Differentially Expressed Proteins in the Liver of HBxTg/p53KO Double Mutant Mouse Using Proteomics

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Abbreviations

B6 mice : C57BL/6J mice

HBxTg mice : C57BL/6J-HBx/HBx transgenic mice

p53KO mice : C57BL/6J- $p53^{-}$/ $p53^{-}$ knock-out mice

CHAPS : 3-[(3-cholamidopropyl)dimthylammonio]-1-propanesulfonic acid

DTT : dithiothreitol

2DE : two-dimensional gel eletrophoresis

IEF : isoelectrofocusing

MALDI-TOF MS : matrix-assisted laser desorption ionization time of flight mass spectrometry

IPG : immobilized pH gradient

Abstract

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Hepatitis B virus (HBV) has been clearly recognized as an etiological factor for hepatocellular carcinoma (HCC). HBV encodes the potentially oncogenic HBx protein. However, little was known for the mechanism underlying HBx-mediated oncogenecity. Functional inactivation of p53 by complex formation with HBx has been regarded as one of possible mechanisms of HCC in HBV-infected liver. Although there have been many attempts to investigate the role of p53 which was one of well-characterized tumor suppressive proteins, *in vivo* relation between p53 and

HBx is not fully understood during hepatocellular carcinogenesis caused by HBx. Therefore, we aimed to elucidate the molecular mechanism of HCC and to discover the effect of functional loss of p53 on the development of HCC by HBx. We generated HBx transgenic and p53 knock-out double mutant mice (HBxTg/p53KO) by the cross mating of HBx transgenic mice (HBxTg) to p53 knock-out (p53KO) mice. Serum ALT value was determined and liver section was observed under H&E staining. We employed two dimensional polyacrylamide gel electrophoresis (2DE) and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) to determine profiles of differentially expressed proteins in the liver of 1-month-old male HBxTg, p53KO and HBxTg/p53KO mice compared to their age-matched normal mice (B6). In HBxTg/p53KO mice, ALT value were significantly increased compared to normal B6 mice and even, both HBxTg and p53KO mice. HBx/p53KO double mutant mice showed swelling of hepatocytes and irregular hepatic cord histopathologically. However, there is not distinct change in the liver of B6, HBxTg and p53KO mice. Cell cycle, cytoskeleton, several metabolisms related proteins were mainly differentially expressed in the liver of HBxTg mice compared to B6 mice. Signal transduction pathway, ATP binding and chaperone activity and lipid binding related proteins were differentially expressed in the liver of p53KO mice compared to B6 mice. Cell cycle, several metabolisms, ATP binding and chaperone activity, transferase activity related proteins were differentially expressed in the liver of HBxTg/p53KO double mutant mice compared to B6 mice. We identified 24 liver proteins, which were differentially regulated in each of HBxTg,

p53KO and HBxTg/p53KO mice compared with B6 mice. They are proteins related with cell cycle regulation, lipid metabolism, fatty acid metabolism, ATP synthesis and several metabolism related proteins. The proteins identified provide insights into the HCC caused by HBx and the regulation of this process related with the functional loss of p53.

Key words: hepatocellular carcinoma (HCC), HBx, p53, HBxTg/p53KO double mutant mice, liver.

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I. Introduction

Hepatocellular carcinoma (HCC) is known as a common malignant tumor that takes the lives of about one million people world wide annually. The major risk factors for human HCC are Hepatitis B virus (HBV), Hepatitis C virus (HCV), p53 mutation, aflatoxin, alcohol abuse and male gender.[1,2] In addition, development of HCC is linked to cirrhosis.[3] Epidemiological data highly indicated that there is a consistent and specific causal association between HBV infection and occurrence of HCC.[4,5] HCC is on the increase in many countries where HCV infection is more

prevalent than HBV infection.[5,6] In Korea, however, there are much more HBV infections than HCV infections. The hepatitis B virus (HBV) genome contains four transcriptional open reading frames designated S, C, P, and X [7,8] . Of the four viral-encoded proteins, Hepatitis B virus X (HBx) has been identified as potentially oncogenic.[9] Since the oncogenic role of HBx in a transgenic mouse model was first demonstrated, there have been controversial results by several researchers. In some studies, HBx induced no pathology in the livers of HBx transgenic mice (HBxTg), but it sensitized liver cells to the other carcinogenic effects.[10,11,12,13] But in other studies, transgenic expression of the HBx was shown to induce liver tumors in transgenic mice.[9,14] HBx protein is a transactivator without DNA-binding activity. [15,16] HBx can also bind to the p53 tumor suppressor protein and interfere with the role of p53 which plays in the cellular response to DNA damage.[17] Initial investigations on the interaction between HBx and p53 demonstrated that the two proteins associate with each other both in vivo and in vitro.[18] Inactivation of p53 by complex formation with HBx has been suggested as a possible mechanism of HCC. [17,19-22] Contrary to the previous reports, HBx has been reported to exert a spontaneous proapoptotic effect on primary cultures of hepatocytes and in the livers of HBxTg mice.[23-25] These observations *in vivo* and *in vitro* suggested that HBx regulates endogenous cellular pathways. However, still the effect of p53 on the HCC caused by HBx remains unclear. More recently, HBxTg/p53KO (HBx/HBx // $p53^{-}/p53^{-}$) double mutant mice were generated by the cross-mating of HBxTg mice with p53 knock-out mice

(p53KO) [26], which is a very useful animal model for studying the relations and the role of p53 on the development of HCC caused by HBx.

