each of the Hep G2 and Chang liver DNA fragments. Homoduplex fragments were prepared by mixing 2 fractions of the Chang liver DNA fragment. Each DNA fragment mixture was suspended in hybridization buffer (35 mM Tris-HCl, pH 7.6, 300 mM NaCl, and 3.5 mM MgCl_2) in a total volume of 150 μ l in a microcentrifuge tube with a screw cap to avoid evaporation. The tubes were boiled for 5 min and were placed immediately in a hybridization oven with constant slow rolling at 68°C for 24 h. The tubes were cooled at room temperature for 30 min, and the DNA was harvested by ethanol precipitation.

T7 endonuclease I treatment For T7 endonuclease I treatment, each 15 μ g of heteroduplex and homoduplex fragments was separately incubated in T7 endonuclease I buffer (20 mM Tris-acetate, pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, and 7.5 U T7 endonuclease I) at 37°C for 30 min in a total volume of 50 μ l. DNA was extracted with phenol/chloroform and chloroform/isoamyl alcohol, followed by ethanol precipitation.

Nuclease S1 treatment After T7 endonuclease I treatment, the DNA was incubated in nuclease S1 buffer (30 mM sodium acetate, pH 5.2, 1 mM ZnSO_4 , 5% (v/v) glycerol, 300 mM NaCl, and 22 U nuclease S1) at 37°C for 10 min in a total volume of 50 μ l. The DNA was extracted twice with phenol/chloroform and once with chloroform/isoamyl alcohol and was then precipitated with ethanol.

T4 DNA polymerase treatment Following nuclease S1 treatment, the DNA was incubated in T4 DNA polymerase buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM MgCl_2 , 1 mM DTT, 100 μ M dNTP, 50 μ g/ml BSA, and 4.5 U T4 DNA polymerase) at 12°C for 15 min in a total volume of 30 μ l. During incubation, the tube was vortexed and centrifuged briefly every 15 min. The DNA was extracted with phenol/chloroform and chloroform/isoamyl alcohol, followed by ethanol precipitation.

Removal of the *EcoRI* adaptors by *Tsp509I* digestion After T4 DNA polymerase treatment, the DNA was incubated in *Tsp509I* buffer containing 10 U *Tsp509I* at 65°C for 70 min in a total volume of 20 μ l. During incubation, the tube was vortexed and centrifuged briefly every 30 min. The DNA sample was extracted with phenol/chloroform and chloroform/isoamyl alcohol, followed by ethanol precipitation. Although the free *EcoRI* adaptors are unable to be cloned with the vectors, the *EcoRI* ends of these adaptors still can ligate into the *EcoRI* ends of the vectors. Thus, removing the free *EcoRI* adaptors with commercial nucleotide removing kit that removes nucleotides smaller than 15 bp, and then reducing the volume of the DNA sample by ethanol precipitation before ligation, should be able to increase the cloning efficiency of the duplex DNA

fragments. If the free adaptors are removed with a small nucleotide removing kit, then there is no need to dephosphorylate the blunt ends (i.e. the *Sma*I-cut ends) of the cloning vectors.

Preparation of vector The pUC 19 plasmids were incubated in *Sma*I buffer (20 mM Tris-acetate, pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, and 1.5 U/ μ g *Sma*I) at 25°C for 1.5 h. The DNA was extracted with phenol/chloroform and chloroform/isoamyl alcohol, followed by ethanol precipitation. The DNA was then dephosphorylated in dephosphorylation buffer containing 0.6 U/ μ g CIP at 37°C for 20 min and at 55°C for another 55 min. Dephosphorylation was stopped by incubating the reaction mixture in proteinase K buffer containing 100 μ g/ml proteinase K at 50°C for 30 min. DNA was extracted 3 times with phenol/chloroform and once with chloroform/isoamyl alcohol and was precipitated with ethanol. The DNA was then incubated in *Eco*RI buffer (100 mM Tris-HCl, pH 7.0, 50 mM NaCl, 10 mM MgCl₂, 0.025% Triton X-100, and 1.5 U/ μ g *Eco*RI) at 37°C for 1.5 h. The DNA was resolved on a 1.5% agarose gel, and the vector DNA band was eluted.

