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• ORIGINAL RESEARCH •

Expression of liver cancer associated gene HCCA3

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Abstract

AIM: **To study and clone a novel liver cancer related gene, and to explore the molecular basis of liver cancer genesis.**

METHODS: **Using mRNA differential display polymerase chain reaction (DDPCR), we investigated the difference of mRNA in human hepatocellular carcinoma (HCC) and paired surrounding liver tissues, and got a gene probe. By screening a human placenta cDNA library and genomic homologous extend, we obtained a full-length cDNA named HCCA3. We analyzed the expression of this novel gene in 42 pairs of HCC and the surrounding liver tissues, and distribution in human normal tissues by means of Northern blot assay.**

RESULTS: **A full-length cDNA of liver cancer associated gene HCCA3 has been submitted to the GeneBank nucleotide sequence databases (Accession No. AF276707). The positive expression rate of this gene was 78.6% (33/42) in HCC tissues, and the clinical pathological data showed that the HCCA3 was closely associated with the invasion of tumor capsule ($P = 0.023$) and adjacant small metastasis satellite nodules lesions ($P = 0.041$). The HCCA3 was widely distributed in the human normal tissues, which was intensively expressed in lungs, brain and colon tissues, while lowly expressed in the liver tissues.**

CONCLUSION: **A novel full-length cDNA was cloned and differentiated, which was highly expressed in liver cancer tissues. The high expression was closely related to the tumor invasiveness and metastasis, that may be the late heredited change in HCC genesis.**

Subject headings Carcinoma, hepatocellular/genetics; DNA, Complementary/genetics; Liver neoplasms/genetics; RNA, messenger/genetics; Gene expression; Polymerase chain reaction

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INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most common fatal malignant tumors in China[1-26]. According to the statistics of our country, primary liver cancer claims 20.40 lives per 100 000 people annually, with 19.98 per 100 000 in cities and 23.59 per 100 000 in rural areas, ranking as the 2nd and the 1st leading cause of cancer death, respectively. Of all the newly enrolled cases in the world each year, 45% are found in the mainland of China. In the southeast areas of high incidence, the situation is even worse with tumors tending to occur in a younger age group. The molecular events for HCC development are very complex, and HCC has proved to be genetically heterogenous neoplasm[27-30]. But to date, the identified genes have not yet fully disclosed the mechanisms of HCC[31-38]. In an attempt to identify HCC susceptible genes, differential display method was employed in this study. In the analysis of altered expression genes between HCC tissues and their nontumor counterparts, we isolated a novel gene named HCCA3 with a full length of cDNA.

MATERIALS AND METHODS

Materials

PCR polymerase is a product of Promega (Madison, USA); isotope α-^{32}P-dATP was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL); Qiaex II gel extraction kit were from Qiagen (Hilden, Germany); pGEM-T vector were from Promega; human placental cDNA library were from Clontech (USA); nitrocellulose were from Amersham Pharmacia Biotech; Prime-a-Gene Labeling System were from Promega; 14-mer anchored oligo (dT) primer (dT_{12}CA) and an arbitrary 10-base olignucleotide A2 (5'-AATCGGGCTG-3') were donated by Professor Pei.

Patients and specimens

Primary HCC and their surrounding liver tissues were obtained from 42 patients who received surgical resection at the Eastern Hepatobilliary Surgical Hospital of the Second Military Medical University, Shanghai, China. These included 41 male and 1 female patients with a median age of 49 years (range 24-72 years, mean age of 49.8 years). Thirty-five (83.3%) patients had serological evidence of hepatitis B virus infection. The serum AFP level was above 25 $\mu g \cdot L^{-1}$ in 23 cases (54.8%). The tumor size was smaller than 5cm (small HCC) in 13 patients and larger than 5 cm in 29. Histologically, 40 patients (95.2%) were complicated with cirrhosis. There were 7 well differentiated (Edmondson's grades I and II) and 35 poorly differentiated (Edmondson's grades III and IV) HCCs. Macroscopic portal vein tumor spread was found in 3 patients, and microscopic surrounding liver vascular cancer thrombi were found in 26. Gross and microscopic intrahepatic adjacent small satellite nodules lesions were found in 28, and tumor capsule invasion of liver cancer in 32. Adult normal tissues were obtained from a healthy young man who died of a traffic accident.