To elucidate factors involved in HCC caused by HBx under the functional loss of p53, we employed two dimensional polyacrylamide gel electrophoresis (2DE) and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and determined profiles of differentially expressed proteins in the liver of 1-month-old male HBxTg, p53KO and HBxTg/p53KO mice compared to its age-matched normal mice (B6). Also serum ALT value was determined and liver section was observed under H&E staining.

II. Materials and methods

1. Laboratory Animals

We used HBxTg mice (C57BL/6J-HBx/HBx transgenic mice) developed by Dr Yu. [14] p53KO mice (C57BL/6J- $p53^{-}$/ $p53^{-}$ knock-out mice) were purchased from the Jackson Laboratory (U.S.A.). [26] HBxTg/p53KO double mutant mice (HBx/HBx//$p53^{-}$/$p53^{-}$) were established by the cross mating of HBxTg mice to p53KO mice. Age matched wild type mice were used as controls from the same background of HBxTg mice. All animals have been maintained in SPF barrier area of the Department of Laboratory Animal Medicine, Yonsei University College of Medicine. The temperature (22±2℃), humidity (50±5%) and light (12D/12N) of animal rooms were controlled constantly. The chloride-added (RO water) water was supplied ad libitum. All animals were sacrificed at the age of 1-month old. Liver was removed, weighted and stored at -70℃ until used.

2. Establishment of HBxTg/p53KO double mutant mice

A. Hybrid between HBxTg and p53KO mice

HBxTg mice were mated with p53KO mice to generate F1 hybrid (HBx/+//p53^{-}/+). F1 pairs were intercrossed each other to generate F2 hybrid when they were 8 weeks old (Fig. 1). We selected HBxTg/p53KO (HBx/HBx//p53^{-}/p53^{-}) double mutant mice from F2 hybrids by genotyping.

B. Genotyping of HBxTg/p53KO double mutant mice

(A) DNA extraction

DNA was isolated from tail as follows. Tail was cut with a sterile razor blade and solubilized in 1 ㎖ lysis buffer (1% SDS, 5 mM EDTA, 10 mM Tris-HCl pH 7.6, 100 mM NaCl, 200 ㎍/㎖ proteinase K). The buffer was incubated in dry oven at 60℃ overnight. After incubation, 400 ㎕ solution (4.21 M NaCl, 0.63 M KCl, 10 mM Tris-HCl) was added with gentle inversion for protein precipitation. The mixture was centrifuged at 13,000 rpm for 30 minutes at 4℃. 300 ㎕ upper aqueous phase containing DNA and 300 ㎕ 100% ethanol were mixed in a microcentrifuge and were incubated in a refrigerator for 2 hours at -20℃. The mixture was centrifuged at 13,000 rpm for 20 minutes at 4℃ and the DNA pellet was washed three times with cold 70% ethanol. The DNA pellet was dried and resuspended in double-distilled water. Purity and concentration of the final samples were evaluated by ultraviolet

spectrophotometer.

(B) PCR

Primer sets for HBx gene and for discriminating HBx homozygote from heterozygote, were obtained from Dr Yu (KRIBB, Taejon, Korea).[14] Primer sets for p53 gene was provided by commercial service.[26] Twenty $\mu\ell$ reaction solution containing 2 μg of the genomic DNA was used for PCR. The primer sequences, annealing temperature and PCR cycles are described in Table 1. After electrophoresis, male mice with genotype of HBxTg/p53KO double mutant mice (HBx/HBx //$p53^{-}$ /$p53^{-}$) were discriminated and sacrificed (Fig. 2).

