Differential expression of genes during aflatoxin B1-induced hepatocarcinogenesis in tree shrews

Yuan Li, Da-Fang Wan, Jian-Jia Su, Ji Cao, Chao Ou, Xiao-Kun Qiu, Ke-Chen Ban, Chun Yang, Liu-Liang Qin, Dan Luo, Hui-Fen Yue, Li-Sheng Zhang, Jian-Ren Gu

AIM: Through exploring the regulation of gene expression during hepatocarcinogenesis induced by aflatoxin B1 (AFB$_1$), to find out the responsible genes for hepatocellular carcinoma (HCC) and to further understand the underlying molecular mechanism.

METHODS: Tree shrews (Tupaia belangeri chinensis) were treated with or without AFB$_1$ for about 90 weeks. Liver biopsies were performed regularly during the animal experiment. Eight shares of total RNA were respectively isolated from 2 HCC tissues, 2 HCC-surrounding non-cancerous liver tissues, 2 biopsied tissues at the early stage (30th week) of the experiment from the same animals as above, 1 mixed sample of three liver tissues biopsied at the beginning (9th week) of the experiment, and another 1 mixed sample of two liver tissues from the untreated control animals biopsied at the 90th week of the experiment. The samples were then tested with the method of Atlas™ cDNA microarray assay. The levels of gene expression in these tissues taken at different time points during hepatocarcinogenesis were compared.

RESULTS: The profiles of differently expressed genes were quite different in different ways of comparison. At the same period of hepatocarcinogenesis, the genes in the same function group usually had the same tendency for up- or down-regulation. Among the checked 588 genes that were known to be related to human cancer, 89 genes (15.1%) were recognized as “important genes” because they showed frequent changes in different ways of comparison. The differentially expressed genes during hepatocarcinogenesis could be classified into four categories: genes up-regulated in HCC tissue, genes with similar expressing levels in both HCC and HCC-surrounding liver tissues which were higher than that in the tissues prior to the development of HCC, genes down-regulated in HCC tissue, and genes up-regulated prior to the development of HCC but down-regulated after the development of HCC.

CONCLUSION: A considerable number of genes could change their expressing levels both in HCC and in HCC-surrounding non-cancerous liver tissues. A few modular genes were up-regulated only in HCC but not in surrounding liver tissues, while some apoptosis-related genes were down-regulated in HCC and up-regulated in surrounding liver tissues. To compare gene-expressing levels among the liver tissues taken at different time points during hepatocarcinogenesis may be helpful to locate the responsible gene (s) and understand the mechanism for AFB$_1$ induced liver cancer.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major malignant tumors with a high mortality worldwide, especially in some areas of Southeast Asia and sub-Saharan. The standardized incidence of HCC in these high-risk regions usually exceeds 100 cases per 100,000 population[1]. HCC is also one of the few malignant tumors with a relatively defined etiology. It has been postulated that chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and exposure to dietary aflatoxins could contribute to an extraordinarily high risk of HCC in some regions[2-5]. However, it is similar to most of other tumors that the molecular mechanism of tumorigenesis is still to be explored.

Tree shrews (Tupaia belangeri chinensis) are small, squirrel-like mammals. Formerly they were considered belonging to the primate order. Currently, they have been classified into a separate order Scandentia, and are considered to be independent of both primates and insectivores[6]. Tree shrews have been used in biomedical researches since early in the 1960’s. Originally most of the researches were on tree shrew’s visual and nervous systems, until Reddy et al.[7] successfully induced liver cancer in tree shrew by aflatoxin B$_1$ (AFB$_1$) in the 1970’s. Latterly, Yan et al.[8,9] reported that tree shrews could be infected with human HBV and there was a synergistic effect between HBV infection and AFB$_1$ intake on inducing tree shrew’ s HCC. Walter et al.[10] reported their results from in vivo and in vitro studies on tree shrews infected with HBV. Tree shrews were then applied in studies on HCC chemoprevention, p53 gene mutation and oncogene expression in hepatocarcinogenesis[11-16]. Since tree shrew is the only known animal that can be infected with human HBV, with the exception of chimpanzee, it has become an animal of interest in researches related to human hepatocarcinogenesis. The
experimental model of tree shrew’s hepatocarcinogenesis was established by Yan et al. early in the 1980’s. One of the characteristics of this model is that the animals can stand repeated liver biopsies during the experimental period. This makes it possible to observe dynamically the sequential changes in liver tissues from the same animal during the course of hepatocarcinogenesis.

