PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• LIVER CANCER •

Differential expression of genes during aflatoxin B_1-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

Yuan Li, Jian-Jia Su, Ji Cao, Chao Ou, Ke-Chen Ban, Chun Yang, Liu-Liang Qin, Dan Luo, Hui-Fen Yue, Department of Experimental Pathology, Guangxi Cancer Institute, Nanning 530021, Guangxi Zhuang Autonomous Region, China
Da-Fang Wan, Xiao-Kun Qiu, Jian-Ren Gu, National Laboratory for Oncogene and Related Genes, Shanghai Cancer Institute, Shanghai 200032, China
Li-Sheng Zhang, Department of Molecular Biology, Guangxi Cancer Institute, Nanning 530021, Guangxi Zhuang Autonomous Region, China
Supported by National Natural Science Foundation of China, NO. 39860072 and NO. 39869001; and Natural Science Foundation of Guangxi Zhuang Autonomous Region, NO. 9817137
Co-first-authors: Yuan Li and Da-Fang Wan
Correspondence to: Dr. Yuan Li, Department of Experimental Pathology, Guangxi Cancer Institute, Nanning 530021, Guangxi Zhuang Autonomous Region, China. li-yuan@public.nn.gx.cn
Telephone: +86-771-5331100 **Fax:** +86-771-5312000
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Abstract

AIM: Through exploring the regulation of gene expression during hepatocarcinogenesis induced by aflatoxin B_1 (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 (30^{th} week) of the experiment from the same animals as above, 1 mixed sample of three liver tissues biopsied at the beginning (0^{th} week) of the experiment, and another 1 mixed sample of two liver tissues from the untreated control animals biopsied at the 90^{th} 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.

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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-Sahara. 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[2-7]. 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[8]. 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*.[9] successfully induced liver cancer in tree shrew by aflatoxin B_1 (AFB_1) in the 1970' s. Latterly, Yan *et al*.[10,11] 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*.[12] 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[13-16]. 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[10]. 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[17-23]. In this study, we used the technique of cDNA array to study gene-expressing patterns of tree shrews' liver at different time points during AFB_1-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 AFB_1 group and control group. Only tree shrews in AFB_1 group were fed with AFB_1 (150 μg/(kg· d), 5 d/w) in milk, from the 1st week to 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℃.

Eight shares 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 with AFB_1 (0th week of the experiment), and 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℃ prior to use.

Table 1 Sources of total RNA samples

Number and type of samples	Animal treatment
HCC tissue	
1	Tree shrew in AFB_1 group died of HCC at the 88th week of treatment with an amount of AFB_1 up to 10.4 mg.
2	Tree shrew in AFB_1 group died of HCC at the 89th week of treatment with an amount of AFB_1 up to 11.3 mg.
HCC-surrounding non-cancerous liver tissue	
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 AFB_1-treatment with a total amount of 3.1 mg AFB_1.
6	Biopsy tissue from the same animal as #2, at the 30th week of AFB_1-treatment with a total amount of 3.2 mg AFB_1.
Liver tissue taken before AFB_1-treatment	
7	Biopsy tissue mixture from 3 animals before they were grouped and treated (0th week of the experiment).
Homochronous control	
8	Biopsy 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-^{32}P-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℃). After washed, the membranes were exposed to the phosphorimage 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℃ 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 $\geqslant$2.0 was considered as gene expression level up-regulated, $\leqslant$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 expressing levels in the 5 types of liver tissue were compared by 6 different ways, namely, liver tissues taken from AFB_1-treated animal before the development of HCC (30^{th} week of the treatment, BC) and before AFB_1 treatment (0^{th} 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, HCC and the liver tissue taken from control group around the same time as HCC (90^{th} 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 6^{th} 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 90^{th} week of the animal experiment, 2 cases showed HCC in AFB_1 group, and the first case of HCC appeared at the 88^{th} week. The average amount of AFB_1 fed to animals in this group was 10.5 mg. All the HCCs were confirmed histopathologically. No HCC developed in control group until the 90^{th} week of experiment.