Methods

RNA extraction and differential display Total cytoplasmatic RNA was extracted by the acid guanidinium thiocyanate-phenol - chloroform extraction methods[39, 40]. The differential display method

was perf ormed as described previously[39-41,42]. Amplification consisted of initial den aturation at 94℃ for 4 min, followed by 40 reaction cycles (60 s at 93℃, 2 min at 40℃, and 90 s at 70℃) and a final cycle at 72℃ for 10 min. PCR fragments were then reamplified by the same primer, separated on a 16 g·L^{-1} agarose gel, purified by Qiaex II gel extraction kit, and subcloned into the pGEM -T vector using standard molecular cloning techniques.

Library screening and DNA sequencing Fragment contained in the PCR clone (length in 350 base pairs) from DDPCR served as probe to screen placental cDNA library, using the standard filter hybridization techniques described[43,44]. At the end of the third screening, we got several plagues containing the target DNA sequence and sequenced them by DNA automated sequencing system. To obtain the cDNA in a full length, genomic homologous screening was used through comparing the cDNA sequence obtained by screening the library with the NCBI GeneBank EST database. We used the PCR assay and sequencing to confirm the correct ness of the cDNA sequence.

Northern blot assay For Northern blot analysis[37,40,44], 40 μg of total RNA was denatured, loaded on a 15 g·L^{-1} agarose gel and ran at 5 V·cm^{-1} for about 3 h. The collected gels were then transferred to nitrocellulose. Hybridization of the filters was performed using specific probe of HCCA3 cDNA fragment (length in 1125 bp) obtained from screening the library. The probe was labeled with 50 μCi A-^{32}P-dATP using Prime-a-Gene labeling kit according to the given protocol. After prehybridization at 42℃ for 3 h, the membranes were hybridized in the same solution containing the labeled probe for 6 h at the same temperature, and exposed to X-ray film for 10 d at-70℃. In order to calibrate relative quantities of loaded RNAs, the blot was rehybridized with a cDNA probe of the â-Actin gene.

Statistical analysis

χ^2 test or Fisher's exact test was used to examine the differences and relationship among groups of patients classified by HCCA3 expression. Differences at $P<0.05$ were judged to be statistically significant.

RESULTS

Differential display analysis and library screening

By DDPCR, we found a differentially expressed gene fragment that exclusively present in the liver cancer lane. This fragment (length in 350 bp) was then subcloned into pGEM-T vector and served as specific probe to screen human placental cDNA library. We have o btained the gene fragment of 1125 bp in length, which shortened nearly 600 bp according to the location of Northern hybridization by screening the library. We also obtained the full-length cDNA of 1706 bp, which was in good agreement with the size of the mRNA species observed by Northern blotting through genomic h omologous extend, along with the EST sequence (GeneBank Accession No. AP001077, length in 197663 bp) of NCBI GeneBank EST databases. The sequence of 1706 bp in length was corrected by PCR assay and sequencing. It was named HCCA3 (HCC associated gene 3, also named STW-2) and submitted to EMBL/GenBank/DDBJ nucleotide sequence databases (Accession No. AF27 6707).

Sequence characteristics of the full-length HCCA3

HCCA3 contains a consensus initiation codon[45,46] at position 681 followed by a single open reading frame of 792 bp encoding 264 amino acids. The 3' untranslated region of 230 bp had a consensus polyadenylation signal (AATAAA) beginning 16 bases upstream the poly (A) tail. Alignment at nucleotide and amino acid level showed no significant homologues with known genes. The deduced protein was estimated to be 29 396 dalton and has a pI of 6.93. Amino acid sequence analysis by GCG sequence analysis software package (version 9.1, Genetics Computer Group, Madison, Wisconsin) and PC/GENE (Version 5.03, Geneva University, Switzerland) showed that there were several putative modification sites. These include two N-glycosylation sites at amino acid of 40 and 67, three N-myristoylation sites at amino acid of 183, 185 and 258, five phosphorylation sites for protein kinase: two protein kinase C (PKC) phosphorylation sites at 82 and 250, three cases in kinase II phosphorylation sites at 8, 189 and 209. No transmembrane domain and signal peptide was found. Further·more, there was no nuclear targeting sequence. The schematic presentation of HCCA3 cDNA is shown in Figure 1.

Normal tissue distribution of HCCA3 Mrna

Northern blot analysis showed that HCCA3 mRNA appears to be widely expressed in human normal tissues (Figure 2). The HCCA3 gene was particularly highly expressed in human lungs, brain and colon, moderately expressed in muscle, stomach, spleen and heart tissues, weakly expressed in small intestines, pancreas, and liver tissues. Among the tissues with positive signals, HCCA3 mRNA was observed in a transcript of approximately 1.7 kb which well corresponded to the size of the cloned cDNA.