cDNA array is a new technology developed in recent years, subsequent to the progress in the human genome project. This new technique can be used to analyze gene expression. It has been applied widely in many research areas including changes of gene expressing level in liver cancer. In this study, we used the technique of cDNA array to study gene-expressing patterns of tree shrews’ liver at different time points during AFB1-induced hepatocarcinogenesis. HCC and its surrounding non-cancerous liver tissues, liver tissues biopsied prior to the development of HCC from the same animals, and normal control animals at start of the experiment and at the time when HCC developed in AFB1-treated animals, were compared. The dynamic changes of gene expression level by cDNA array during hepatocarcinogenesis are reported here.

MATERIALS AND METHODS

Animal experiment and collection of liver tissue samples

Adult tree shrews were purchased from Kunming Institute of Zoology, Chinese Academy of Sciences (Yunnan, China). Their body weights ranged from 100 to 160 g. After acclimatized to the facilities, all the animals were screened for sera alanine aminotransferase (ALT) and sera hepatitis B virus surface antigen (HBsAg), as well as histopathology of liver biopsies. The healthy animals were then divided into AFB1 group and control group. Only tree shrews in AFB1 treated group were used in the experiment, under ketamine hydrochloride anesthesia. Tumors developed in AFB1-treated animals, were compared. The healthy animals were then divided into AFB1 treatment groups. The healthy animals were then divided into AFB1 treatment groups and control group respectively, 2 from the 30th week of the experiment, and another 2 from the 90th week of the experiment. All the animals throughout the experiment were housed in suspended, stainless steel wire cages individually, under a controlled environmental condition with a 12 h light/dark photoperiod. They had free access to tap water and a diet containing rice, corn, wheat bran, soybean, egg, whole milk powder, sugar, salt, vitamins and minerals, etc. They were also fed with reconstituted powdered milk and fruit daily.

Liver biopsies were performed regularly during the experiment, under ketamine hydrochloride anesthesia. Tumors and their corresponding non-cancerous liver tissues were taken when animals were sacrificed. All tissue samples were immediately frozen by liquid nitrogen and stored at -80°C.

Eight drops of total RNA were prepared from the tissue samples, 2 from two cases of HCC and 2 from the non-cancerous counterparts respectively, 2 from the 30th week (before HCC developed) biopsies of the same animals respectively, 1 mixed sample by three biopsies taken before the animals were grouped and treated (0th week of the experiment), another 1 mixed sample by two biopsies taken at the 90th week (around the same time as HCC tissues were taken) from the control group. More detailed information of the samples is presented in Table 1. All liver tissues analyzed in this study were form female tree shrews.

Preparation of total RNA

Total RNAs were extracted from the specimens of liver tissue by a phenol/chloroform-based method. Briefly, 0.5 g of frozen tissue was pulverized in liquid nitrogen, lysed by adding 17.5 ml of denaturation solution (Trizol, GIBCO BRL), followed by phenol/chloroform extraction and isopropyl alcohol precipitation. The samples were then purified by DNase I and RNase inhibitor followed again by extraction and precipitation as above. Two µl and 5 µl RNA from each sample were taken respectively for checking concentrations by measuring OD at 260 nm and 280 nm, and for checking quality by electrophoresis on a denaturing formaldehyde/agarose/EtBr gel. The purified total RNA samples were stored at -80°C prior to use.

Table 1 Sources of total RNA samples

| Number and type of samples         | Animal treatment                     |
|-----------------------------------|--------------------------------------|
| HCC tissue                        |                                      |
| 1 Tree shrew in AFB1, group died  |                                       |
| 2 Tree shrew in AFB1, group died  |                                       |
| 3 Autopsy tissue from the same animal and at the same time as #1. |                                      |
| 4 Autopsy tissue from the same animal and at the same time as #2. |                                      |
| Liver tissue taken before HCC developed |                                      |
| 5 Biopsy tissue from the same animal as #1, at the 30th week of AFB1-treatment with a total amount of 3.1 mg AFB1. |                                      |
| 6 Biopsy tissue from the same animal as #2, at the 30th week of AFB1-treatment with a total amount of 3.2 mg AFB1. |                                      |
| Liver tissue taken before AFB1-treatment |                                      |
| 7 Liver tissue mixture from 3 animals before they were grouped and treated (0th week of the experiment). |                                      |
| Homochronous control              |                                      |
| 8 Liver tissue mixture from 2 animals in control group at the 90th week of the experiment. |                                      |