The A_{260}/A_{280} 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	Genbank	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	X12795	v-erbA related protein 3 (EAR3)			↑ (3.56)	↑ (7.45)	↑ (4.80)	
Cell Cycle Regulators (1/41, 2.4%)								
A6i	L26584	cell division cycle 25 homolog (CDC25)				↑ (3.43)		↑ (5.92)
Ion channels & transport proteins (0/3)								
Modulators, effectors & intracellular transducers (5/88, 5.7%)								
B1f	M62424	thrombin receptor (TR)	↓ (0.15)	↑ (5.64)		↑ (10.75)		↑ (6.52)
B1l*	X74764	neurotrophic tyrosine kinase receptor-related 3		↑ (5.50)	↑ (2.63)	↑ (14.05)	↑ (25.36)	
B2j	D21878	bone marrow stromal antigen 1 (BST-1)	↓ (0.15)		↑ (11.14)			↑ (3.40)
B4l	U07707	epidermal growth factor receptor substrate 15 (EPS15)	↓ (0.40)			↑ (2.87)		↑ (2.18)
B5m*	M65066	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	M11717	heat shock 70-kDa protein 1 (HSP70.1)	↑ (3.54)	↓ (0.26)		↓ (0.26)		↓ (0.17)
Apoptosis-associated proteins (15/64, 23.4%)								
C1g	M14694	p53 cellular tumor antigen			↓ (0.35)			↓ (0.10)
C1n	L07414	TNF-related activation protein (TRAP)			↓ (0.42)			↓ (0.24)
C3c	M25627	glutathione S-transferase A1 (GSTA1)				↓ (0.29)		↓ (0.23)
C3j	M13228	N-myc proto-oncogene	↑ (181.47)		↓ (0.30)		↑ (148.98)	↓ (0.18)
C3k	U25994	receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIPK1)	↑ (4.61)		↓ (0.25)			↓ (0.06)
C3n	L11015	tumor necrosis factor C (TNFC)			↓ (0.24)			↓ (0.08)
C4c*	U15172	NIP1 (NIP1)			↓ (0.08)		↓ (0.16)	↓ (0.20)
C4d*	U15174	BCL2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3)			↓ (0.31)		↓ (0.34)	↓ (0.21)
C4f	U23765	BCL2 homologous antagonist/killer 1 (BAK1)			↓ (0.28)			↓ (0.12)
C4h	U29680	BCL2-related protein A1 (BCL2A1)		↑ (8.19)	↓ (0.16)			↓ (0.09)
C4k	U45880	inhibitor of apoptosis protein 3 (IAP3)	↑ (4.53)		↓ (0.42)			↓ (0.23)
C4m*	U57059	TNF-related apoptosis inducing ligand (TRAIL)	↓ (0.26)				↓ (0.20)	↓ (0.40)
C5c	Y09392	WSL protein+TRAMP+Apo-3+ death domain receptor 3 (DDR3)			↓ (0.39)			↓ (0.20)