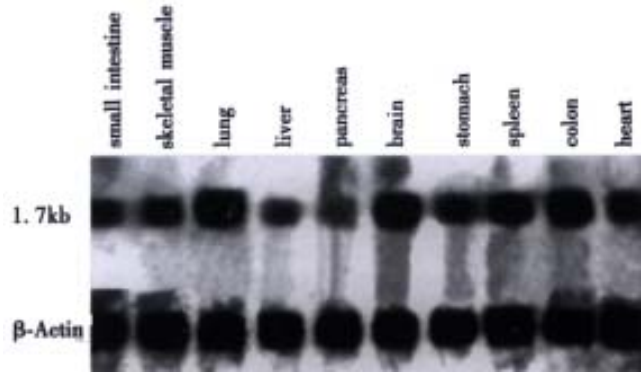

Figure 2 Northern blot analysis of HCCA3 mRNA in human adult normal tissues. Upper panel showed HCCA3 mRNA was highly expressed in human lungs, brain and colon, moderately expressed in muscle, stomach, spleen and heart tissues, weakly expressed in small intestines, pancreas, and liver tissues. Equal a mounts of total RNA loading as indicated by rehybridizing with (-actin cDNA probe (lower panel).

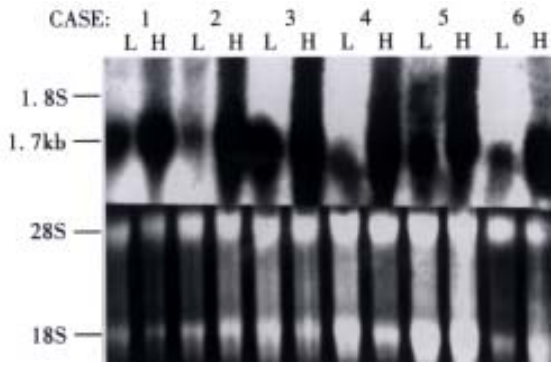

Figure 3 Differential expression of HCCA3 analyzed by Northern b lot. Upper panel shows that HCCA3 mRNA was highly expressed in HCC tissues (H), and lowly in paired surrounding liver tissues (L). A signal transcript of 1.7 kb is shown in six tumors. 28S and 18S rRNAs were used for evaluating the quality and quantity of RNA loading (lower panel).

