RESEARCH COMMUNICATION

Novel PNLIPRP3 and DOCK8 Gene Expression and Prognostic Implications of DNA Loss on Chromosome 10q25.3 in Hepatocellular Carcinoma

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Abstract

Our previous study of gene alterations in 29 hepatocellular carcinoma (HCC) using AP-PCR amplified with 59 different 10-mer arbitrary primers and gene cloning, indicated DNA alterations by DNA fingerprints from 34 primers. Among these, the altered DNA fragment from primer U-8 predominated (62%). The aim of this report is to identify the gene alterations on chromosomal banding and gene expression in these patients, including the association of these alterations with patient demographic data. Seven different sequences, mapped to chromosomes 5q33.3, 7q31.33, 7q34, 9p24.3, 10q25.3, 13q31.3, and 16p11.2, were identified by gene cloning and nucleotide sequencing. Novel PNLIPRP3 gene over-expression and DOCK8 gene under-expression were observed in 41% and 44% of these patients, respectively, which point to an association of these genes and the development of HCC. Likewise, allelic loss on chromosome 10q25.3 was associated with shorter survival among HCC patients (P=0.03); this indicated that allelic loss on chromosome 10q25.3 may serve as a prognostic marker in patients with HCC.

Key Words: Genetic alterations - AP-PCR - 10q25.3 - HCC - prognostic factor

Introduction

Hepatocellular carcinoma (HCC) arises from hepatocytes and accounts for 80% of all primary liver cancers. HCC is almost always lethal, survival from time of diagnosis often being less than six months; only 10% of patients survive 5 years or more (Khuhaprema et al., 2007). The development of hepatocellular carcinoma is associated with chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), chronic exposure to the mycotoxin aflatoxin B1, and excessive alcohol intake (Buendia, 2000). Recently, certain genetic alterations have been associated with HCC, including mutations of p53, Rb1, beta-catenin, IGF2R, SMAD2/4 (Nishida et al., 1993; Zhang et al., 1994; De Souza et al., 1995; de La Coste et al., 1998; Yakiciier et al., 1999; Zhu et al., 2004; Elmileik et al., 2005), DNA amplification and over-expression of cyclin D1 gene (Nishida et al., 1994) and epigenetic changes in p16 gene hypermethylation (Fukai et al., 2005; Hsu et al., 2006). Gene gains on chromosomes 8q, 1q, 6p, and 17q, and allelic losses on chromosomes 4q, 8p, 16q, 17p, 13q, and 6q, have also been found in HCC (Marchio et al., 1997). However, the molecular mechanism of hepatocarcinogenesis remains largely unknown.

In the current study, the 2,000 bp amplified tumor fragment from primer U-8 was selected for cloning and sequencing. Quantitative real-time polymerase chain reaction was used to quantify copy-number alterations in HCC. The mRNA expression levels of DOCK8 (dedicator of cytokinesis 8) and PNLIPRP3 (pancreatic lipase-related protein 3), located on chromosomes 9p24.3 and 10q25.3, respectively, were also analyzed by real-time reverse transcription-PCR. The correlation of the genetic alterations and gene expression level with clinic-pathological features and overall survival of patients, were then analyzed.

Materials and Methods

Tumor specimens

Twenty-nine paired samples of HCC and normal liver tissues were collected from Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. This research was approved by the Ethics Committee, Faculty...
of Medicine, Khon Kaen University (HE471214). No therapeutic treatment (chemotherapy or radiation) was instituted before the operation. All tissues samples were frozen in liquid nitrogen at -80°C for DNA and RNA extraction. Hematoxylin & eosin-stained samples from each tumor block were examined microscopically. Differential grading was performed according to Edmonson and Steiner (1954), with classification into 3 groups; well differentiated (grade I), moderately differentiated (grades II-III), and poorly differentiated (grade IV). Tissues with > 80% tumor cells and corresponding normal liver tissues from the same patients were used in this study.