C5f	U39613	cysteine protease ICE-LAP3			↓ (0.13)			↓ (0.03)
C5h	U71364	cytoplasmic antiproteinase 3 (CAP3)		↑ (44.85)	↓ (0.28)			↓ (0.13)
DNA synthesis, repair & recombinant proteins (15/34, 44.1%)								
C5k*	L07540	replication factor C 36-kDa subunit (RFC36)	↓ (0.24)	↑ (6.73)	↓ (0.26)		↓ (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)
C6l	J04088	DNA topoisomerase II alpha (TOP2A)		↑ (4.00)	↓ (0.33)			↓ (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)		↓ (0.44)	↓ (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)		↑ (6.02)	↓ (0.27)			↓ (0.12)
C7k	U63139	DNA damage repair & recombination protein 50 homolog (RAD50)			↓ (0.18)			↓ (0.08)
C7m	X84740	DNA ligase III (LIG3)		↑ (4.23)	↓ (0.21)			↓ (0.08)
C7n	X90392	muscle-specific DNase I-like (DNL1L)		↓ (5.36)	↓ (0.22)			↓ (0.12)
Transcription factors & DNA-bingding proteins (8/98, 8.2%)								
D3a	D26155	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 2 (SMARCA2)	↓ (0.19)	↑ (3.85)		↑ (4.58)		↑ (2.55)
D4b*	M36542	octamer-binding transcription factor 2 (OCT2)		↑ (2.64)		↑ (8.32)	↑ (7.33)	↑ (3.30)
D4e	M62810	mitochondrial transcription factor 1 (MTTF1)				↑ (8.04)		↑ (25.07)
D4j	M76541	transcriptional repressor protein yin & yang 1 (YY1)	↓ (0.32)	↑ (2.73)		↑ (3.74)		↑ (3.10)
D5a*	M83234	nuclease-sensitive element DNA-binding protein (NSEP)			↑ (2.59)	↑ (3.24)	↑ (2.75)	↑ (5.21)
D5f	M96824	nucleobindin 1 (NUCB1)	↓ (0.24)	↑ (2.64)		↑ (3.00)		↑ (2.37)
D6h*	U10324	nuclear factor NF90				↑ (3.69)	↑ (2.34)	↑ (2.90)
D6l	U14575	activator of RNA decay (ARD-1)	↑ (72.46)	↑ (2.53)			↑ (93.75)	
Growth factor & chemokine receptors (1/25, 4.0%)								
E1h	L06623	endothelin receptor type B (ETB)	↓ (0.37)	↑ (4.46)		↑ (2.07)		↑ (2.78)
Interleukin & interferon receptors (0/18)								
Hormone receptors (0/4)								
Neurotransmitter receptors (0/11)								
Cell surface antigens & adhesion proteins (3/40, 7.5%)								
E5l	M23197	myeloid cell surface CD33 antigen		↑ (6.68)		↑ (5.75)		↑ (3.26)
E6f*	M74777	dipeptidyl peptidase IV (DPP IV); T-cell activation CD26			↓ (0.32)		↓ (0.36)	↓ (0.28)
E7b	M35198	integrin beta 6 (ITGB6)		↑ (5.44)	↓ (0.44)			↓ (0.36)
Growth factors, cytokines & chemokines (20/64, 31.3%)								
F1f	X03438	granulocyte colony-stimulating factor (GCSF)	↑ (8.95)	↑ (3.32)	↓ (0.05)	↓ (0.18)		↓ (0.06)
F1j*	M16552	thrombomodulin (THBD)			↓ (0.10)		↓ (0.24)	↓ (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)	↑ (9.09)		↓ (0.40)			↓ (0.26)
F2f	L12260	neuregulin 1 (NRG1)	↑ (21.23)		↓ (0.10)		↑ (3.76)	↓ (0.13)