Test ligation Test ligation was done by ligating a small aliquot of the vectors with the *Eco*RI adapter that possessed two phosphorylated ends. The ligation mixture was transformed into high efficiency XL1-Blue competent cells. Both white colonies and blue colonies were observed in plates containing IPTG, X-gal, ampicillin, and tetracycline, and 65% of the colonies were white. The plates of the control ligation only grew blue colonies. The control ligation contained the same materials as in the test ligation but without the addition of the *Eco*RI adapter.

Cloning of sequence variations The DNA fragments were incubated in T4 DNA ligation buffer containing 0.1 μ g of *Sma*I/CIP/*Eco*RI-treated pUC 19 vectors and 50 U/ μ l T4 DNA ligase at 16°C for 16 h. The ligation mixture was transformed into high efficiency XL1-Blue competent cells, and cells were spread on plates containing IPTG, X-gal, ampicillin, and tetracycline. Sequences of the inserts were identified by BLAST in NCBI.

PCR PCR reactions were done as described previously (Jiang and Liao, 2004). The PCR-amplified bands were eluted and cloned into pGEM-T-easy vectors (Promega Madison, USA). The identities of the PCR products were demonstrated by DNA sequencing.

Results and Discussion

The strategy of the method is outlined in Fig. 1. Human cell lines derived from hepatoma, the Hep G2 cells, and from normal liver, the Chang liver cells, were used as an example for large-scale cloning of the possible SNPs between a tumor cells and a normal cells. Chromosomal DNA extracted from cells were cleaved with the restriction enzyme, *Tsp*509I. *Tsp*509I recognizes the sequence, AATT, which is a very common sequence in mammalian genomes. *Tsp*509I digestion generated DNA fragments ranging from about 20 to 1,500 bp, and the most abundant were those around 200 bp in length (Fig. 2).

The ends of the *Tsp*509I-cleaved DNA fragments were dephosphorylated with CIP to suppress self-ligation before these ends were ligated with the *Eco*RI adaptors. Ligation of two DNA fragments involves the formation of new bonds between phosphate residues located at the 5' termini of double-stranded DNA and the adjacent 3'-hydroxyl moieties; therefore there were two single-strand nicks in each DNA fragment after ligation of the CIP-treated DNA with the *Eco*RI adaptors. *Escherichia coli* DNA polymerase I is a DNA-dependent DNA polymerase with inherent 5' to 3' exonuclease, 3' to 5' exonuclease, and 5' to 3' polymerase activities (Kelley and Stump, 1979). Thus, *E. coli* DNA polymerase I was used to remove the dephosphorylated 5' termini and to repair the nicks in the *Eco*RI adaptor-ligated DNA fragments. The 3' to 5' exonuclease activity of *E. coli* DNA polymerase I may produce nicks in the DNA (Sambrook *et al.*, 1989). Thus, nicks in the DNA fragments were repaired with T4 DNA ligase.

Heteroduplex fragments were prepared by melting and re-annealing Hep G2 DNA fragments with equal amounts of Chang liver DNA fragments. Homoduplex fragments were prepared by melting and re-annealing Chang liver DNA fragments. The homoduplex fragments were used as the control in the experiments. The duplex DNA fragments were treated with the resolvase, T7 endonuclease I, at a concentration of 7.5 U/reaction to scan the mismatched base pairs in the hybrids.

Resolvase has been shown to cleave single-base-pair mismatches in model experiments with synthetic DNA (Kosak and Kemper, 1990). With the use of designed primers and the PCR technique, the two most extensively studied resolvases, T7 endonuclease I and T4 endonuclease VII, are widely employed in the detection of mutations of many known genes (Mashal *et al.*, 1995; Youil *et al.*, 1995). Resolvase can detect all eight types of mismatches (A/A, T/T, C/C, G/G, A/C, A/G, T/C, and T/G) as well as insertions and deletions (Kleff and Kemper, 1988; Solaro *et al.*, 1993). Resolvase binds to non-perfectly matched DNA and then cleaves it within six nucleotides 3' or 5', respectively. A counter-nick may then be placed on the other side of the mismatch within six nucleotides on the opposite-strand DNA. The rates and frequencies of the formations of these two nicks are not necessarily the same, and the locations of these two nicks on the DNA are not necessarily symmetrical about the mismatch. Thus, both single-strand nicks and staggered double-strand breaks may be observed after T7 endonuclease I or T4 endonuclease VII digestion (Parsons and Kemper, 1990; Parkinson and Lilley, 1997).

