

# Primarily screening and analyzing ESTs differentially expressed in rats' primary liver cancer

Dandan Liu<sup>1</sup>, Lijuan Zhi<sup>2</sup>, Mingxia Ma<sup>1</sup>, Dan Qiao<sup>1</sup>, Meijuan Wang<sup>1</sup>, Yawei Wang<sup>1</sup>, Baijie Jin<sup>1</sup>, Anqi Li<sup>1</sup>, Guting Liu<sup>1</sup>, Yiqing Zhang<sup>1</sup>, Yanyan Song<sup>1</sup>, Hongxu Zhang<sup>1</sup>

<sup>1</sup>College of Life Science, Henan Normal University, Xinxiang 453007, China; <sup>2</sup>Xinxiang University, Xinxiang 453000, China

Corresponding to: Hongxu Zhang. College of Life Science, Henan Normal University, Xinxiang 453007, China. Email: hxzh5571@sina.com.cn.

**Objective:** To screen and analyze key express sequence tags (ESTs) which were differentially displayed in every period of SD rats' primary hepatic carcinoma and reveal the molecular mechanism of carcinogenesis.

**Methods:** Using diethylnitrosamine (DENa) as a cancerigenic agent, animal models with different phases of primary hepatic cancer were constructed in SD rats. Rats were respectively sacrificed at d 14, d 28, d 56, d 77, d 105 and d 112 after the rats received DENa by gavage, then the livers were harvested. One part of the livers was classified according to their pathological changes, while the other was reserved for molecular mechanism studies on hepatocarcinogenesis. The differentially expressed genes were isolated from both normal and morbid tissues by mRNA differential display technique (DDRT-PCR). After the fragments were sequenced, bioinformatics were used to analyze the results.

**Results:** Twelve differentially expressed cDNA fragments were obtained. Nine fragments had the homology with known cDNA clones, especially EST-7 was similar to BN/SsNHsdMCW mitochondrion gene and the identity was 100% which suggested EST-7 may be the part of BN/SsNHsdMCW mitochondrion gene. In contrast, other three fragments (EST-1, EST-3 and EST-5) had extremely low identity to any genes registered in GENBANK databases.

**Conclusions:** BN/SsNHsdMCW mitochondrion gene was expressed in different periods of hepatocarcinogenesis. Moreover, EST-1, EST-3 and EST-5 were suggested to contribute to the development of rat hepatocarcinogenesis, and thus may be candidates of new targets of oncogenes or cancer suppressor genes.

**Key Words:** Animal models of primary liver cancer; DDRT-PCR (differential display reverse transcription PCR); ESTs (express sequence tags); mitochondrion gene



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## Introduction

The development of primary liver cancer is a complicated procedure involving multiple factors and polygene mutations. Its incidence and mortality rank second among all cancers in China. It is often symptomless, usually occult in onset, and has nonspecific symptoms even in part of advanced liver cancer, so it is difficult to find out whether a person has liver cancer or not in very early stage (1). About 10% of primary liver cancer comes from the malignancy

of biliary epithelium, indicating that the biliary epithelium has a greater potential to develop into cancer than hepatocytes. Moreover, intrahepatic cholangiocarcinoma (ICC) has been described as being difficult to distinguish from hepatocellular carcinoma (HCC) and exhibits a pseudoglandular or poorly differentiated morphology, so the rate of early detection and diagnosis is greatly reduced (2). Although, it has been reported that ICC is associated with the inactivation of certain tumor suppressor genes such as *p53*, adenomatous polyposis coli (*APC*) and *smad-4* as well as

mutations in oncogenes such as *K-ras*, *c-myc* and *c-erb-2* (3), the mechanisms related to the overall process remain largely unknown. In addition, the present studies mainly focused on the molecular mechanism of hepatic cancer, and most of the experimental materials were obtained from clinical patients with moderate and advanced liver cancer. As a result, that greatly limited the systematic studies of molecular mechanisms in early stage of canceration, and the comprehensive analyses were lacked at gene level during the carcinogenesis.