F2g	L12261	heregulin-beta3		↑ (6.10)	↓ (0.10)			↓ (0.14)
F2h	L36052	thrombopoietin (THPO)		↑ (7.26)	↓ (0.04)	↓ (0.24)		↓ (0.06)
F2m	M22491	bone morphogenetic protein 3 (BMP3)			↓ (0.05)			↓ (0.08)
F3a	M24545	small inducible cytokine subfamily A member 2 (SCYA2)			↓ (0.36)			↓ (0.34)
F3d	M30704	amphiregulin (AR)	↑ (3.69)		↓ (0.17)	↓ (0.41)		↓ (0.12)
F3i	M57502	small inducible cytokine subfamily A member 1 (SCYA1)			↓ (0.22)			↓ (0.27)
F3l	M60718	hepatocyte growth factor (HGF)			↓ (0.20)			↓ (0.30)
F4c	M74178	hepatocyte growth factor-like protein	↓ (0.31)	↑ (5.11)		↑ (4.62)		↑ (2.28)
F4e	M92934	connective tissue growth factor (CTGF)	↑ (3.86)	↑ (6.00)	↓ (0.30)		↑ (5.44)	↓ (0.24)
F4h	U43142	vascular endothelial growth factor C (VEGFC)	↑ (8.75)	↑ (3.82)	↓ (0.14)		↑ (4.81)	↓ (0.15)
F4i	X02530	small inducible cytokine subfamily B member 10 (SCYB10)	↑ (5.60)	↑ (4.14)	↓ (0.14)		↑ (3.17)	↓ (0.15)
F4k	X06234	calgranulin A (CALA)			↓ (0.21)			↓ (0.21)
F4n	X51943	AFGF + HBGF-1 + ECGF-beta			↓ (0.10)			↓ (0.19)
F5e	X79929	OX40 ligand (OX40L)	↑ (5.42)		↓ (0.11)	↓ (0.31)		↓ (0.09)
Interleukins & interferons (12 / 20, 60.0%)								
F5j	J04156	interleukin 7 (IL7)		↑ (3.62)	↓ (0.25)			↓ (0.27)
F5k	A14844	interleukin 2 (IL2)	↑ (11.82)		↓ (0.14)	↓ (0.16)		↓ (0.13)
F5l	X02851	interleukin 1 alpha (IL1-alpha)			↓ (0.19)			↓ (0.24)
F5n	M14743	interleukin 3 (IL3)		↑ (11.18)	↓ (0.08)			↓ (0.06)
F6a	M13982	interleukin 4 (IL4)	↑ (2.87)		↓ (0.26)			↓ (0.22)
F6b*	X04602	interleukin 6 (IL6)		↑ (3.37)	↑ (2.73)	↑ (9.47)	↑ (12.41)	
F6d	M28622	interferon beta 1 (IFN-beta 1)			↓ (0.17)			↓ (0.16)
F6g	L06801	interleukin 13 (IL13)	↓ (0.20)	↑ (5.04)		↑ (5.90)		↑ (2.36)
F6i	M57765	interleukin 11 (IL11)		↑ (4.15)	↓ (0.11)	↓ (0.40)		↓ (0.10)
F6j	M65290	interleukin 12 beta subunit (IL12-beta)	↑ (2.71)		↓ (0.19)			↓ (0.18)
F6k	M65291	interleukin 12 alpha subunit (IL12-alpha)	↑ (3.78)		↓ (0.17)			↓ (0.14)
F6l	U14407	interleukin 15 (IL15)		↑ (3.36)	↓ (0.24)			↓ (0.30)
Hormones (7 / 14, 50.0%)								
F7d	A06925	prorelaxin H2 (RLN2)			↓ (0.23)			↓ (0.16)
F7f	J04040	glucagon (GCG)		↑ (5.75)	↓ (0.17)			↓ (0.12)
F7i	M31159	insulin-like growth factor-binding protein 3 (IGFBP3)			↓ (0.35)			↓ (0.35)
F7j	M68867	cellular retinoic acid-binding protein II (CRABP2)		↑ (2.84)	↓ (0.27)			↓ (0.23)
F7l	X58022	corticotropin-releasing factor-binding protein	↑ (5.00)	↑ (2.97)	↓ (0.12)	↓ (0.36)		↓ (0.16)
F7m	U08098	estrogen sulfotransferase (STE; EST)	↑ (5.62)	↑ (3.44)			↑ (12.98)	
F7n	X54469	preprotachykinin beta (beta-PPT)	↑ (15.57)		↓ (0.07)	↓ (0.21)		↓ (0.09)
Total amount		89 / 588, 15.1%	32	36	70	29	20	83
Up-regulation			20(62.5)	35(97.2)	5(7.1)	18(62.1)	13(65.0)	15(18.1)
Down-regulation			12(37.5)	1(2.8)	65(92.9)	11(37.9)	7(35.0)	68(81.9)

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.

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[14]. 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 AFB_1-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 AFB_1 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[11,24], which demonstrated the tree shrew model of AFB_1-induced HCC was reliable.

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 AFB_1-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 (SMARCA 2) 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 AFB_1-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 tissues which were higher than that in BC. This type of genes included the genes in the functional group of modulators, effectors and intracellular transducers, and transcription factors and DNA-binding proteins, *etc*. This pattern of change might imply that these genes were up-regulated after the initiation of hepatocarcinogenesis but kept at a persistent level in the late stage. Moreover, it strongly indicated that a tremendous change in HCC-surrounding liver tissues at molecular level, implying the HCC-surrounding non-cancerous liver tissue was precancerous rather than normal.

Type III, genes that were down-regulated in HCC tissue *vs* NCL tissue. They included several groups of genes. (1) Genes related to apoptosis. Most of apoptosis-inducing genes were down regulated but a few of anti-apoptosis genes such as IAP were exceptionally down-regulated (0.4) too. The overall trend caused by this pattern of gene expression change, however, favored cell survival. This phenomenon was also seen in some other studies[27-29]. (2) Genes for DNA-repairing were generally down-regulated. The down-regulation of this group of genes might imply the alteration of response of HCC cells to the signals from the surrounding environment. (3) Genes for growth factor, cytokines and chemokines. (4) Genes for interleukins, interferons and hormones.