DNA extraction
Genomic DNA was isolated by Proteinase K digestion and salting-out method (Miller et al., 1988). DNA was loaded in agarose gel electrophoresis, and stained with ethidium bromide to check purity.

RNA isolation and complementary DNA synthesis
Total RNA was extracted from 22 HCCs and their corresponding normal liver tissues using Trizol reagent, according to the instruction manual (Invitrogen, Carlsbad, CA, USA). mRNA was isolated by Oligotex mRNA purification kit (Qiagen, Gmbh, Germany). Reverse transcription reactions were conducted according to the manufacturer’s instructions using the SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (Invitrogen, Carlsbad, CA, USA).

AP-PCR analysis
The DNA fingerprinting of tumor amplified from primer U-8 was performed according to methods previously described (Saelee et al., 2008). The reactions were performed in a final volume of 25 ml, containing 100 ng genomic DNA, 1x PCR buffer, 0.2 mM of each dNTP, 2.5 mM MgCl₂, 0.4µM U-8 primer (5'-GGC GAA GGT T-3') and 1 unit of Taq DNA polymerase (Amersham Pharmacia Biotech, USA). PCR amplification was carried out in a Perkin-Elmer 97˚C GeneAmp PCR system for 45 cycles of denaturation at 95˚C for 1 min, followed by annealing at 36˚C for 1 min, and extension at 72˚C for 2 min. Twenty-five microliters of PCR product were electrophoresed in 1.4% agarose gel, stained with ethidium bromide, and photographed under UV light.

Cloning and sequencing
The 2,000 bp DNA band amplified in tumor DNA (Figure 1) was eluted from agarose gel, purified and cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). The recombinant plasmid isolated from each clone was restriction-digested by EcoR I restriction enzyme. Plasmid DNA containing the insert-fragment was sequenced using forward or reverse M13 sequencing primer (Macrogen Inc., Korea).

Bioinformatics analysis
Information on the DNA sequences in each clone were identified by comparison with known human nucleotide sequences in the Genome database using the BLAST program at www.ncbi.nlm.nih.gov; these DNA sequences were also tested for DNA repeat sequences by using the RepeatMasker program at www.repeatmasker.org.

Quantitative real-time PCR
To detect DNA variations on chromosomes 5q33.3, 7q31.33, 7q34, 9p24.3, 10q25.3, 13q31.3, and 16p11.2 in HCC tumors, sequence-specific primers were designed using the Primer 3 program; the sequences are shown in Table 1 (Bioservice, Bangkok, Thailand). All reactions were amplified by AP-PCR. Amplification bands showed in cases 15, 16, 18, 20, and 21. An arrow indicates an altered band of 2,000 bp (bp = base pair, M = GeneRuler™ 100 bp DNA Ladder Plus).

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Sequence (5’-3’)</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5q33.3</td>
<td>F CGA GAT TGA GCC ATT GCA</td>
<td>380</td>
</tr>
<tr>
<td></td>
<td>R AAG GTT ATG TAG ATG CCT GA</td>
<td></td>
</tr>
<tr>
<td>7q31.33</td>
<td>F AGA GGA GAC AGT AGT TGA GA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R CAC ACC AAA TCC ATG AGG A</td>
<td>196</td>
</tr>
<tr>
<td>7q34</td>
<td>F GAG GAA GAA ATG GAG GGT AAC TG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R CCT CAG TCA TTT CCT TGA TTG AC</td>
<td>174</td>
</tr>
<tr>
<td>9p24.3</td>
<td>F GAG GAT ATT GGA AAC AAG CAC AC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R GTG TGA AGC TTA GCA ACC AGA AC</td>
<td>181</td>
</tr>
<tr>
<td>10q25.3</td>
<td>F AAG GTA CAC TGA GCT GGT A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R TGA ATC TCT TGT AGA CAG CA</td>
<td>200</td>
</tr>
<tr>
<td>13q31.3</td>
<td>F ACT GCG ACA GTC TTA GTG AAA GC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R ATG TAT GCA AGG GTC AGA TCA AG</td>
<td>222</td>
</tr>
<tr>
<td>16p11.2</td>
<td>F GCT TAG CTT TTC TTA GAG CA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R CTG AGG CTA AGA ATG TCC A</td>
<td>199</td>
</tr>
</tbody>
</table>