HCCA3 mRNA expression in HCC and its clinical significance

HCCA3 mRNA expression was noticed in 78.6% (33/42) patients, which was intensively expressed in HCC tissues (Figure 3), while lowly expressed in the surrounding liver tissues. To investigate the potential biological role of HCCA3 in the development of HCC, we further investigated its expression in HCC with extensive samples and compared with the clinically pathological parameters. The mRNA expression level of HCCA3 was associated with the invasion of liver cancer capsule and the adjacant small satellite nodules lesions ($P< 0.05$), but not with tumor size, tumor differentiation, serum AFP, hepatitis B vrius (HBV) infection and microscopic vascular cancer cell thrombi ($P>0.05$). The results are listed in Table 1.

```
                                                                    35
GGCAGGAGGGAAAAGGTTATGGGTCGGCCCGGCGGT
CTCTCCCAGCACCCGCGACTCTCACCTTGCTCAGCTGCTGGTGGA                       80
TGAGCTGCTTCAGCTCGGAGGCTTTCTCCGGAGGCAGCGACATGC                      125
TGGCAGCCGGCGTCTCCCCGCCGCTTCTCCCCGCCTCAGATGCCC                      170
TAACTGCGCGGCCCCAGCCGGGCCAGGGAGCGTTAGGAGCGACTG                      215
GAGCACAAAGCGCCGCAGCCGTTCGCCTAGCGCAGCTCCCGGGGG                      260
ACGCAACGCCGCGTCAGGCCGGGGGGCTGACCTGGAAATGAGGCCC                      305
CGGCGGCGGCGGCGCCAACTGTTTTCAAACAGTGGCGGACAAACA                      350
GGGCTTGGGGCTGGCCCGCACGCTGCCTGATCGTTTCCGCCCGCC                      395
GCTCCACCTCCCCGCGGGCCCCGCACCCCGAGACCTCAGCGCACC                      440
CCATCGCCTTGCTTGCCAGGGTCTCCGAAAGCGCTGCTGGCCCCT                      485
CTTCGCGGCCACCCGGCGGCCTCTTTCCGCCCTCTGAACCGGCAG                      530
TTAGCTGGCAGCCTCAAGGGCCCGGCGCCCAGGGACTCAAAGGAC                      575
CCTCCCGCGCCCCGCGAGGCTCCGGGGGTCTCGGGCTTCCGCCTTC                     620
TTGCTGCCCTCGTTCTTGCCAGGGCCGCGGTTAGTCCCTGCTGGC                      665
CACCCCACTGCGACC[ATG]TTCGTTCCCTGCGGGGAGTCGGCCCCC                    710

1                                   M   F   V   P   C   G   E   S   A   P
GACCTTGCCGGCTTCACCCTCCTAATGCCAGCAGTATCTGTTGGA                      755
11  D   L   A   G   F   T   L   L   M   P   A   V   S   V   G
AATGTTGGCCAGCTTGCAATGGATCTGATTATTTCTACACTGAAT                      800
26  N   V   G   Q   L   A   M   D   L   I   I   S   T   L   N
AATGTTGGCCAGCTTGCAATGGATCTGATTATTTCTACACTGAAT                      800
41  M   S   K   I   G   Y   F   Y   T   D   C   L   V   P   M
GTTGGAAACAATCCATATGCGACCACAGAAGGAAATTCAACAGAA                      890
56  V   G   N   N   P   Y   A   T   T   E   G   N   S   T   E
CTTAGCATAAATGCTGAAGTGTATTCATTGCCTTCAAGAAAGCTG                      935
71  L   S   I   N   A   E   V   Y   S   L   P   S   R   K   L
GTGGCTCTACAGTTAAGATCCATTTTTATTAAGTATAAATCAAAG                      980
86  V   A   L   Q   L   R   S   I   F   I   K   Y   K   S   K
CCATTCTGTGAAAAACTGCTTTCCTGGGTGAAAAGCAGTGGCTGT                     1025
101 P   F   C   E   K   L   L   S   W   V   K   S   S   G   C
GCCAGAGTCATTGTTCTTTCAAGCAGTCATTCATATCAGCGTAAT                     1070
116 A   R   V   I   V   L   S   S   S   H   S   Y   Q   R   N
GATCTGCAGCTTCGTAGTACTCCCTTCCGGTACCTACTTACACCT                     1115
131 D   L   Q   L   R   S   T   P   F   R   Y   L   L   T   P
TCCATGCAAAAAAAGTGTTCAAAATAAAATAAAGAGCCTTAAGTGG                    1160
146 S   M   Q   K   S   V   Q   N   K   I   K   S   L   N   W
GAAGAAATGGAAAAAAAGCCGGTGCATTCCTGAAATAGATGATTCC                    1205
161 E   E   M   E   K   S   R   C   I   P   E   I   D   D   S
GAGTTTTGTATCCGCATTCCGGGAGGAGGTATCACAAAAACACTC                     1250
176 E   F   C   I   R   I   P   G   G   G   I   T   K   T   L
TATGATGAAAGCTGTTCTAAAGAAATCCAAATGGCAGTTCTGCTG                     1295
191 Y   D   E   S   C   S   K   E   I   Q   M   A   V   L   L
AAATTTGTTTCAGAAGGGGACAACATCCCAGATGCATTAGGTCTT                     1340
206 K   F   V   S   E   G   D   N   I   P   D   A   L   G   L
GTTGAGTATCTTAATGAGTGGCTTCAGATACTCAAACCACTTAGC                     1385
221 V   E   Y   L   N   E   W   L   Q   I   L   K   P   L   S
GATGACCCCACAGTATCTGCCTCACGGTGGAAAATACCAAGTTCT                     1430
236 D   D   P   T   V   S   A   S   R   W   K   I   P   S   S
TGGAGATTACTCTTTGGCAGTGGTCTTCCCCCTGCACTTTTCTGA                     1475
251 W   R   L   L   F   G   S   G   L   P   P   A   L   F   *
TCTAATTTCTGTTTTATACCTTATACCCAAAAACACTTACTACCAA                    1520
CACAGCTGTTAAACATTCTATACAAAAAAAATTGTATGATCTGGTA                    1565
TTAGGAAATTACTTTCACAGTAAATATCAAAGAAAAAAAGATTAAG                    1610
GGTCTCTTTGCCATGCTTTTCATCATATGCACCAAATGTAAATTT                     1655
TGTACAATAAAAATTTTATTTCCTAAGTAAAAAAAAAAAAAAAAAAAAAA                1706
