Detection of p53 Gene Mutations by Single Strand Conformation Polymorphisms Analysis in Various Human Liver Cancer Cell Lines Using a Non-radioactive Mini Gel Electrophoresis System

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Abstract

Previous studies have reported that human liver cancer cells derived from patients with hepatocellular carcinoma (HCC), for example, Alexander (PLC/PRF/5), have p53 gene mutations in different codons. Detection of these mutations by single strand conformation polymorphisms (SSCP) has been reported useful in p53 mutation studies in HCC. The aim of this study was to determine whether SSCP analysis using a mini gel electrophoresis system, followed by visualisation of electrophoretic band using silver staining, was able to detect the p53 gene mutations. Exons 3, 5, 6, 7, 8, 9, 10 and 11 of the p53 gene of five human HCC cell lines (Alexander, HLE, HLF, HuH7 and HepG2) and two normal liver cell lines (Chang and WRL-68) were amplified using polymerase chain reaction (PCR), and the PCR products were later subjected to SSCP analysis. Our results showed that the migration patterns for exon 7 in the HCC cell lines, Alexander, HLE and HLF, were different from those of the normal cell lines, Chang and WRL-68. However, the cell lines HepG2 and HuH7 demonstrated the same migration patterns as those of the two normal cell lines. For exon 6, all of the cell lines except HuH7 showed the same mobility shifts with the presence of a band. For exons 3, 5, 8, 9, 10 and 11, the migration patterns for all HCC cell lines were as those of the normal cell lines. Further examination by direct sequencing confirmed the presence of mutations in the respective HCC cell lines in exons 6 and 7 of the p53 gene. In conclusion, p53 gene mutations could be detected and differentiated using our SSCP protocol.

Keywords: p53 gene mutations, hepatocellular carcinoma cell lines, SSCP

Introduction

Hepatocellular carcinoma (HCC) is one of the five most common cancers in the world, accounting for about 1 million cases annually [1]. It is the seventh most common cancer in males and the ninth in females [2]. The highest incidence of HCC is encountered in the countries of South East Asia and sub-Saharan Africa, Taiwan, and southeast China, while the lowest rate of HCC is found in Western countries, South America and the Indian subcontinents [3]. The remarkable geographical distribution in the incidence of HCC was found to have no association with race or genetic factors, but to be more closely related to environmental agents, particularly the prevalence of chronic hepatitis B (HBV) infection and/or exposure to dietary aflatoxin B₁, AFB₁, a hepatocarcinogen [4].

HCC is one of the many tumours which display p53 tumour suppressor gene mutations at relatively high frequency [5, 6]. Guanine to thymine transversion at codon 249 of the p53 gene was detected in up to 50% of HCCs from Qidong, China [5] and Mozambique [6]. Interestingly, infection of HBV and exposure to dietary AFB₁ are high at these locations [5, 6] and therefore, the combination of these two HCC risk factors seems necessary for generating the G → T transversion hotspot mutation [7]. However, there are also other p53 mutations detected at different codons. These mutations are observed from locations with either low frequency of HBV infection or low exposure to AFB₁, or from countries of other HCC risk factors and etiology [8, 9]. These results show that the spectrum of p53 gene mutations in HCC have strong association with its risk factors and etiology. We are interested to investigate the type of p53 mutations in HCC patients in Malaysia. However, we report here the results obtained from our efforts to optimise the method for detection of p53 mutation using a non-radioactive PCR-SSCP analysis in a mini gel electrophoresis system, Mini-PROTEAN® 3 Cell (Bio-Rad, USA), with detection of the electrophoretic bands by silver staining for both the HCC and normal liver cell lines.
Materials and Methods