Table 1. Sequence of PCR primers

Genotype		Primer Sequence	Tm	Cycles
+/+, +/p53^{-} or p53^{-}/p53^{-}	p1	5'-CTTGGGTGGAGAGGCTATTC-3'	64℃ (-0.5) 58℃	12 + 25
	p2	5'-AGGTGAGATGACAGGAGATC-3'		
	p3	5'-ATAGGTCGGCGGTTCAT-3'		
	p4	5'-CCCGAGTATCTGGAAGACAG-3		
HBx/? or +/+	p1	5'-TTCTCATCTGCCGGTCCGTG-3'	53℃	34
	p2	5'-CTCTCTTTGCGCTCCCTGGG-3'		
HBx/HBx or HBx/+	p1	5'-GAAAACACACTCACTGTTCAGAG-3'	54℃	34
	p2	5'-GGGTCAATGTCCATGCCCCA-3'		

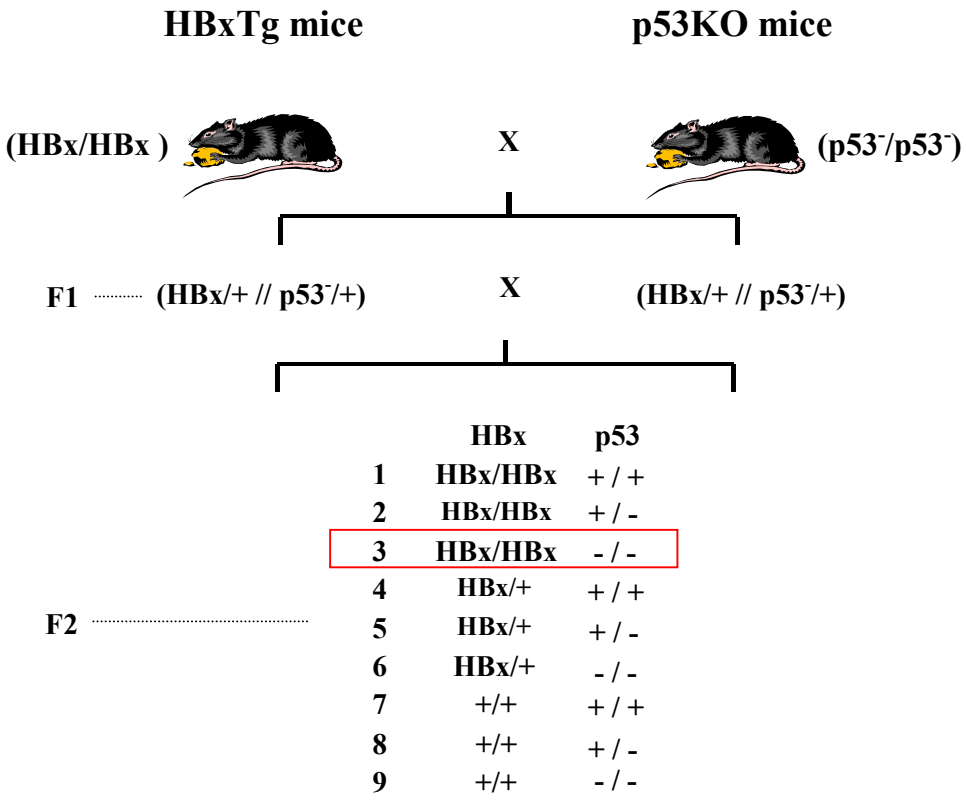


Figure 1. Establishment of HBxTg/p53KO double mutant mice. The homozygotic HBxTg mice (HBx/HBx) were mated with the homozygotic p53KO mice ($p53^{-}/p53^{-}$) to generate F1 hybrid (HBx/+//$p53^{-}$/+). F1 hybrid pairs were intercrossed each other to generate F2 hybrid. We selected HBxTg/p53KO (HBx/HBx//$p53^{-}/p53^{-}$) double mutant mice from F2 hybrids.