Type IV, genes that were up-regulated before the appearance of HCC 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 I, genes that up-regulated both in HCC *vs* NCL and NCL *vs* BC.
Modulators, effectors & intracellular transducers
neurotrophic tyrosine kinase receptor-related 3
cAMP-dependent protein kinase type I beta regulatory subunit (PRKAR1B)
Type II, genes that had similar expressing level in HCC and NCL but higher than that in BC.
II-1. Modulators, effectors & intracellular transducers
thrombin receptor (TR)
epidermal growth factor receptor substrate 15 (EPS15)
II-2. Transcription factors & DNA-bingding 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)
transcriptional repressor protein yin & yang 1 (YY1)
nucleobindin 1 (NUCB1)
II-3. Growth factor & chemokine receptors
endothelin receptor type B (ETB)
II-4. Cell surface antigens & adhesion proteins
myeloid cell surface CD33 antigen
II-5. Growth factors, cytokines & chemokines
hepatocyte growth factor-like protein
Type III, genes down-regulated in HCC *vs* NCL.
III-1. Apoptosis-associated proteins
p53 cellular tumor antigen
TNF-related activation protein (TRAP)
N-myc proto-oncogene
receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIPK1)
tumor necrosis factor C (TNFC)
NIP1 (NIP1)
BCL2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3)
BCL2 homologous antagonist/killer 1 (BAK1)
inhibitor of apoptosis protein 3 (IAP3)
WSL protein + TRAMP + Apo-3 + death domain receptor 3 (DDR3)
cysteine protease ICE-LAP3
III-2. DNA synthesis, repair & recombination proteins
nuclear factor IV
xeroderma pigmentosum group B complementing protein (XPB; ERCC3)
70-kDa thyroid autoantigen (TLAA)
replication protein A 70-kDa subunit (RPA70)
excision repair cross-complementing rodent repair deficiency complementation group 1 (ERCC1)
colon cancer nonpolyposis type 2 protein (COCA2)
UV excision repair protein RAD23 homolog B (hHR23B; XPC repair-complementing complex 58-kDa protein)
growth arrest & DNA damage-inducible protein 153 (GADD153)
DNA damage repair & recombination protein 50 homolog (RAD50)
III-3. Growth factors, cytokines & chemokines

thrombomodulin (THBD)
hepatoma-derived growth factor (HDGF)
T-cell activation protein 2 (AT2)
neuroleukin (NLK)
neuregulin 1 (NRG1)
bone morphogenetic protein 3 (BMP3)
small inducible cytokine subfamily A member 2 (SCYA2)
hepatocyte growth factor (HGF)
calgranulin A (CALA)
AFGF + HBGF-1 + ECGF-beta

III-4. Interleukins & interferons
interleukin 2 (IL2)
interleukin 1 alpha (IL1-alpha)
interleukin 4 (IL4)
interferon beta 1 (IFN-beta 1)
interleukin 12 beta subunit (IL12-beta)
interleukin 12 alpha subunit (IL12-alpha)

III-5. Hormones
insulin-like growth factor-binding protein 3 (IGFBP3)

Type IV, genes up-regulated before HCC appearance but down-regulated after HCC developed.

IV-1. DNA synthesis, repair & recombination proteins
replication factor C 36-kDa subunit (RFC36)
DNA topoisomerase II alpha (TOP2A)
DNA damage repair & recombination protein 52 homolog (RAD52)
DNA ligase III (LIG3)
muscle-specific DNase I-like (DNL1L)

IV-2. Growth factors, cytokines & chemokines
granulocyte colony-stimulating factor (GCSF)
heregulin-beta3
thrombopoietin (THPO)
connective tissue growth factor (CTGF)

IV-3. Interleukins & interferons
interleukin 7 (IL7)
interleukin 3 (IL3)
interleukin 6 (IL6)
interleukin 11 (IL11)

IV-4. Hormones
cellular retinoic acid-binding protein II (CRABP2)
corticotropin-releasing factor-binding protein

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 AFB_1- 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 AFB_1 and HBV, the study on tree shrew' s hepatocarcinogenesis induced by both AFB_1 and HBV is now in progress, and the preliminary result was published recently[34].

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