Nuclease S1 can induce double-strand breaks in DNA nicks as well as being capable of hydrolyzing single-stranded DNA (Chaudhry and Weinfeld, 1995). Thus, the T7 endonuclease I-treated DNA was treated with nuclease S1 (22 U/reaction) at 37°C for 10 min to induce double-strand breaks in DNA nicks generated by T7 endonuclease I and also to hydrolyze single-stranded regions of the staggered double-strand breaks

Fig. 1. Strategy for large-scale cloning of the possible single nucleotide polymorphism sequences between a tumor cell line and a normal cell line. Chromosomal DNA was cleaved with *Tsp509I* or its isoschizomers. The DNA fragments were dephosphorylated and ligated with the *EcoRI* adaptors. Nicks between adaptors and DNA fragments were repaired by *E. coli* DNA polymerase I and T4 DNA ligase. The heteroduplex fragments were prepared by melting and re-annealing DNA fragments of two cell lines. The homoduplex fragments were prepared by melting and re-annealing DNA fragments of a single cell line. The duplex fragments were scanned for mismatches by resolvase. Resolvase produces nicks or staggered double-strand breaks within 6 nucleotides around the mismatches. Nuclease S1 and T4 DNA polymerase treatment creates ligatable blunt DNA ends at the nicks or the breaks. The *EcoRI* adaptors in duplex fragments were cleaved with *Tsp509I* digestion. Each duplex fragment containing SNP now possesses a phosphorylated blunt end and a phosphorylated *EcoRI* compatible cohesive end and thus can be cloned with the *SmaI*-dephosphorylation-*EcoRI*-treated vectors. Each normal duplex fragment now possesses only two phosphorylated *EcoRI*-compatible cohesive ends and thus cannot be cloned with the vectors. Alternatively, the cleaved *EcoRI* adaptors can be removed with a small nucleotide removing kit. In this case, there is no need to dephosphorylate the *SmaI* cut-ends of the vectors. Clones carrying inserts can be recognized by blue/white selection. The symbol (◆) represents sequence variation. Please see the text for further details.

Fig. 2. Analysis of chromosomal DNA fragments generated by *Tsp509I* digestion. Hep G2 and Chang liver chromosomal DNA were digested with *Tsp509I* and analyzed by 2% agarose gel electrophoresis. Lane 1, marker DNA (100 bp DNA ladders, Geneaid, Taoyuan, Taiwan); lane 2, *Tsp509I*-cleaved Hep G2 chromosome DNA; lane 3, *Tsp509I*-cleaved Chang liver chromosome DNA; lane 4, Hep G2 chromosome DNA; lane 5, Chang liver chromosome DNA.

generated by T7 endonuclease I at the sites of SNPs.

Each DNA fragment possesses two *EcoR*I-compatible ends. After having been treated with T7 endonuclease I and nuclease S1, each DNA fragment containing SNP possesses an *EcoR*I-compatible end and a blunt end near the site of the SNP; on the other hand, each normal DNA fragment contains only two *EcoR*I-compatible ends. This makes it possible to clone the SNP fragments differentially. To achieve this, the pUC 19 vector was cut with *Sma*I, dephosphorylated by CIP, and cleaved with *EcoR*I. These treatments create a pUC 19 vector containing a phosphorylated *EcoR*I-compatible cohesive end and a dephosphorylated blunt end. Thus, the DNA fragments containing SNPs can be cloned with the *Sma*I-CIP-*EcoR*I-treated pUC 19 vectors; while the DNA fragments of the normal genes cannot be cloned with the vectors.