Express sequence tags (ESTs) have also been found to be useful for screening new unknown genes, and the differential and quantitative analysis of expression patterns and for the evaluation of gene expression profiles in cancers. The analysis using EST sequences has an advantage of selecting new candidate genes (2). In a previous study, using ESTs, we identified some novel genes that offered valuable information related to the development of NIH mice's fore stomach carcinoma (4).

In this study, the SD rat liver cancer model, the pathological changes of which were much similar to human primary liver cancer, was built by diethylnitrosamine (DENa) gavage, then mRNA differential display reverse transcription PCR (DDRT-PCR) was used to primarily screen differentially expressed ESTs. The aim of this study was to study the related genes in carcinogenesis and the development of liver cancer, discuss the molecular mechanisms, and provide the important theory basis and references for tumor clinical early diagnosis and therapies.

## Materials and methods

### Experimental animals

Ninety male SD rats (SPF III,  $112 \pm 25$  g, 4 weeks old) were purchased from the Experimental Animal Center of Henan Province (Zhengzhou), and randomly divided into two groups: the induced group (70 rats) and the control group (20 rats). The animal experiments were approved by the Institutional Animal Care and Use Committee of the College of Life Science, Henan Normal University.

### Inducing drugs and primers

DENa was purchased from Sigma (St. Louis, USA). RNase-free DNase I and Trizol™ reagent were respectively bought from Promega (Madison, USA) and Tiangen (Beijing, China). RevertAid™ First Strand cDNA Synthesis

Kit was purchased from Fermentas (Berlington, Canada). Taq DNA polymerase, dNTPs and pMD18-T Vector were all purchased from TaKaRa (Dalian, China). DNA gel purification kit was bought from SunBiotech (Beijing, China).

The third generation primer sequences designed by Liang *et al.* were used as degenerate primers. There were 3 down anchor primers and 8 up random primers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers: 5'-GTGATGCTGGTGCTGAGTATGTC-3' and 5'-CAGTCTTCTGAGTGGCAGTGATG-3'. Primers were all synthesized by Shanghai Sangon (Shanghai, China).

### Establishment of animal models

In the induced group, SD rats' hepatocarcinoma was induced by gavage method. 1% DENa aqueous solution was administered at the dose of 70 mg/kg bw once a week for 15 weeks, then on normal diet. The control group was treated by gavage as the same dose of 0.9% physiological saline. Based on the relevant rules and guidelines about the experimental animals in China, cervical dislocation as the most humane way was used in this study. Rats were sacrificed by six times at d 14, d 28, d 56, d 77, d 105 and d 112 respectively. Then rats' livers were made into paraffin sections, stained by hematoxylin-eosin (HE) and observed in different pathological periods by two senior pathologists of medical college.

### RNA isolation and purification

Total RNA was extracted with the recommendations of the Trizol Reagent manufacturer. After measuring  $OD_{260}/OD_{280}$ , 1.5% agarose gel electrophoresis was used to test whether RNA was degraded, then total RNA was treated by RNase-free DNase I to get rid of genome DNA.

### Reverse transcription and cDNA identification

First-strand DNA was prepared from pure total RNA using 3 different down anchor primers respectively and RevertAid™ first strand cDNA synthesis kit. GAPDH, house keeping gene, was amplified by PCR with cDNA as templates while the quality of cDNA was tested.

### PCR amplification

The PCR reactions were performed using all combinations

**Table 1** The sequences of anchor and random primers

Primes	Sequences
H-T <sub>11</sub> A	5'-AAGCTTTTTTTTTTTTA-3'
H-T <sub>11</sub> C	5'- AAGCTTTTTTTTTTTC-3'
H-T <sub>11</sub> G	5'- AAGCTTTTTTTTTTTTG-3'
H-AP <sub>1</sub>	5'-AAGCTTGATTGCC-3'
H-AP <sub>2</sub>	5'-AAGCTTCGACTGT-3'
H-AP <sub>3</sub>	5'-AAGCTTGCTCAG-3'
H-AP <sub>4</sub>	5'-AAGCTTCTCAACG-3'
H-AP <sub>5</sub>	5'-AAGCTTAGTAGGC-3'
H-AP <sub>6</sub>	5'-AAGCTTGACCCAT-3'
H-AP <sub>7</sub>	5'-AAGCTTAACGAGG-3'
H-AP <sub>8</sub>	5'-AAGCTTTTACCGC-3'