Probe synthesis, labeling, purification and hybridization

Atlas™ human cDNA expression array (catalog No. 7740-1, lot No. 9090199) kits were purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). There were 588 of human cDNAs for the known genes related to different kinds of human tumors, along with 9 housekeeping cDNAs as positive control for normalizing mRNA abundance. The 588 cDNAs were functionally divided into 16 classes. Each sequence of cDNA was 200-600 bp in length and spotted in duplicate with 10 ng of cDNA/dot on the array membrane. Some information of the genes and their functional categories are shown in Table 2. Probe synthesis, labeling, purification and hybridization were performed according to the manufacturer’s instructions. Briefly, cDNA probes were synthesized from 5 µg aliquots (2 µl) of the total RNA samples by the gene-specific CDS primer mix supplied with the Kits, labeled with α-35P-dATP (10.0 mCi/ml, NEN Life Science Company, USA) by the method of reverse transcription and purified through column chromatography. Atlas array membrane was prehybridized for 2 hours and hybridized to the cDNA probes overnight (7 rpm, 68°C). After washed, the membranes were exposed to the phosphorimaging system (BIO RAD, GS-525, USA) for 4 hours, the hybridized intensity of each dot was scanned and calculate. The membranes were finally exposed to Kodak X-ray film with intensifying screen for 24-72 hours at -80°C and then developed.

Data analysis

The intensity of each hybridized dot on each membrane was calibrated according to the formula: calibrated intensity of dot = (original intensity of dot - background intensity of membrane)/ (intensity of housekeeping gene ubiquitin C - background intensity). The background intensity was taken from the middle of each membrane. The ratio of intensity between two compared dots ≥2.0 was considered as gene expression level up-regulated, ≤0.5 as down-regulated. If the same comparison of the two tissue specimens (i.e., the two cases of HCC...
compared to their corresponding non-cancerous liver tissues respectively) had the same pattern of change (i.e., both up- or down-regulated), the average ratio was considered as "validated value". Otherwise the ratio was considered as "invalidated value" perhaps due to individual variations or some other unknown factors.

The gene expression levels in the 5 types of liver tissue were compared by 6 different ways, namely, liver tissues taken from AFB1-treated animal before the development of HCC (30th week of the treatment, BC) and before AFB1 treatment (0th week of the experiment, BA), HCC-surrounding non-cancerous liver tissue (NCL) and its corresponding BC, HCC and its corresponding NCL, HCC and its corresponding BC, HCC and BA, and the liver tissue taken from control group around the same time as HCC (90th week of the treatment, C). Among these 6 ways of comparison, the first three were compared according to the possible sequence of hepatocarcinogenesis (normal liver → liver before HCC developed → HCC-surrounding non-cancerous liver → HCC), the last four were for comparing HCC tissues with the other 4 types of liver tissues. The genes showing changes at least twice among the first three ways of comparison or thrice among the last four ways of comparison, or in the 6th way plus any one of the other three among the last four ways of comparison were considered as "important genes".

All the data of hybridized dot's intensity were analyzed with Excel software (Microsoft).

RESULTS

Up to the 90th week of the animal experiment, 2 cases showed HCC in AFB1 group, and the first case of HCC appeared at the 88th week. The average amount of AFB1 fed to animals in this group was 10.5 mg. All the HCCs were confirmed histopathologically. No HCC developed in control group until the 90th week of experiment.

The A300/A260 ratio for the 8 shares of total RNA was between 1.8-2.2. The electrophoresis showed intactness of all isolated RNA samples.

The intensity of ubiquitin C on each hybridized membrane was most distinct and steady among the 9 housekeeping genes. Generally, the profiles of differentially expressed genes were quite different in different ways of comparison, and the genes in the same function group usually had the same tendency for up- or down-regulation in a given type of comparison. Among the checked 588 genes, 89 (15.1%) were considered as "important genes" according to the criteria mentioned above. Table 2 shows the detailed information for these genes.