T4 DNA polymerase can remove protruding single-stranded 3' termini with its 3' to 5' exonucleolytic activity and fills in recessed 3'-hydroxyl termini with its 5' to 3' polymerizing activity; therefore T4 DNA polymerase treatment generates ligatable blunt DNA ends (Sambrook *et al.*, 1989). Thus, the DNA fragments were treated with T4 DNA polymerase to repair frayed termini generated by nuclease S1. Before cloning, *EcoR*I adaptors in the duplex DNA fragments were cleaved by *Tsp509I* digestion. Because the blunt ends of the *EcoR*I adaptors are dephosphorylated, the free *EcoR*I adaptors cannot be cloned with the *Sma*I-CIP-*EcoR*I-treated vectors. Since cloned insert fragment that

Fig. 3. Analysis of inserts of some of the isolated clones by 2% agarose gel electrophoresis. Lane 1, marker DNA (1-kb plus DNA ladders, Invitrogen); lanes 2~16, DNA fragments of pUC 19 plasmids digested with *Hind*III and *Tsp509I*. pUC 19 plasmids were isolated from the bacterial cultures of the white colonies of the heteroduplex samples. Bracket indicates inserts.

smaller than 15 bp is difficult to identify which gene it belongs. And although the free *EcoR*I adaptors are unable to be cloned with the vectors, the *EcoR*I ends of these adaptors still can ligate into the *EcoR*I ends of the vectors. Thus, removing the free *EcoR*I adaptors with commercial nucleotide removing kit that removes nucleotides smaller than 15 bp, and then reducing the volume of the DNA sample by ethanol precipitation before ligation, should be able to increase the cloning efficiency of the duplex DNA fragments. Because the purpose of the dephosphorylation of the blunt ends of the cloning vector is to inhibit the cloning of the free *EcoR*I adaptors. If the free *EcoR*I adaptors are removed with a small nucleotide removing kit, then there is no need to dephosphorylate the blunt ends of the cloning vectors. The phosphorylation in the blunt ends of the vectors also can increase the efficiency of ligation. Clones containing inserts can be recognized by blue/white selection. Restriction enzyme analyses of some of the plasmids isolated from the white clones of the heteroduplex DNA samples are shown in Fig. 3. The sizes of these cloned inserts ranged from about 20 to 350 bp.

Various doses of T7 endonuclease I (3, 7.5, and 15 U/reaction) and nuclease S1 (10, 22, and 35 U/reaction) and different enzyme incubation times were tested. The most optimum condition was with 7.5 U/reaction T7 endonuclease I followed by 22 U/reaction nuclease S1 treatment. The condition of 7.5 U/reaction T7 endonuclease I digestion followed by 22 U/reaction nuclease S1 treatment were independently repeated 3 times, and all replicates produced satisfactory positive results. The numbers of white colonies of the heteroduplex DNA samples were significantly higher than those of the control homoduplex DNA samples. Numbers of white colonies were $5,617 \pm 353$ (mean $\pm$ standard deviation (SD)) and 636 ± 87 for heteroduplex and control homoduplex DNA samples, respectively. Clones isolated from the homoduplex control samples may reflect sequence variations (i.e. gene polymorphisms and repetitive sequences) of genes in a cell line.

Thirty isolated clones were sequenced and sequences were identified by BLAST in NCBI. Seven sequences belong to

Fig. 4. Comparisons of the clone 16 insert sequence (A) and the PCR-amplified Chang liver (B) and Hep G2 (C) chromosomal DNA sequences that covering the region of the clone 16 insert sequence. The *Sma*I end and the *Eco*R1 end of the pUC 19 vector, and the *Tsp*509I recognition sequences in the Hep G2 and the Chang liver PCR sequences are indicated. The *Tsp*509I sites of the PCR product sequences that can be ligated into the *Eco*R1 cloning site of the pUC 19 vector are indicated by arrows. The site of the double-T deletion in the Hep G2 chromosomal PCR sequence is indicated by the symbol (Δ). The sites of the “differential cloning end” in the Hep G2 and the Chang liver chromosomal PCR sequences are indicated by the symbol ($\blacklozenge$). Please see the text for further details.