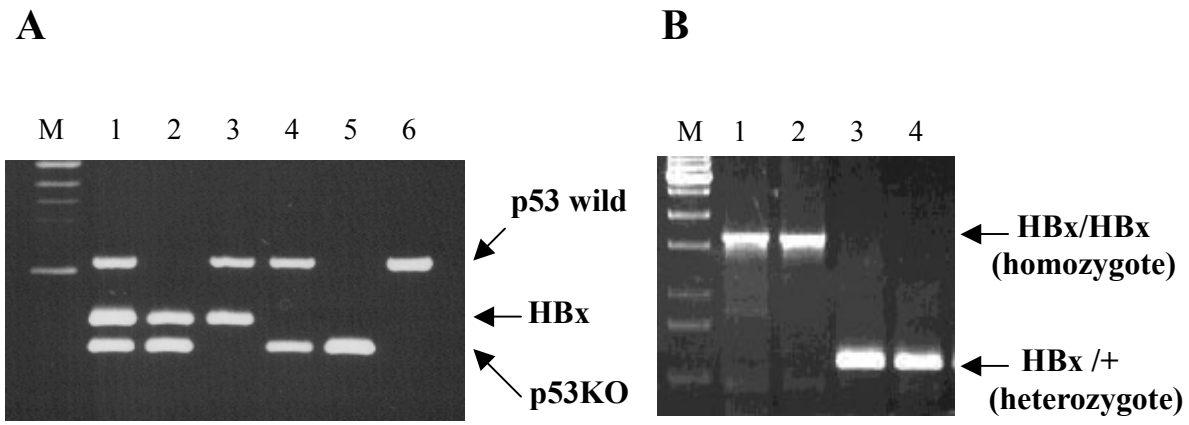


Figure 2. Genotyping of HBxTg/p53KO double mutant mice. (A) PCR genotyping of HBx transgene and p53 knock-out. M; DNA size marker. Lane 1,2 and 3 showed the introduction of HBx transgene. Lane 4,5 and 6 showed wild type of HBx. Lane 4 showed the heterozygotic knock-out of p53. Lane 5 showed the homozygotic knock-out of p53. Lane 6 showed wild type p53 (B) PCR genotyping for heterozygote and homozygote of HBx transgene. M; DNA size marker. Lane 1 and 2 showed the homozygotic transgene of HBx. Lane 3 and 4 showed the heterozygotic transgene of HBx.

3. Liver Function Test

Under anesthesia with ethyl-ether, blood was collected by heart puncture from mice. Serum level of alanine aminotransferase (ALT) was determined by commercial service provided by Ewon Reference Laboratory, Seoul, Korea.

4. Histology of liver

Liver samples were fixed in 10% neutral buffered formalin for 24 hours. After serial processing of dehydration by graded alcohol series and clearing, tissues were embedded in paraffin. Tissues were cut into 4 ㎛ sections. Slides were stained with hematoxylin & eosin.

5. Proteomic analysis

A. Sample preparation

Liver tissue of 100 ㎎ was homogenized under liquid nitrogen. Homogenized liver tissue was lysed with 800 ㎕ lysis buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS, 2% Pharmalyte pH 3-10, 100 mM DTE) and centrifuged at 50000 rpm at 4℃ for 1 hr.

The supernatant was transferred into new eppendorf tube. After then the supernatant mixed DNase (Roche 104132, 2.5 ㎎/㎖) and Rnase (Roche 019141, 2.5 ㎎/㎕) and incubated for 30 min on 20℃. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Switzerland). Solubilized protein samples were divided into 1 mg aliquots and stored at -80℃.

B. 2D-gel electrophoresis

2D-PAGE was performed as previously described. [27] The 1 mg aliquots proteins were diluted in lysis buffer to total volume 450 ㎕. The sample was applied to the 240 mm, immobilized, nonlinear pH gradient strips of pH 3-10 (IPG Drystrips, Amersham Pharmacia Biotech. Uppsala, Sweden), which was rehydrated for at least 10 hrs. After rehydration, the strips were focused at 30 V for 3 hrs, 100 V for 1 hr, 200 V for 1 hr, 500 V for 1 hr, 1,000 V for 1 hr and finally at 8,000 V for 11 hrs so as to obtain approximately 90,000 Vhr (IPG phore, Amersham Pharmacia Biotech). Once IEF was completed, the strips were equilibrated in 6M urea containing 20% v/v glycerol, 2% w/v SDS and 0.01% w/v BPB with 10mM TBP (Tributyl phosphine, Fluka Chemie, Buchs, Switzerland). SDS-PAGE was performed using 8-18% SDS polyacrylamide separating gel without a stacking gel using the Ettan Dalt system (Amersham Pharmacia). The second dimensional was carried out overnight at 3W/gel at 20℃. The gels were stained with Coomassie G-250 (Bio-Rad, USA).

C. Protein image analysis

The stained gels were scanned using GS800 photometer (Bio-Rad, U S A) and analyzed with the Melanie III (SIB, Swiss). For comparison of spot density, the digitized image was compared by matching method. Intensity levels were normalized between gels as a proportion of the total protein intensity detected for the entire gel. Differentially expressed spots among groups by > 3-fold and < 1/3-fold were analyzed and annotated.

D. Protein identification by MALDI-TOF MS

MALDI-TOF MS was performed using the Applied Biosystems Voyager DE-PRO spectrometer (Applied Biosystems, Forste city, USA), equipped with a 337 nm nitrogen laser. The instrument was operated in accelerating voltage 20 kV, positive ion reflection mode, voltage grid 74.5 %, guide wire voltage 0 %, delay-time 120 ns. The spectra were internally calibrated using the trypsin autolysis products (842.51 [M+H] and 2211.11 [M+H]), and the proteins were identified by searching in Swiss-Prot and NCBI database using MS-FIT (Protein Prospector, UCSF, San Francisco, CA, USA). All the searches were analyzed with a 50 ppm mass tolerance. For the detection of post-translational modifications, the molecular weight of each spots were analyzed on 3 different gels with MALDI-TOF.[28]

II. Results

1. Liver weight and liver function test

The liver weight in HBxTg, p53KO and HBxTg/p53KO double mutant mice were significantly increased compared to B6 mice ($p<0.05$, Fig. 3). Serum ALT level of HBxTg/p53KO double mutant mice was significantly higher than that those of B6, HBxTg and p53KO mice ($p<0.05$, Fig. 4).