of one random primer and one anchor primer (Table 1). cDNA was diluted to 1 mg/ $\mu$ L, and PCR was performed in 20 mL reaction system (1 $\times$  reaction buffer, 1.5 mmol/L MgCl<sub>2</sub>, 0.1 mmol/L dNTPs, 1 mmol/L anchor primer, 0.2 mmol/L random primer, 1 mL cDNA and 1U Taq polymerase) for 1 cycle (94 °C, 5 min), 5 cycles (94 °C 30 s, 40 °C 2 min, 72 °C 30 s), 35 cycles (94 °C 30 s, 55 °C 2 min, 72 °C 30 s) and 1 cycle (72 °C, 7 min).

#### Denaturated polyacrylamide gel electrophoresis (PAGE) and secondary PCR

The PCR products (15  $\mu$ L) were denatured at 95 °C for 5 min and 6% denaturated PAGE was carried out. Then, differential DNA bands were displayed by silver staining method. After comparison and analysis, differential bands were cut from the gel and placed for more than 1 h at 37 °C. After centrifugation, supernatants were used to amplify with the same primer set and conditions, but 0.2 mmol/L dNTPs substituted for 0.1 mmol/L dNTPs. The secondary PCR products were separated with 1.5% agarose gel electrophoresis, purified and retrieved.

#### Cloning and sequencing

The secondary PCR products were cloned into pMD18-T vector and transformed into *E. coli* DH-5 $\alpha$ . The fragments were sequenced by Beijing SunBiotech.

#### Bioinformatics analysis

BLASTN 2.2.18 program was used for homologous comparison in GENBANK, and the information of the related homologous ESTs could be obtained. Eventually, the sequences and related proteins were analyzed.

## Results

### Pathological classification

According to the histological and cytological changes in hepatocarcinogenesis (5,6), the rat livers from animal models could be divided into the following types: normal (N), light inflammation (LI), heavy inflammation (HI), nodus hyperplasia (NH), untypical nodus hyperplasia (UNH) and ICC.

### RNA isolation

The bands of 28S and 18S of total RNA were all clear while the ratio of 28S/18S was 2:1, and there was no protein contamination (Figure 1). Furthermore, OD<sub>260</sub>/OD<sub>280</sub> was 1.8-2.0. The results showed the extracted total RNA was intact and had high purity.

### cDNA identification

In electrophoresis picture, one bright and clear band about 300 bp showed that successful reverse transcription and cDNA had high quality (Figure 2). There was no band in lane C with RNA as the template. It suggested RNA was not contaminated by genome DNA.

### Differential expressed cDNA isolation and reamplification

In Figure 3, the comparison showed that the abundance of certain DNA bands between normal and pathological stages was displayed in a pattern of up-regulation, down-regulation, generation or loss. Among them, 101 differential expressed bands were targeted. After retrieving and re-amplification, 55 differential expressed bands were obtained eventually (Figure 4).

### DNA sequencing and bioinformatic analysis

Twelve genes were transformed into *E. coli* DH-5 $\alpha$  and sequenced. After homological analysis, 3 fragments were new unknown genes and 9 were known genes. Among them, EST-7 was identical to rat mitochondria gene BN/SsNHsdMCW, and the encoded protein was unknown (Tables 2,3).