F, Forward; R, Reverse

Figure 1. AP-PCR analysis of HCC with primer U-8. Corresponding normal DNA (N) and tumor DNA (T) were amplified by AP-PCR. Amplification bands showed in cases 15, 16, 18, 20, and 21. An arrow indicates an altered band of 2,000 bp (bp = base pair, M = GeneRuler™ 100 bp DNA Ladder Plus).

Figure 2. Amplification Plots on Chromosome 10q25.3 for DNA loss. SYBR Green I Fluorescence signal versus cycle number of specific primer on chromosome 10q25.3 and single copy gene of α-globin was used as reference control in tumor DNA and corresponding normal DNA.
were carried out in a total volume of 10 µl/capillary. Each reaction mixture contained 20 ng of template DNA, 1x LightCycler FastStart DNA Master SYBR Green I (contains Taq DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP) and 1 mM MgCl₂), 4 mM MgCl₂, 0.5 mM sense and antisense strand primers. The PCR amplifications were performed in a LightCycler Instrument (Roche Applied Science, Germany). The touchdown PCR was then started at 95°C for 10 min, followed by 50-cycle amplification, consisting of 10 s at 95°C, annealing for 5 s at temperatures decreasing from 68 to 58°C (5q34), at 70 to 66°C (7q31.33), at 70 to 62°C (7q34, 9p24.3, 13q31.3, 16p11.2) and at 68 to 58°C (10q25.3) during the first 15 cycles (with 1°C decremental steps in cycles 2 to 15) and 10 s at 72°C. An α-globin gene was used as the reference DNA control (Figure 2). Each amplification reaction was checked for the absence of nonspecific PCR products by melting curve analysis and agarose-gel electrophoresis. The comparative Ct (threshold cycle) method was used to determine gene expression levels. Tumor gene expression <0.8-fold was assigned as under-expression for DOCK8, whereas, gene expression >1.4-fold was over-expression for PNLIPRP3.

### Statistical analysis

The correlation of DNA alterations on 7 different chromosomes (5q33.3, 7q31.33, 7q34, 9p24.3, 10q25.3, 13q31.3, and 16p11.2), and DOCK8 & PNLIPRP3 expression levels with clinic-pathological characteristics-age, gender, tumor differentiation, tumor size, metastasis, treatment, serum HBsAg and anti-HCV--were analyzed by χ²-test or Fisher’s exact test. Survival analysis was analyzed for patients followed up for at least 200 weeks, or until death, post-surgery. One patient who died within 4 weeks, and one patient who was lost to follow-up, were excluded. Thus, only 27 patients were available for survival analysis.

Univariate analysis of Kaplan-Meier survival curve and log-rank tests were used to analyze overall survival. Multivariate analysis by Cox regression model was used to assess which prognostic factor(s) from univariate analysis significantly influenced on overall survival—tumor size, metastasis, treatment, genetic alterations, and gene expression level. A correlation was considered significant with a P value <0.05.

### Results

**AP-PCR and quantitative real-time PCR analysis**

Seven different sequences, mapped to chromosomes 5q33.3, 7q31.33, 7q34, 9p24.3, 10q25.3, 13q31.3, and 16p11.2, were revealed from gene cloning and nucleotide sequencing. Quantitative real-time PCR was used to validate genetic alterations on these chromosome sequences. The results showed that 6 (21%), 9 (31%), 8 (28%), 4 (14%), 5 (17%), 5 (17%), and 8 cases (28%), respectively, harbored DNA amplification. Meanwhile, 5 (17%), 5 (17%), 7 (24%), 10 (35%), 7 (24%), 6 (21%), and 10 cases (35%) exhibited DNA loss on these chromosomes, respectively. DNA alterations were observed on one or more chromosomes in 27 of 29 cases (93%).