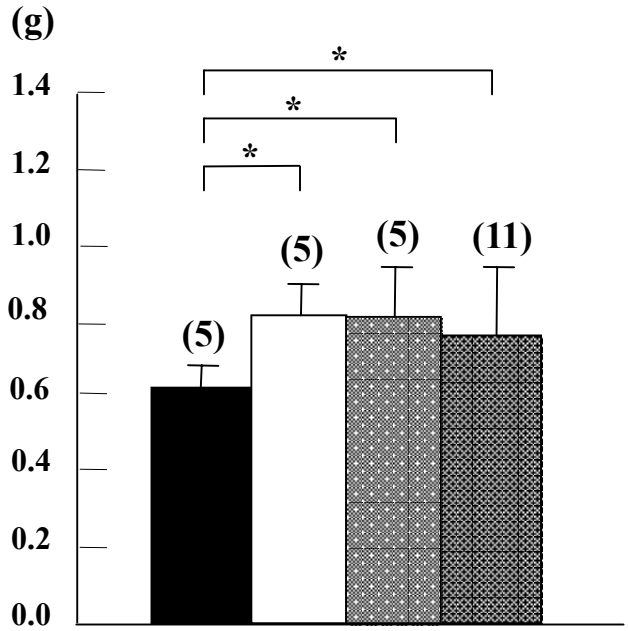


Figure 3. The changes of liver weight in B6, HBxTg, p53KO and HBxTg/p53KO double mutant mice. Data were expressed as means ± S.D. The liver weight in HBxTg, p53KO and HBxTg/p53KO double mutant mice were significantly increased compared to B6 mice (*: $p<0.05$). However, there is not significant difference in the liver weight among HBxTg, p53KO and HBxTg/p53KO double mutant mice. Number of parentheses indicated the number of animals used. (■ B6 mice, □ HBxTg mice, ▩ p53KO mice, ▩ HBxTg/p53KO double mutant mice)

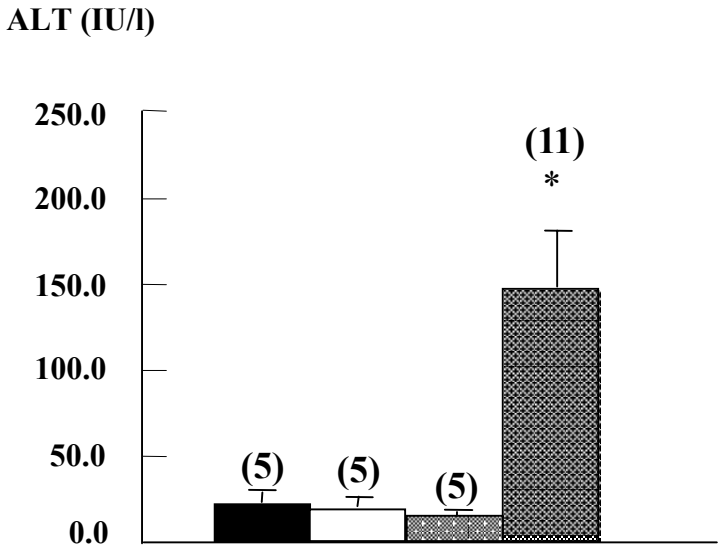


Figure 4. Serum value of alanine aminotransferase in B6, HBxTg, p53KO and HBxTg/p53KO double mutant mice. Data were expressed as means ± S.D. The ALT value was significantly increased in HBxTg/p53KO double mutant mice compared to B6, HBxTg and p53KO mice (*: $p<0.05$). (■ B6 mice, □ HBxTg mice, ▩ p53KO mice, ▩ HBxTg/p53KO double mutant mice)

2. Histology of liver

We observed liver stained with by hematoxylin & eosin. Consequently, in 1 month of age, the livers of HBxTg mice were characterized by a generalized variation in the nuclear size of the hepatocytes, compared to B6 mice (Fig. 5A and B). Around the central vein there were hepatocytes with large and light nuclei, while hepatocytes with small and dark nuclei were observed around the portal vein in HBxTg mice (Fig. 5B). In 1-month-old HBxTg/p53KO double mutant mice, the livers were characterized by a generalized variation in the size of the hepatocytes and their nuclei (Fig. 5D). Around the central vein there were hepatocytes with large nuclei showing chromatin clumping, while hepatocytes with small nuclei were observed around the portal vein (Fig. 5D).