## Discussion

Liver cancer consists of three types: HCC, ICC and

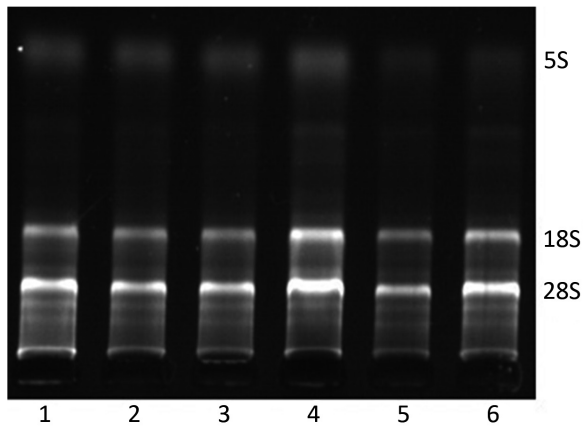


Figure 1 Results of RNA isolation

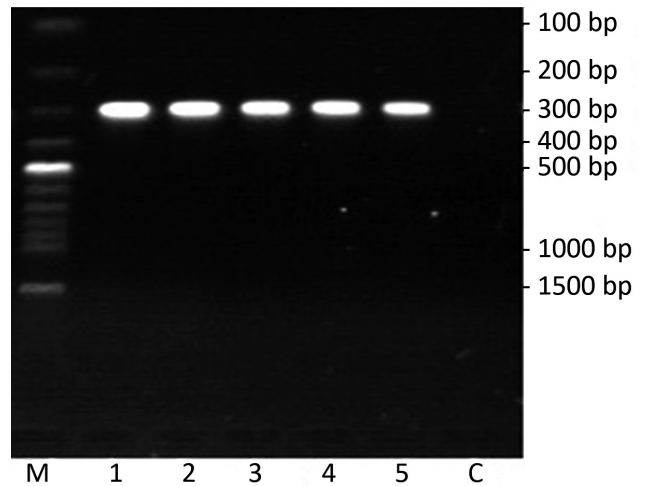


Figure 2 Results of cDNA identification. M. molecular weight standard; C. the negative control amplified with RNA as the template

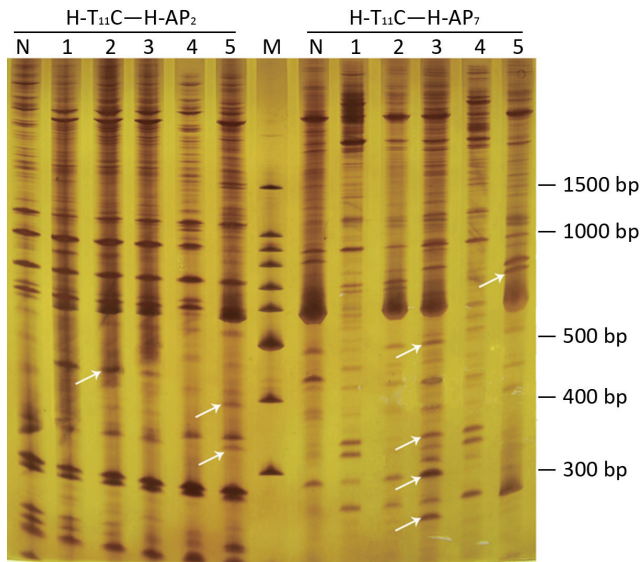


Figure 3 Differentially expressed genes among differential pathological stages. Parts of the differentially expressed bands were showed by arrows. N. normal liver; 1. light inflammation; 2. heavy inflammation; 3. nodus hyperplasia; 4. untypical nodus hyperplasia; 5. intrahepatic cholangiocarcinoma; M. molecular weight standard

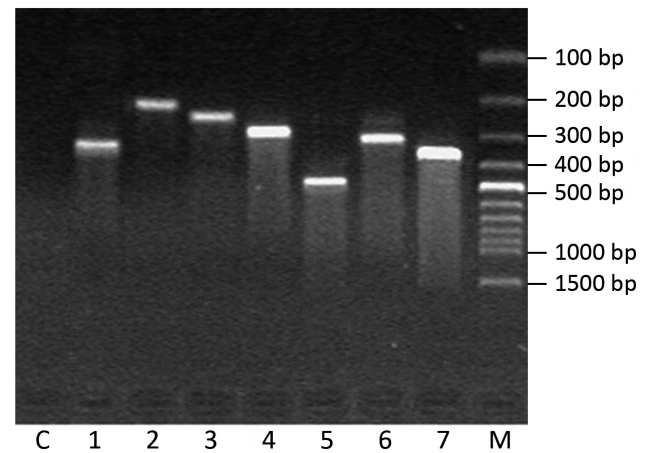


Figure 4 Parts of the interesting DNA bands were reamplified and showed by agarose gels. M, molecular weight standard; C, the negative control amplified with H<sub>2</sub>O as the template