**Bioinformatics analysis**

Information about the nucleotide sequences from the 7 different clones was retrieved from the BLAST program (www.ncbi.nlm.nih.gov). The first clone mapped to chromosome 5q33.3 (NW 922784.1), located about 60,745 bp upstream of T-cell immunoglobulin and mucin domain containing 4 (TIMD4) and 5,241 bp downstream of hepatitis A virus cellular receptor 1 (HAVCR1). The second clone mapped to chromosome 7q31.33 (NW 923640.1), located about 195,331 bp upstream of hypothetical protein LOC730130 and 248,674 bp upsteam of hypothetical protein LOC730130.
downstream of hypothetical protein LOC136157. The third clone mapped to chromosome 7q34 (NW 923651.1), located about 13,012 bp upstream of glutathione s-transferase kappa 1 (GSTK1) and 3,228 bp downstream of transmembrane protein 139 (TMEM139). The fourth clone mapped to chromosome 9p24.3 (NW 924062.1), located in intron 3 of dedicator of cytokinesis 8 (DOCK8). The fifth clone mapped to chromosome 10q25.3 (NW 924884.1), located in intron 4 of pancreatic lipase-related protein 3 (PNLIPRP3). The sixth clone mapped to chromosome 13q31.3 (NW 925517.1), located about 2,225,565 bp upstream of SLIT and NTRK-like family, member 5 (SLITRK5) and 1,500,192 bp downstream of glypican 5 (GPC5). The seventh clone mapped to chromosome 16p11.2 (NW 926362.1), located about 61,189 bp upstream of similar to kinase suppressor of ras 1 (LOC645146) and 66,910 bp downstream of similar to kinase suppressor of ras 1 (LOC645146) and 66,910 bp downstream of similar to kinase suppressor of ras 1 (LOC645146).

These fragments were tested for the presence of DNA repeat sequences by the RepeatMasker program (www.repeatmasker.org.). The repeat sequences on chromosomes 5q33.3, 7q31.33, 7q34, 9p24.3, 10q25.3, and 16p11.2 were similar to ALUs (22%), LINE1 (11%), ALUs (58%), ALUs (20%), LINE1 (68%) and ALUs (45%), respectively. No repeat sequence was found on chromosome 13q31.3.

PNLIPRP3 and DOCK8 expression level
The DNA alterations on chromosomes 9p24.3 and 10q25.3 mapped to intron 3 of the DOCK8 and intron 4 of the PNLIPRP3 gene, respectively. These gene expressions were determined by real-time reverse transcription-PCR; 8 of 18 (44%) cases presented DOCK8 under-expression. Whereas 9 of 22 cases (41%) exhibited PNLIPRP3 over-expression.

Statistical analysis
The associations between these DNA alterations on 7 different chromosomes, 5q33.3, 7q31.33, 7q34, 9p24.3, 10q25.3, and 16p11.2, and PHLIPRP3 over-expression, including DOCK8 under-expression, and the demographic data of these patients were analyzed statistically. There were no significant correlations between DNA alterations on these chromosomal regions and clinico-pathological data (see Tables 2 and 3). The PNLIPRP3 gene was significantly over-expressed in the chemotherapeutic treatment group (Table 2). Survival analysis, based on of Kaplan-Meier log-rank tests and multivariate Cox regression analysis-- for tumor size, metastasis, treatment, allelic loss, and gene expression--showed DNA loss on chromosome 10q25.3 to be an independent prognostic factor for poor survival among...
Prognostic Implications of DNA Loss on Chromosome 10q25.3 in Hepatocellular Carcinoma

HCC patients (median survival time 14 wks vs 103 wks) with P = 0.03, risk ratio =8.26, 95% CI = 1.21-56.38, as shown in Figure 3. Associations of PNLIPRP3 over-expression and DOCK8 under-expression and survival time were not statistically significant (see Table 4).