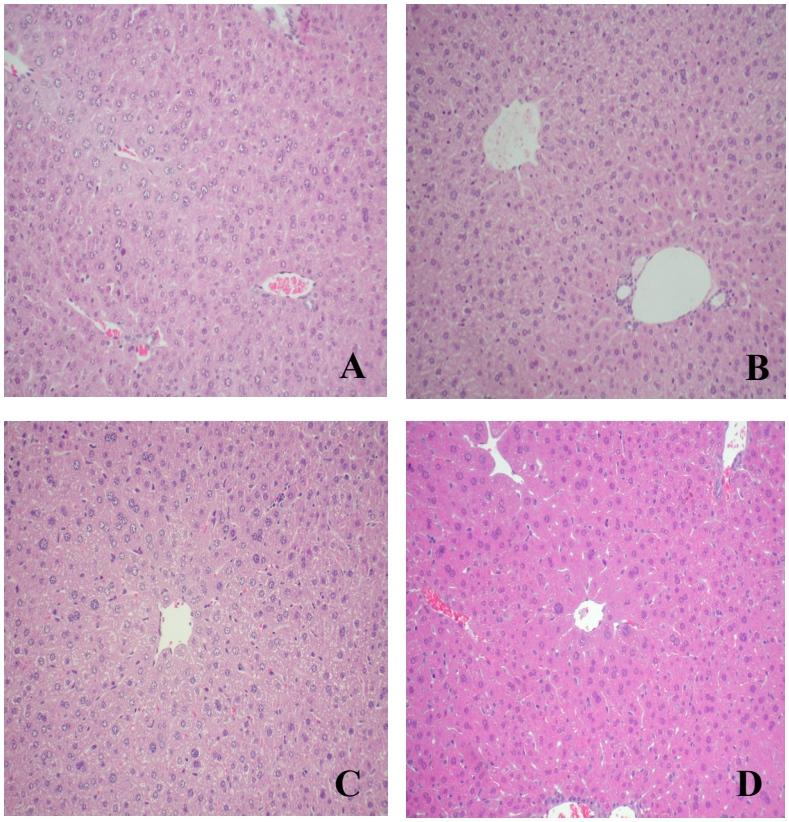


Figure 5. Histopathological findings of liver by hematoxylin and eosin staining.

HBx/p53KO double mutant mice showed swelling of hepatocytes and irregular hepatic cord histopathologically. However, there is not distinct change in the liver of B6 HBxTg and p53KO mice.

A. Liver of 1-month-old B6 mouse

B. Liver of 1-month-old HBxTg mouse

C. Liver of 1-month-old p53KO mouse

D. Liver of 1-month-old HBxTg/p53KO double mutant mouse

3. Proteomic analysis of liver

A. Identification of HBx overexpression specifically expressed proteins in the liver by 2-DE and MALDI-TOF MS

We identified profiles of differentially expressed liver proteins in HBxTg, p53KO and HBxTg/p53KO double mutant mice compared to normal B6 mice. Liver proteins were extracted and analyzed by 2D-PAGE. After 2D gels were visualized by Coomassie blue staining, 2-DE was executed three times for each type of tissue sample from each mouse (Fig. 6). Within each pair (e.g., B6 vs. HBxTg, B6 vs. p53KO, B6 vs. HBxTg/p53KO), the percent volume of the protein spots was compared between each pair using Melanie III. The protein spots that changed > 3 fold up-regulation or < 1/3 fold down-regulation were selected. The selected spots were cut out from the gel and subjected to in-gel digestion with trypsin and peptide fingerprinting by MALDI-TOF MS. The peptide mass data were identified by MS-FIT. In this way, we found up- and down-regulated proteins each pair (Table 2, 4 and 6).

It was 54 proteins changed > 3-fold up-regulation or < 1/3-fold down-regulation in the liver of HBxTg compared to B6 mice (Table 2). 54 proteins were found to be affected by HBx transgene. 54 proteins search for protein function by http://www.ebi.ac.uk/ego/ (Table 3). Among them, oxidoreductase activity

(metabolism resulting in cell Growth) related proteins were 9. For example, aldehyde dehydrogenase family 7 member A1, adehyde dehydrogenase mitochondrial precursor, malate dehydrogenase, 2-oxoisovalerate dehydrogenase alpha subnuit, peroxisomal bifunctional enzyme, sepiapterin reductase were up-regulated proteins, dimethylglycine dehydrogenase, NADH-ubiquinone oxidoreductase 75kDa subunit and peroxiredozin 1 were down-regulated proteins. ATP binding and chaperone activity related proteins were 9. For example, 60kDa heat shock protein mitochondrial precursor, seryl-tRNA synthetase, Stress-70 mitochondrial precursor were up-regulated proteins. 26S proteasome non-ATPase regulatory subunit 13, 26S protease regulatory subunit 6B, S-adenosylmethionine synthetase alpha and beta forms, ATP synthase beta chain mitochondrial precursor, glutathione synthetase, methylcrotonyl-CoA carboxylase alpha chain mitochondrial precursor were down-regulated proteins. Lipid binding and transport related proteins were 5. For example, apolipoprotein E precursor, nonspecific lipid transfer protein mitochondrial precursor, SEC14-like protein 2 were up-regulated proteins. Microsomal triglyceride transfer protein large subunit and PCTP-like protein (PCTP-L) were down-regulated protein. Hydrolase activity related proteins were 3. For example, proteasome subunit alpha type 4, proteasome subunit alpha type 5, transitional endoplasic reticulum ATPase were up-regulated proteins. Calcium ion binding related proteins were 2. Calreticulin precursor was up-regulated protein. Transketolase was down-regulated protein. Cell cycle regulation and signal transduction related proteins were 3.They were zinc finger protein 189 that be related with DNA replication and ribonuclease/angiogenin