Table 2: Differential expressions of important genes

| Coordinate | Gene Name | 1 BC/ BA | 2 NCL/ BC | 3 HCC/ NCL | 4 HCC/ BC | 5 HCC/ BA | 6 HCC/ C |
|------------|-----------|---------|-----------|-----------|---------|---------|---------|
| Oncogenes & tumor suppressors (1/ 57, 1.8%) | | | | | | | |
| A1i | v-erbA related protein 3 (EAR3) | ↑ (3.56) | ↑ (7.45) | ↑ (4.80) | | | |
| Cell Cycle Regulators (1/ 41, 2.4%) | | | | | | | |
| A6i | cell division cycle 25 homolog (CDC25) | ↑ (3.43) | ↑ (5.92) | | | | |
| Ion channels & transport proteins (1/ 3) | | | | | | | |
| B1f | thymosin receptor (TR) | ↓ (0.15) | ↑ (5.64) | ↑ (10.75) | ↑ (6.52) | | |
| B1l* | neurotrophic tyrosine kinase receptor-related 3 | ↑ (5.50) | ↑ (2.63) | ↑ (14.05) | ↑ (25.36) | | |
| B2j | bone marrow stromal antigen 1 (BST-1) | ↓ (0.15) | ↑ (11.14) | | ↑ (3.40) | | |
| B4l | epidermal growth factor receptor substrate 15 (EPS15) | ↓ (0.40) | ↑ (2.87) | | (2.18) | | |
| B5m* | cAMP-dependent protein kinase type I beta regulatory subunit (PRKAR1B) | ↓ (0.02) | ↑ (4.03) | ↑ (11.35) | ↑ (51.59) | | |
| Stress response proteins (1/ 7, 14.3%) | | | | | | | |
| B7m | heat shock 70-kDa protein 1 (HSP70.1) | ↑ (3.54) | ↓ (0.26) | ↓ (0.26) | ↓ (0.17) | | |
| A apoptosis-associated proteins (15/ 64, 23.4%) | | | | | | | |
| C1g | p53 cellular tumor antigen | ↓ (0.35) | ↑ (0.39) | | (0.10) | | |
| C1n | TNF-related activation protein (TRAP) | ↓ (0.42) | | | (0.24) | | |
| C3c | glutathione S-transferase A1 (GSTA1) | ↓ (0.29) | ↓ (0.23) | | | | |
| C3j | N-myc proto-oncogene | ↓ (181.47) | ↓ (0.30) | ↑ (148.98) | ↓ (0.18) | | |
| C3k | receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIPK1) | ↑ (4.61) | ↑ (0.25) | ↓ (0.06) | | | |
| C3n | tumor necrosis factor C (TNFC) | ↓ (0.24) | ↓ (0.08) | | | | |
| C4c* | NIP1 (NIP1) | ↓ (0.08) | ↓ (0.16) | ↓ (0.20) | | | |
| C4d* | BCL2/ adenovirus E1B 19-kDa-interacting protein 3 (BNIP3) | ↓ (0.31) | ↓ (0.34) | ↓ (0.21) | | | |
| C4f | BCL2 homologous antagonist/ killer 1 (BAK1) | ↓ (0.28) | ↓ (0.12) | | | | |
| C4h | BCL2-related protein A 1 (BCL2A1) | ↑ (8.19) | ↓ (0.16) | ↓ (0.09) | | | |
| C4k | inhibitor of apoptosis protein 3 (IAP3) | ↑ (4.53) | ↑ (0.42) | ↓ (0.23) | | | |
| C4m* | TNF-related apoptosis inducing ligand (TRAIL) | ↓ (0.26) | ↓ (0.20) | ↓ (0.40) | | | |
| C5c | WSL protein+TRAMP+Ap-3+ death domain receptor 3 (DDR3) | ↓ (0.39) | ↓ (0.20) | | | | |
| Gene Name | Accession | Description | Fold Change | P-value | Q-value |
|-----------|-----------|-------------|-------------|---------|---------|
| C5f       | U39613    | cysteine protease ICE-LAP3 | 1.13 | 0.03 |
| C5h       | U71364    | cytoplasmic antiproteinase 3 (CAP3) | 44.85 | 0.02 | 0.13 |
| DNA synthesis, repair & recombinant proteins (15/34, 44.1%) |
| C5k*      | L07540    | replication factor C 36-kDa subunit (RFC36) | 0.24 | 0.26 | 0.17 |
| C6a       | M30938    | nuclear factor IV | 0.25 | 0.07 |