Cell lines

Human HCC cell lines HLE, HLF and HuH7 were purchased from Health Science Research Resources Bank (HSRRB), Japan, while Alexander (PLC/PRF/5) and HepG2 cell lines were purchased from the American Type Culture Collection (ATCC), USA. Two normal human liver cell lines, Chang and WRL-68, also purchased from ATCC were used as controls. Cell lines HLE, HLF and HuH7 were cultured in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (5% for HLF), 20 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cell lines Alexander and HepG2 were cultured in Earl’s Modified Eagles Medium (EMEM), with added 10% heat-inactivated fetal bovine serum, 20 mM HEPES, 20mM NaHCO3, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids (NEAA), 100 U/mL penicillin and 100 µg/mL streptomycin. Chang cell line was maintained in EMEM with all the supplements as the cell lines Alexander and HepG2, but without 1 mM sodium pyruvate, while cell line WRL-68 was maintained in the same medium and supplements as the cell line Chang, but without 0.1 mM NEAA. All cell lines were grown to 80-90% confluency in a humidified incubator at 37ºC in 5% CO2, and these cells were harvested for genomic DNA extraction with 0.25% trypsin.

Genomic DNA extraction

Genomic DNA was isolated from cell pellets (2 x 10^4 number of cells) using High Pure PCR Template Preparation Kit according to the manufacturer’s recommendations (Roche, USA).

Table 1: Annealing temperature and time for polymerase chain reaction of the studied exons of the p53 gene.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Annealing Temperature (°C)</th>
<th>Annealing Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>5 (5’ end)</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td>5 (3’ end)</td>
<td>66</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>66</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>66</td>
<td>30</td>
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<td>8</td>
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<td>9</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>47</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>64</td>
<td>30</td>
</tr>
</tbody>
</table>

Direct DNA Sequencing

Samples that showed different electrophoretic mobility when compared to the normal liver cell lines were electrophoresed on 2% agarose gel and purified using QIAquick® Gel Extraction Kit (QIAGEN, USA) according to the manufacturer’s recommendations. The samples were sequenced by the dideoxy chain termination method using the same primers as in the PCR. DNA sequencing was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA) using ABI PRISM®BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA).

PCR-SSCP analysis and silver staining

Hot start PCR was performed on extracted genomic DNA of the cell lines to amplify exons 3, 5, 6, 7, 8, 9 and 11 of the p53 gene. PCR fragments were generated from ~1µg of genomic DNA in a 50 µL mixture containing: 200 µM dATP, dCTP, dGTP and dTTP; 1.5 mM MgCl2; 10 pmol of each primer (primers used are as reported previously [10]); 10mM Tris-HCl (pH 9.0); 50 mM KCl; 0.1% Triton®X-100; and 5 unit Taq polymerase in Buffer B (Promega, USA). PCR was carried out using PTC-100 Programmable Thermal Controller (MJ Research Inc, USA), and DNA template was initially denatured for 4 min at 94°C, followed by 35 PCR cycles as follows: denaturation for 30 sec at 94°C; annealing for 20 to 30 sec at various temperature ranging between 47 to 66°C depending on the exons (Table 1); and elongation for 30 sec at 72°C. After the last cycle of amplification, elongation was continued at 72°C for another 3 min. One-tenth of the amplified product was diluted with 10 µL of loading buffer consisting of 95% formamide, 10 mM NaOH and 0.05% bromophenol blue. The diluted samples were heat-denatured at 95°C for 10 min, before being placed in ice for 5 min. The samples were then applied to 12% non-denaturing polyacrylamide gel, prepared using MiniPROTEAN® 3 Cell Set (Bio-Rad, USA), with gel size of 8 cm (width) X 7.3 cm (height). The width of gel spacer and comb used was 0.5 mm. The gel was then electrophoresed at 130V for 2 hr at 4°C. The difference in electrophoretic mobility of the samples was detected using PlusOne™ Silver Staining Kit (Amersham Pharmacia Biotech, UK) according to the manufacturer’s recommendations.