inhibitor 1 which is related with ribonuclese inhibitor. These proteins are mainly related with cell cycle regulation. Up-regulated Heterogeneous nuclear ribonucleoproteins A2/B1 and Down-regulated poly (A) polymerase beta were proteins related with RNA processing. Up-regulated guanie nucleotide-binding protein beta subunit-like protein and Down-regulated Pyruvate kinase were proteins related with kinase activity. Down-regulated 10-formyltetrahydrofolate dehydrogenase and thiosulfate sulfurtransferase were proteins related with transferase activity. Down-regulated keratin type I cytoskeletal 18 and keratin type II cytoskeletal 18 were proteins related with cytoskeleton. Remaining 8 were aflatozin biosynthesis, isomerase activity, protein biosynthesis, cell mobility, GTP binding, aminoacylase activity, lyase activity and ligase activity related proteins. Function of 4 proteins could not search and 3 proteins were RIKEN cDNA.

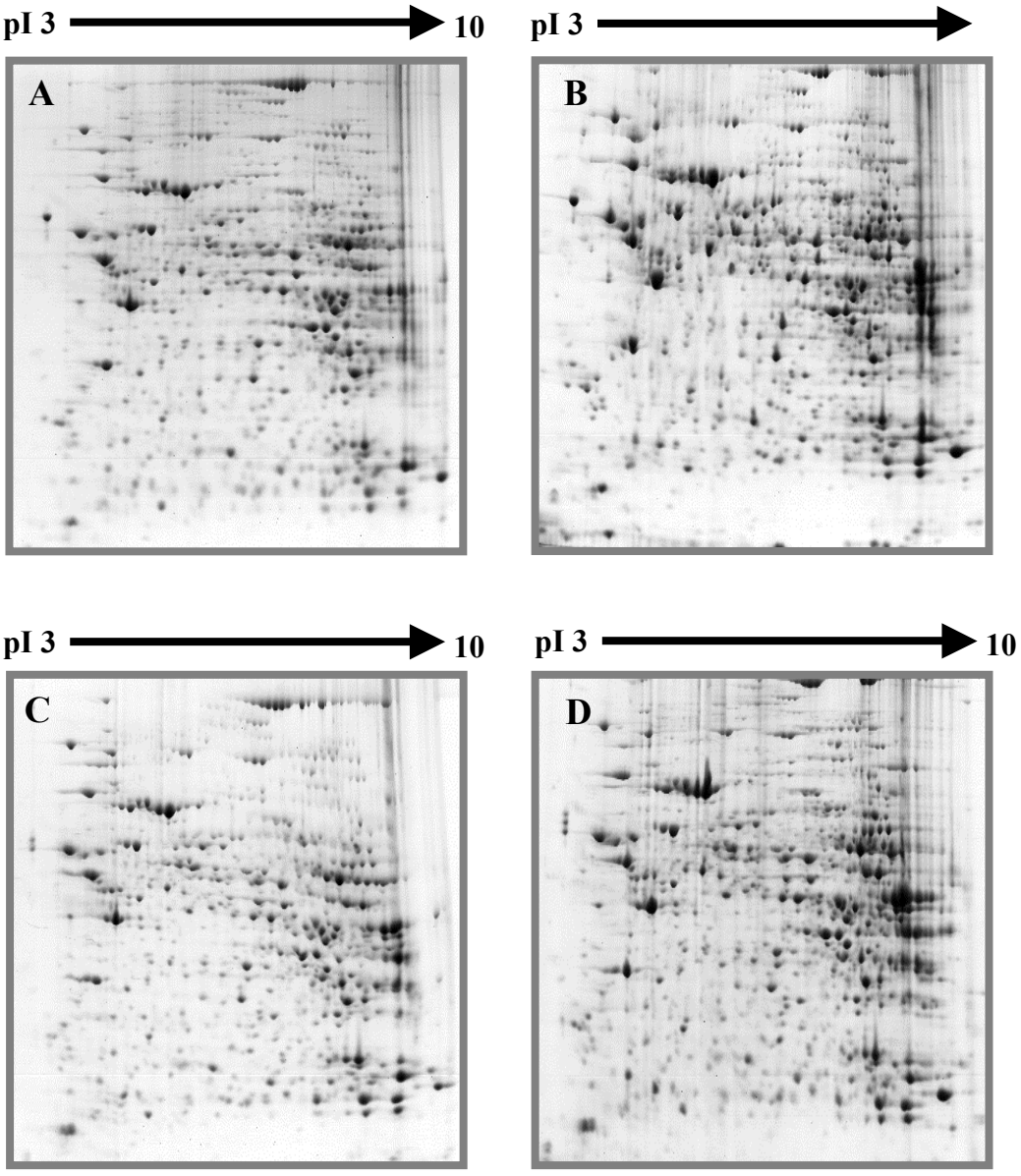

pI 3
10
A
pI 3
B
pI 3
10
C
pI 3
10
D

Figure 6. 2-DE images of master gel in the liver of B6, HBxTg, p53KO and HBxTg/p53KO double mutant mice visualized by Coomassie blue staining. Proteins from the whole liver were extracted and separated on pH2 to 10 nonlinear immobilized pH-gradient strips, followed by a 8-18% SDS polyacrylmaide gel. The gel was stained with Coomassie blue G-250. A. B6 mice, B. HBxTg mice, C. p53KO mice, D. HBxTg/p53KO double mutant mice.

Table 2. Lists of up- and down-regulated proteins in the liver of HBxTg mice compared to B6 mice

B6 vs HBxTg					
Protein name	**Accession #**	**Sequence coverage(%)**	**M.W (Da)//pI**	**Species**	**Regulation**
Aflatoxin B1 aldehyde reductase 1	27527042	32	37677/6.4	MUS MUSCULUS	Up
Aldehyde dehydrogenase family 7 member A1 (Antiquitin 1)	Q9DBF1	26	55645/6	MOUSE	Up
Aldehyde dehydrogenase, mitochondrial precursor (ALDH class 2) (AHD-M1) (ALDHI) (ALDH-E2)	P47738	23	56538/7.5	MOUSE	Up
Apolipoprotein E precursor (Apo-E)	P08226	39	35867/5.6	MOUSE	Up
Calreticulin precursor (CRP55) (CALREGULIN) (HACBP) (ERP60)	P14211	27	47995/4.3	MOUSE	Up