| C6b       | M31899    | xeroderma pigmentosum group B complementing protein (XPB-ERCC3) | 0.06 | 0.04 |
| C6c       | M32865    | 70-kDa thyroid autoantigen (TLAA) | 0.44 | 0.20 |
| C6e       | M63488    | replication protein A 70-kDa subunit (RPA70) | 0.25 | 0.07 |
| C6i       | J04088    | DNA topoisomerase II alpha (TOP2A) | 4.00 | 0.30 |
| C7a       | M13194    | excision repair cross-complementing rodent repair deficiency complementation group 1 (ERCC1) | 0.26 | 0.09 |
| C7b       | M13267    | cytosolic superoxide dismutase 1 (SOD1) | 0.39 | 0.24 |
| C7c       | U07418    | colon cancer nonpolyposis type 2 protein (COCA2) | 0.30 | 0.10 |
| C7d*      | D21090    | UV excision repair protein RAD23 homolog B (hHR23B; XPC repair-complementing complex 58-kDa protein) | 0.26 | 0.08 |
| C7g       | S40706    | growth arrest & DNA damage-inducible protein 153 (GADD153) | 0.29 | 0.15 |
| C7i       | U12134    | DNA damage repair & recombination protein 52 homolog (RAD52) | 0.27 | 0.12 |
| C7k       | U63139    | DNA damage repair & recombination protein 50 homolog (RAD50) | 0.18 | 0.08 |
| C7m       | X84740    | DNA ligase III (LIG3) | 0.21 | 0.08 |
| C7n       | X90392    | muscle-specific DNase I-like (DNL1) | 0.22 | 0.12 |
| Transcription factors & DNA-binding proteins (8/98, 8.2%) |
| D3a       | D26155    | SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 2 (SMARCA2) | 0.19 | 0.55 |
| D4b*      | M36542    | octamer-binding transcription factor 2 (OCT2) | 2.64 | 3.30 |
| D4e       | M62810    | mitochondrial transcription factor 1 (MTTF1) | 0.84 | 25.07 |
| D4j       | M76541    | transcriptional repressor protein yin & yang 1 (YY1) | 2.73 | 3.10 |
| D5a*      | M83234    | nuclease-sensitive element DNA-binding protein (NSEP) | 2.59 | 5.21 |
| D5f       | M96824    | nucleobindin 1 (NUCB1) | 2.64 | 2.37 |
| D6h*      | U10324    | nuclear factor NF90 | 3.69 | 2.90 |
| D6i       | U14575    | activator of RNA decay (ARD-1) | 7.46 | 93.75 |
| Growth factor & chemokine receptors (1/25, 4.0%) |
| E1h       | L06623    | endothelin receptor type B (ETB) | 0.37 | 2.78 |
| Interleukin & interferon receptors (0/18) |
| Hormone receptors (0/4) |
| Neurotransmitter receptors (0/11) |
| Cell surface antigens & adhesion proteins (3/40, 7.5%) |
| E5i       | M23197    | myeloid cell surface CD33 antigen | 6.68 | 3.26 |
| E6f**     | M74774    | dipeptidyl peptidase IV (DPP IV); T-cell activation CD26 | 0.32 | 0.28 |
| E7b       | M35198    | integrin beta 6 (ITGB6) | 5.44 | 0.36 |
| Growth factors, cytokines & chemokines (20/64, 31.3%) |
| F1f       | X03438    | granulocyte colony-stimulating factor (GCSF) | 9.96 | 0.06 |
| F1j*      | M16552    | thrombomodulin (THBD) | 0.10 | 0.23 |
| F2a       | D16431    | hepatoma-derived growth factor (HDGF) | 0.39 | 0.28 |
| F2c       | J04130    | T-cell activation protein 2 (AT2) | 0.17 | 0.20 |
| F2e       | K03515    | neuroleukin (NLK) | 0.40 | 0.26 |
| F2f       | L12260    | neuregulin 1 (NRG1) | 21.23 | 3.76 |