combined hepatocellular and cholangiocarcinoma. The incidence of HCC is the highest and 15% of liver cancer cases all over the world are ICC (7). As one subtype of liver cancer, researches and reports about ICC are still rare either in domestic or foreign on its clinical diagnosis and molecular mechanism (8-10).

Among the twelve differential expressed fragments obtained, EST-7 is highly homologous (100%) with rat mitochondria gene BN/SsNHsdMCW and considered as

the part of BN/SsNHsdMCW sequence. In 2006, Li (11) was studying rats with cardiomyopathy and found that mitochondria gene BN/SsNHsdMCW was up-regulated after bone marrow mesenchymal stem cell transplantation. BN/SsNHsdMCW is considered to be relevant to mitochondria synthesis. When it and other related genes are up-regulated, numbers of mitochondria increase and cardiac function would be improved. There are multiple variants of mitochondria gene in rat genetic models of hypertension,

**Table 2** Homology results of 12 sequences according to the rat genome database

Serial number	Homology results (just highest homology)		
	Accession	Definition	Identity
EST-01	No significant similarity found		-
EST-02	NW_047694.1	Rattus norvegicus chromosome 4 genomic contig, reference assembly	98%
EST-03	No significant similarity found		-
EST-04	NW_047513.2	Rattus norvegicus chromosome 18 genomic contig, reference assembly	100%
EST-05	No significant similarity found		-
EST-06	NW_047492.2	Rattus norvegicus chromosome 17 genomic contig, reference assembly	99%
EST-07	AC_000022.1	Rattus norvegicus strain BN/SsNHsdMCW mitochondrion, complete genome	100%
EST-08	NW_047760.2	Rattus norvegicus chromosome 6 genomic contig, reference assembly	99%
EST-09	NW_047562.2	Rattus norvegicus chromosome 1 genomic contig, reference assembly	98%
EST-10	NW_047829.1	Rattus norvegicus chromosome Un genomic contig, reference assembly	99%
EST-11	NW_047430.2	Rattus norvegicus chromosome 14 genomic contig, reference assembly	100%
EST-12	NW_047341.1	Rattus norvegicus chromosome 10 genomic contig, reference assembly	95%

**Table 3** Homology results of correlative proteins according to the rat genome database

Serial number	Homology results (just highest homology)		
	Accession	Definition	Identity
EST-01	No significant similarity found		-
EST-02		Stam binding protein (associated molecule with the SH3 domain of STAM)	98%
EST-03	No significant similarity found		-
EST-04		Aldehyde dehydrogenase family 7, member A1	100%
EST-05	No significant similarity found		-
EST-06		Similar to inter-alpha-inhibitor H2 chain	99%
EST-07	No significant similarity found		-
EST-08		EGL nine homolog 3 (C. elegans)	99%
EST-09		Coronin, actin binding protein 1A	98%
EST-10		Ubiquitin A-52 residue ribosomal protein fusion product 1	99%
EST-11		Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	100%
EST-12		Similar to Protein kinase C alpha type (PKC-alpha) (PKC-A)	95%

the lack of any sequence variants provides conclusive evidence that divergence in blood pressure is exclusively programmed through their nuclear genomes (12). Moreover, variants of mitochondria gene are closely related with type I and II diabetes (13-15). Mitochondria have play a crucial role in carcinogenesis and development of many cancers (16-20), but most of the present researches are focusing on variants or the instability of mitochondria genes, and few direct evidence between BN/SsNHsdMCW and carcinogenesis is available.