Results

PCR-SSCP analysis

Our results showed that for exon 7 of the p53 gene, three HCC cell lines, Alexander, HLE and HLF demonstrated different migration patterns as compared to both normal liver cell lines, Chang and WRL-68 (Figure 1). This observation suggests that the HCC cell lines carry mutated p53 gene at exon 7. As shown in Figure 1, both HLE and HLF cell lines showed similar migration patterns which were distinguishable from that of the Alexander cell line. This implies that
the mutation occurred in the cell lines HLE and HLF was different from the one in the Alexander cell line. The cell lines HepG2 and HuH7, however, had the same migration patterns as normal liver cell lines, thus suggesting there is no mutation present in exon 7 of both the cell lines.

Direct DNA Sequencing

The different electrophoretic mobility between the Alexander cell line and the normal liver cell lines, Chang and WRL-68, in our PCR-SSCP analysis, is attributed to the p53 hotspot mutation of HCC, G:C to T:A transversion at position 3 of codon 249 in exon 7 carried by the Alexander cell line (Figure 4). The disparity shown between the migration pattern of the cell lines HLE and HLF as compared to both of the normal liver cell lines is due to the changes of G:C → C:G at position 2 of codon 249.

The results of PCR-SSCP analysis for exon 6 of the p53 gene in various the human cell lines are shown in Figure 2. All the HCC cell lines, except for HuH7, have the same mobility shift with the presence of a band on the polyacrylamide gel. Both of the normal cell lines also generated the same migration patterns, indicating all the six cell lines had no mutation within the exon 6 of the p53 gene. However, this band was absent in the cell line HuH7, hence, implying the presence of mutation in exon 6 of the cell line.

For exons 3, 5, 8, 9, 10 and 11, the results from our PCR-SSCP analysis showed that the migration patterns for all cell lines are similar (Figure 3, showing the results from PCR-SSCP analysis of exon 5 as an example). These observations suggest that there was no mutation in any of the exons of all the cell lines.
244 of exon 7 in the p53 gene (Figure 5). On top of that, we also established that both HLE and HLF cell lines carried the same mutations, contradicting earlier report showing that HLE cell line carried the G:C → C:G changes at position 3 of codon 249 of exon 7 in the p53 gene [13]. Our sequencing results also showed that the lost of the electrophoretic band in the HuH7 cell line is due to the presence of mutation A:T → G:C at position 2 of codon 220 in exon 6 of the p53 gene (Figure 6). We also sequenced some of the exons of both the HCC and normal liver cell lines which did not show electrophoretic mobility shift by PCR-SSCP analysis, and the results confirmed these exons have wild type p53 sequence (results not shown).

The cell line Alexander was derived from a South African patient, and has been reported to have a G:C to T:A transversion at position 3 of codon 249 of the p53 gene [5, 6]. The different migration pattern using our PCR-SSCP protocols was attributed to the presence of mutations in codon 249 of the p53 gene of the Alexander cell line. Our sequencing result also confirmed the presence of the p53 hotpot mutation in the Alexander cell line.

The cell lines HLE and HLF were derived from two different nodules of a Japanese patient’s liver. Both these cell lines have been reported to carry different mutations in the p53 gene: G:C to C:G transversion, at position 3 of codon 249 in the cell line HLE, and G:C to C:G transversion at position 2 of codon 244 in the cell line HLF [13]. However, analysis using our PCR-SSCP protocol showed that both the HLE and HLF cell lines had the same migration patterns, and this could be due to the identical G:C to C:G transversion. Nevertheless, sequencing results showed that both cell lines actually carry the same mutation, G:C to C:G transversion at position 2 of codon 244 of the p53 gene.

We also confirmed that the samples which did not show presence of mutation using PCR-SSCP analyses, indeed have wild type sequences in the respective exons of the p53 gene. On top of that, we have demonstrated that the cell lines Alexander, HLE, HLF and HuH7 could be used as positive controls in screening for p53 mutations in human HCC samples using PCR-SSCP analysis.

In conclusion, our results showed that the PCR-SSCP analysis using a mini gel electrophoresis system in combination with silver staining was able to detect different types of p53 mutations in the HCC cell lines, besides not detecting any false positive. This optimised non-radioactive PCR-SSCP technique will enable rapid and efficient screening for mutations in the p53 gene of HCC patients.

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**References**