Discussion

In the current study, we used AP-PCR, a PCR-based DNA fingerprinting technique first developed by Welsh and McClelland (1990), to investigate genetic changes in HCC. DNA fingerprinting generates an information-rich and unbiased fingerprint of genomic DNA, without the necessity for nucleotide sequence information (Welsh et al., 1990). This technique has been widely used for detecting genetic changes, including DNA rearrangement, DNA addition or deletion and ploidy changes in cells (Welsh et al., 1995).

Genetic alterations detected by this method have been identified in various cancers, including breast cancer (Singh et al., 2001; Paketoot et al., 2007), colorectal and pancreatic carcinomas (Peinado et al., 1992; Achille et al., 1996), lung cancer (Kawakami et al., 1998), ovarian cancer (Pongstaporn et al., 2006), cholangiocarcinoma (Chariyalertsak et al., 2005; Chuensumran et al., 2007), as well as in HCC (Xian et al., 2005; Saelee et al., 2008).

Several reports have shown DNA loss on chromosomes 10q in many malignancies, e.g., allelic loss on chromosome 10q21–q24 and 10q25.2–q26.3 has been reported in endometrial cancers (Peiffer et al., 1995). Small chromosome deletions in 10q25.1 have been found in medulloblastomas (Rossi et al., 2006). Loss of heterogeneity (LOH) on chromosome 10q25-25.3 has been studied in advanced prostate cancer (Chu et al., 2003) and on chromosome 10q in HCC (Piao et al., 1998; Fujiwara et al., 2000). This report identified allelic loss on chromosome 10q25.3 from one HCC patient with a worse HCC prognosis, which indicated that the gene located in this chromosomal region might be associated with the development of HCC and clinical manifestations among patients.

The gene expression of the PNLIPRP3 gene located in this region was further analyzed since the nucleotide sequences identified on chromosome 10q25.3 mapped on intron 4 of this gene. Interestingly, PNLIPRP3 over-expression was significant in the chemotherapeutic patient group. Functions of this gene have been proposed to participate in a lipid metabolic process. Proteins are expected to have molecular functions in catalytic activity and triacylglycerol lipase activity, and to localize extracellularly. This may be the first report of a novel PNLIPRP3 over-expression in HCC. Although PNLIPRP3 over-expression in HCC was significant in the chemotherapeutic patient group, the therapeutic function of this gene for HCC patients should be studied further.

We further analyzed the expression level of DOCK8, which mapped to chromosome 9p24.3. No correlation was found between DOCK8 expression level and clinicopathological data or survival analysis. The DOCK family of proteins has been shown to play a role in regulating the migration, morphology, adhesion, and growth of cells (Takahashi et al., 2006). It has been reported that the DOCK8 gene is associated with the progression of low-grade glioma (Idbaih et al., 2008). A homozygous deletion of DOCK8 was found in a lung-cancer cell line, and DOCK8 down-regulation was found to be involved in human lung carcinogenesis (Takahashi et al., 2006). This is the first research of DOCK8 expression in HCC. The results suggest that the function of these genes in HCC and other cancers should be studied further.

In conclusion, genetic alterations in Thai HCC patients were identified by AP-PCR. This study showed for the first time that allelic loss on chromosome 10q25.3 may be associated with hepatocellular carcinogenesis, and could be serve as a prognostic or therapeutic target for HCC patients with poor survival prognoses. Moreover, the altered mRNA expression level of DOCK8 and PNLIPRP3 may be involved with the pathogenesis of HCC.

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References


Pensri Saelee et al


506