ISSN 1007-9327      CN 14-1219/ R      World J Gastroenterol    February 15, 2004   Volume 10   Number 4
**DISCUSSION**

Tree shrew is a kind of animals classified more closely to human being than the common laboratory animals. Park _et al_. reported that tree shrew’s wild-type p53 showed 91.7% and 93.4% homologies with human p53 nucleotide and amino acid sequences respectively, and 77.2% and 73.7% homologies respectively with mice. With limited information of tree shrew’s genome and no commercial gene chip for tree shrew was currently available, in this study we used human cDNA-array kit to explore tree shrew’s gene expression patterns during AFB1-induced hepatocarcinogenesis. The results of hybridization implied that the method used in this study was practicable. Besides, the incidence of HCC (10/15 and 0/12 respectively for AFB1 group and control group at the 135th week of the experiment, data unpublished) and the liver histopathological changes in this study were similar to those of our former experiments, which demonstrated the tree shrew model of AFB1-induced HCC was reliable.

| Gene     | Accession | Expression Pattern | Ratio (Fold Change) |
|----------|-----------|--------------------|---------------------|
| heregulin-beta3 | L12261 | up-regulation | (6.10) |
| thrombopoietin (THPO) | L36052 | down-regulation | (0.10) |
| bone morphogenetic protein 3 (BMP3) | M22481 | up-regulation | (7.26) |
| small inducible cytokine subfamily A member 2 (SCYA2) | M24545 | down-regulation | (0.05) |
| amphiregulin (AR) | M30704 | up-regulation | (3.69) |
| small inducible cytokine subfamily A member 1 (SCYA1) | M57502 | down-regulation | (0.22) |
| hepatocyte growth factor (HGF) | M60718 | down-regulation | (0.20) |
| hepatocyte growth factor-like protein | M74178 | up-regulation | (5.11) |
| vascular endothelial growth factor C (VEGFC) | U43142 | up-regulation | (8.75) |
| calgranulin A (CALA) | X06234 | down-regulation | (0.21) |
| AFGF + HBGF-1 + ECGF-beta | X51943 | up-regulation | (11.18) |
| OX40 ligand (OX40L) | X79929 | up-regulation | (5.42) |
| prorelaxin H2 (RLN2) | A06925 | down-regulation | (0.23) |
| glucagon (GCG) | J04040 | up-regulation | (5.75) |
| insulin-like growth factor-binding protein 3 (IGFBP3) | M31159 | down-regulation | (0.17) |
| cellular retinoic acid-binding protein II (CRABP2) | M68867 | up-regulation | (2.84) |
| corticotropin-releasing factor-binding protein | X58022 | up-regulation | (5.00) |
| estrogen sulfotransferase (STE; EST) | U08098 | up-regulation | (5.62) |
| preprotachykinin beta (beta-PPT) | X54469 | down-regulation | (0.17) |

1 BA: liver tissue from animals before AFB1 treatment (0th week of the experiment). BC: liver tissue from the same HCC animals after AFB1 treatment for 30 weeks. HCC: hepatocellular carcinoma tissue from the same animal after AFB1 treatment. NCL: HCC-surrounding non-cancerous liver tissues from the same HCC animal. C: liver tissue from untreated animals taken at the time when HCC developed in AFB1 treated animals. 2 The numbers followed the name of functional classes are the amount of the important genes identified in this class and their percentage. 3 : up-regulation; ↓: down-regulation. The numbers followed are the averages of the two checked samples. 4*: Genes might be of more importance.
In the former reports on differentially expressed genes of liver cancer, the usual method was to compare the levels of gene expression between HCC tissues and its surrounding tissues\[17-23,25,26\]. These so-called “normal” HCC-surrounding tissues, however, frequently involve the changes of hepatitis and/or liver cirrhosis. The present study, by using biopsy tissue from a given animal at different time points during AFB1-induced hepatocarcinogenesis, showed a dynamic profile of gene expression not reported previously. It is notable that a set of genes were up- or down-regulated in HCC-surrounding liver tissue but without any differences with the HCC counterpart. This phenomenon further indicated that the HCC-surrounding liver tissue was no longer normal even in terms of molecular biology. In these HCC-surrounding liver tissues, some growth factors, effectors or their receptors such as thrombin receptor, and some transcriptional factors such as SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 2 (SMARCA2) were up-regulated, but the genes related to apoptosis were down-regulated. These changes inevitably resulted in abnormality of the tissue response to the environment, and kept in anti-apoptosis condition. Apparently, the HCC-surrounding liver tissue was at a precancerous status at molecular level. Actually, there were only a few genes that were continuously up- or down-regulated during hepatocarcinogenesis. This study also showed that some genes were up-regulated during the early stage but down-regulated during the late stage of hepatocarcinogenesis, the reason of this phenomenon awaits further elucidation.

By comparing the 5 types of tissues taken at different time points, the genes differentially expressed during the course of AFB1-induced hepatocarcinogenesis could be categorized into the following 4 types.