In this study, EST-7 is expressed highly in normal livers, down-regulated in LI, and up-regulated in HI and

NH. Although its expression declines again in UNH, it is highly expressed in ICC. It shows that liver cell malignant transformation is closely related to complex changes of mitochondria synthesis which may directly provide the energy for hepatocarcinogenesis. In early stage, the toxicity of DENA could activate the rat's stress reaction, mitochondria synthesis decreases as well as energy metabolism. Finally, that might cause low expression of EST-7 in LI. In HI, more cells are malignant and more energy is required for rapid growth, so synthesis and numbers of mitochondria must be increased to promote the energy metabolism by up-regulating EST-7. With

serious lesion, cachexia induced by liver evil proliferation seriously interferes in the normal metabolism of the body, so the mitochondria synthesis and energy metabolism are all decreased in UNH. In advanced stage, tumor growth, invasion and metastasis require more mitochondria to generate more energy. Tumor cells could up-regulate the synthesis genes to supply adequate energy for immortal growth. The protein encoded by BN/SsNHsdMCW and its mechanism should be further studied in detail.

In addition, EST-7 is also completely homologous with Ac2047 which is down-regulated in rat liver regeneration. It suggests that EST-7 may play certain role in the stress reaction, division and proliferation of liver cells, and mitochondria function may be also promoted by this gene to generate more energy for cell proliferation.

After blasting rat genome database, EST-12 is homologous (95%) with PCK-alpha on chromosome 10. Khan (21) found down-regulated PCK-alpha may be very important in carcinogenesis involved with Ras mutation. When Bianchi (22) researched on distribution of T-cell signaling molecules in human myeloma, he found the expression of PCK-alpha declined greatly. In this study, down-regulation of EST-12 in every stage further shows PCK-alpha is closely related to carcinogenesis. It is speculated that PCK-alpha may change the activities of regulation proteins by regulating some tumor suppressor genes. Down-regulation of PCK-alpha may cause abnormal phosphorylation of regulation proteins, and the related suppressor genes are restrained and not activated to transform. At last, the negative control of cell proliferation is lost and that can promote carcinogenesis with simultaneous *ras* mutation (23-25). But the mechanisms and genes regulated by PCK-alpha have not been elucidated.

The related proteins of other seven ESTs are respectively associated molecule with the SH3 domain of STAM (AMSH), aldehyde dehydrogenase family 7, inter-alpha-inhibitor, EGLN3, coronin and actin binding protein 1A, ubiquitin A-52 residue ribosomal protein fusion product 1 and tyrosine 3/tryptophan 5-monooxygenase activation protein (Table 3). They are closely related to the carcinogenesis, invasion and metastasis. For example, AMSH and tyrosine 3/tryptophan 5-monooxygenase activation protein are kinases/regulatory factors which take part in cell signal transduction pathways by "on-and-off" switching other factors of the pathway, then promotive and inhibitive signals of cell growth are transmitted into cell nucleus to activate or restrain transcription of genes bound up with cell growth and proliferation, thus

cell dedifferentiation and malignant transformation are interfered by them (26,27). Inter-alpha-inhibitor is an important factor in cell apoptosis. Its abnormal expression would make tumor cells escape from cell death program and immune surveillance for immortal division and proliferation. Aldehyde dehydrogenase family 7 is a member of cell metabolism enzyme families. During carcinogenesis, its normal characteristics will be changes, and that inevitably results in metabolism chaos and abnormal expressions of related proteins which could feed back to physiological functions of other cells (28-31). Moreover, coronin and actin binding protein 1A is related to organize cytoskeleton. It can keep or change the stabilities of cytoskeleton by changing actin conformations, induce cell morphological and biochemical changes and participate in carcinogenesis, invasion and metastasis (32-34).

Since EST-1, EST-3 and EST-5 have no homology with any known genes, they are considered as new genes. Their whole sequences, conformations of related proteins and functions in tumors will need more researches in the future.

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