repetitive sequences. There are many repetitive sequences in the human genome. It is difficult to verify SNPs in repetitive sequences, because PCR amplification of a repeat region often produces multiple products. The sequences of three isolated clones were compared with their corresponding Hep G2 and Chang liver chromosomal DNA PCR product sequences to validate the capability of this method in the isolation of sequence variations between tumor and normal cells. All of the Hep G2 chromosomal DNA PCR product sequences analyzed possess SNPs near the sites corresponding to the “differential cloning end” of the inserts of the isolated clones. Because of space limitations, only clone 16 and its corresponding Hep G2 and Chang liver chromosomal DNA PCR product sequences are shown in here to describe the capability of this method in the cloning of SNP sequences.

The clone 16 was isolated from the heteroduplex DNA sample, and its sequence is shown in Fig. 4A. Clone 16 contains a 145-bp insert, and the sequence of this insert is identical to bases 107,640 to 107,785 of the RP11-137L15 gene (GenBank

accession no. AC023991). Hep G2 and Chang liver chromosome DNA regions covering the sequence of the clone 16 insert were amplified by PCR. The amplified 621-bp PCR product corresponds to bases 107,439 to 108,060 of the RP11-137L15 gene. DNA sequencing analysis showed that the sequences of the Hep G2 and the Chang liver PCR products were identical except that there was a double-T deletion in the Hep G2 PCR product sequence. The site of this double-T deletion corresponds to base 107,791 of the RP11-137L15 gene, and this site is 5 bases toward the “differential cloning end” which is located at base 107,786 the gene. Each Hep G2 and Chang liver PCR product sequence possesses two *Tsp*509I recognition sites (Fig. 4 B and C). In the RP11-137L15 gene sequence, there are also two *Tsp*509I recognition sites in bases 107,640 and 107,873. The two *Tsp*509I sites in bases 107,640 and 107,873 of the RP11-137L15 gene provided the *Eco*R1-compatible end for cloning of the SNPs that located from bases 107,640 to 107,873 of the gene.

The 145-bp insert was ligated to the *Eco*R1 end of the pUC

19 vector with its *Tsp509I* end, and was ligated to the *SmaI* end of the pUC 19 vector with its “differential cloning end”. The “differential cloning end” was the site that T7 endonuclease I recognized and cut, and which was near the SNP site of the sequence. These results demonstrate the success of this method in the cloning of SNP sequences between cells, and also demonstrate that the sites of SNPs can be localized to within 6 nucleotides.

The average density of SNPs was estimated 1 per 1.9 kb in the human genome (The International SNP Map Working Group, 2001). The SNPs are not evenly distributed in the human genome: 90% of contiguous 20-kb windows contain one or more SNPs, as do 63% of 5-kb windows and 28% of 1-kb windows, and 4% of genome sequence fall in gaps between SNPs of >80 kb (The International SNP Map Working Group, 2001). It should be noted that the average density of SNPs are calculated based on SNP data collected from multiple individuals, hence the average density of SNPs between an affected individual and a comparable normal individual, or between the tumor and the normal cells (or tissues) of a same individual, must be much less than 1 per 1.9 kb. Thus, the differential cloning method should be superior to method by randomly sequencing the plasmids of a whole-genome library for identifying sequence differences between tumor and normal cells by most ordinary laboratories. Since there will be a high percentage of plasmids in the whole-genome library that don't contain sequence differences, and most laboratories don't have sufficient financial support to sequencing all the clones of both the tumor cell and the normal cell whole-genome libraries.

The numbers of SNPs identified are approaching 3 million; nevertheless identification of new SNPs from diverse kinds of diseases will still be helpful for disease gene discovery. Moreover, analyzing the sequence differences between the tumor cells and the normal cells of a same individual at a large-scale should be important for the discovery of oncogenes. This method needs no expensive or complex equipment, and the techniques of the method should be easy for most researchers. Thus, this method may be able to facilitate the discovery of genetic factors that associated with cancer formation and progression.

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