Type I, genes that were continuously up-regulated in HCC vs HCC-surrounding non-cancerous liver tissue (NCL) and NCL vs liver tissue taken before HCC developed (BC). This type of genes was rare. Only two were found among the 588 checked genes, namely genes for neurotrophic tyrosine kinase receptor-related protein 3 and cAMP-dependent protein kinase type I beta regulatory subunit (PRKAR1B). Their ratio for HCC vs NCL was 2.6 and 11.4 respectively, and the ratio for NCL vs BC was 5.5 and 4.0 respectively. The gene for nuclease-sensitive element DNA-binding protein (NSEP) probably would also be included into this type.

Type II, genes that had similar expressing levels in HCC and NCL but down-regulated after the development of HCC, including genes related to DNA-repair, genes related to growth factor and cytokines, genes for interleukins, and genes related to hormones. Their up-regulation might putatively imply the acute phase response of related genes to genotoxicity. The down-regulation might imply the defective expression of related genes during carcinogenesis, or some other up-regulated genes might functionally compensate for these genes. Table 3 shows the detailed information about these four types of genes.

### Table 3 Four types of differentially expressed genes

| Type | Description | Genes |
|------|-------------|-------|
| I    | Genes that were continuously up-regulated both in HCC vs NCL and NCL vs BC. | NSEP |
| I-1  | Modulators, effectors & intracellular transducers | neurotrophic tyrosine kinase receptor-related 3, cAMP-dependent protein kinase type I beta regulatory subunit (PRKAR1B) |
| I-2  | Transcription factors & DNA-binding proteins | SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 2 (SMARCA2), octamer-binding transcription factor 2 (OCT2), mitochondrial transcription factor 1 (MTTF1) |
| I-3  | Growth factor & chemokine receptors | endothelin receptor type B (ETB) |
| I-4  | Cell surface antigens & adhesion proteins | myeloid cell surface CD33 antigen |
| I-5  | Growth factors, cytokines & chemokines | hepatocyte growth factor-like protein |
| II   | Genes that had similar expression level in HCC and NCL but higher than that in BC. | |
| II-1 | Apoptosis-associated proteins | p53, tumor necrosis factor |
| II-2 | DNA damage repair & recombination proteins | nuclear factor IV, xeroderma pigmentosum group B complementing protein (XPB; ERCC3) |
| II-3 | DNA synthesis, repair & recombination proteins | 70-kDa thyroid autoantigen (TLAA), replication protein A (RPA70), excision repair cross-complementing rodent repair deficiency complementation group 1 (ERCC1), colon cancer polyposis type 2 protein (COCA2) |
| III  | Genes down-regulated in HCC vs NCL. | |
| III-1 | Apoptosis-associated proteins | p53, tumor necrosis factor |
| III-2 | DNA damage repair & recombination proteins | nuclear factor IV, xeroderma pigmentosum group B complementing protein (XPB; ERCC3) |
| III-3 | Growth factors, cytokines & chemokines | growth arrest & DNA damage-inducible protein 353 (GADD153), DNA damage repair & recombination protein 30 homolog (RAD50) |
It has to be noticed that cDNA array, the method used in this study has some limitations. First, this method only shows mRNA but not the protein expression level of related genes. Second, even if the expression level of mRNA may equally reflect the expression level of protein, it can not represent the functions of these gene products such as phosphorylation/dephosphorylation, methylation, acetylation or glycosylation. The status of protein phosphorylation is very critical for cellular signal transduction, and there is accumulating evidence that suggests the relationship between the status of methylation and activation of gene in hepatocarcinogenesis[30-33]. Therefore, the genes showing differential expressions at mRNA level must be further studied on their protein expression level and their modified status. The present study, however, provides a preliminary clue to the differential expressions of mRNA during AFB1-induced hepatocarcinogenesis, particularly at different stages of carcinogenesis from the same animals. Since the development of most human HCCs in China are attributed to the synergistic effects of AFB1 and HBV, the study on tree shrew’s hepatocarcinogenesis induced by both AFB1 and HBV is now in progress, and the preliminary result was published recently[34].

ACKNOWLEDGMENT
The authors express their appreciation to Drs. Geng-Sun Qian and Yi-Qian Wu at Shanghai Cancer Institute for providing meaningful advice and discussion regarding the microarray work.

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Edited by Wang XL