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Figure 1 Nucleotide sequence and predicted amino acid sequence of HCCA3. HCCA3 nucleotide sequence (1706 bp) and deduced amino acid sequence (264 amino acid). The franking sequence of the predicted initiating methionine which satisfied the Kozak criterion is shown in the box. A consensus polyadenylation signal sequence (AATAAA) be ginning 16 bases upstreams the poly(A) tail shown in black line. The asterisk indicates the stop codon. EMBL / GenBank™ / DDBJ accession number is AF276707.

Table 1 Relationship between HCCA3 mRNA expression and clinic opathological features in HCC

Clinicopathology	*n*	Positive *n* (%)
Tumor size (cm)		
<5	10	6 (60.0)
>5	32	27 (84.4)
Tumor differentiation		
Well	7	4 (57.1)
Poor	35	29 (82.9)
Serum AFP ($\mu g \cdot L^{-1}$)		
⩽25	>19	15 (79.0)
>25	23	18 (78.3)
HBV infection		
Positive	35	28 (80.0)
Negative	7	5 (71.4)
Capsule invasion		
Positive	32	28 (87.5)
Negative	10	5 (50.0)[a]
Cancer thrombi		
Positive	26	21 (76.9)
Negative	16	12 (82.4)
Satellite lesions		
Positive	28	25 (89.3)
Negative	14	8 (57.1)[a]

[a]$P<0.05$, *vs* positive.

DISCUSSION

Differential display polymerase chain reaction (DDPCR) method is a useful tool for detecting and characterizing altered gene expression in eukaryotic cells[39,41,47,48]. Using this technique, we have successfully isolated a gene named HCCA3 with a transcript of 1706 bp. A consensus initia tion coden is at 681 bp and the franking sequence of the predicted initiating me thionine coincides with Kozak[45,46,49] criterion because the nucleot ides at -3, +4 site of start codon are purine, and there is one stop codon at the upstream sequence. The length of HCCA3 cDNA agrees with the size of mRNA by Northern b lot analysis. No significant homologues with known genes at nucleotide and amino acid levels were found[47-50]. No signal peptides searched by GCG and PC/GENE software were found, suggesting that HCCA3 was not a secretive protein[51,52]. This protein also has no transmembrane domain and nuclear targeting sequence indicating that it may not be located on cell member or within nucleus. The putative protein of HCCA3 revealed several phosphorylation sites for protein kinase C and casein kinase II. It is generally accepted that protein phosphorylation-deph osphorylation plays a role in the regulation of essentially all cellular functions, and there is evidence that deregulation of protein phosphorylation is involved in several human cancers[22,53,54]. HCCA2 as a possible oncoprotein may be functionally abnormal, and phosphorylative deregulation may be a mechanism. The function of HCCA3 protein may also largely depend on its phosphorylation status[22,54]. However, this needs further studies.

Studies on the expression of HCCA3 can reveal its potential biological significance[22,36,37,55]. By Northern blot analysis, we noted that HCCA3 mRNA was expressed widely in normal human tissues, indicating that HCCA3 is a normal cellular gene which may be involved in the physical process of the distributed tissues. Although HCCA3 mRNA was lowly expressed in normal liver tissues, it was significantly expressed in HCC tissues, showing that the high expression of HCCA3 might participate in the process of liver on cogenesis[36,37]. Because HCCA3 is a normal cellular gene, increased expression of HCCA3 in HCC implys the genetic abnormality and acts as an oncogene in the dev elopment of HCC. In 42 patients with HCC, HCCA3 mRNA was detected in 33 (78.6%), which was highly expressed in HCC tissues, suggesting that HCCA3 is a very common molecular event involved in the pathogenesis of HCC. These findings indicate that HCCA3 mRNA is overexpressed preferentially in HCC and can serve as a tumor biomarker for HCC[7,18,23,36,37]. The three patients with macroscopic portal vein tumor thrombi all had significantly high expression of HCCA3 mRNA, implying that HCCA3 is a late incidence in HCC carcinogenesis. Compared with pathological features,HCCA3 mRNA expression was associated with the invasion of tumor capsule and the adjacant small satellite nodules lesions ($P<0.0$ 5), indicating that HCCA3 mRNA expression is a factor of HCC invasiveness and metastasis[37,38]. Although the function of HCCA3 is still unknown, our results suggest that the up-regulation of expression of HCCA3 mRNA may play an important role in the development and/or progression of hepatocellular carcinoma[36-38,42,45]. This finding demonstrated that it is possible to identify the pr eviously unknown, differential gene expression from a small amount of clinical samples[39-42,56]. Information about such alteration of HCCA3 in gene expression could be useful in elucidating the genetic events in HCC pathogenesis, developing new diagnostic markers, or determining novel therapeutic targets.

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