gi|13385656|ref|NP_080428.1| RIKEN cDNA 0610010D20 [Mus musculus]	13385656	33	34644/7.6	UNREADABLE	Up
gi|19527178|ref|NP_598721.1| RIKEN cDNA 9130231C15 [Mus musculus]	19527178	18	61941/5.7	UNREADABLE	Up
gi|21312204|ref|NP_077219.1| RIKEN cDNA 2810435D12 [Mus musculus]	21312204	39	28027/6.3	UNREADABLE	Up
gi|6754472|ref|NP_004847.2| kinesin family member 23 isoform 2; mitotic kinesin-like 1; kinesin-like 5 (mitotic kinesin-like protein 1) [Homo sapiens]	6754472	11	98106/8.7	UNREADABLE	Up
Guanine nucleotide-binding protein beta subunit-like protein 12.3 (P205) (Receptor of activated protein kinase C 1) (RACK1) (Receptor for activated C kinase)	P25388	52	35077/7.6	HUMAN	Up
60 kDa heat shock protein, mitochondrial precursor (Hsp60) (60 kDa chaperonin) (CPN60) (Heat shock protein 60) (HSP-60) (Mitochondrial matrix protein P1) (HSP-65)	P19226	36	60956/5.9	MOUSE	Up
Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2 / hnRNP B1)	O88569	45	35993/8.7	MOUSE	Up
Malate dehydrogenase, cytoplasmic	P14152	31	36477/6.2	MOUSE	Up
Nonspecific lipid-transfer protein, mitochondrial precursor (NSL-TP) (Sterol carrier protein 2) (SCP-2) (Sterol carrier protein X) (SCP-X) (SCPX)	P32020	17	59158/7.2	MOUSE	Up

Protein name	Accession #	Sequence coverage(%)	M.W (Da)//pI	Species	Regulation
2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial precursor (Branched-chain alpha-keto acid dehydrogenase E1 component alpha chain) (BCKDH E1-alpha)	P11960	24	50165/7.7	RAT	Up
Peroxisomal bifunctional enzyme (PBE) (PBFE) [Includes: Enoyl-CoA hydratase ; 3,2-trans-enoyl-CoA isomerase ; 3-hydroxyacyl-CoA dehydrogenase]	Q9DBM2	21	78244/9.3	MOUSE	Up
Proteasome subunit alpha type 4 (Proteasome component C9) (Macropain subunit C9) (Multicatalytic endopeptidase complex subunit C9) (Proteasome subunit L)	Q9R1P0	30	29471/7.6	MOUSE	Up
Proteasome subunit alpha type 5 (Proteasome zeta chain) (Macropain zeta chain) (Multicatalytic endopeptidase complex zeta chain)	Q9Z2U1	30	26411/4.7	MOUSE	Up
Ribonuclease/angiogenin inhibitor 1	16307569	33	49817/4.7	MUS MUSCULUS	Up
SEC14-like protein 2 (Alpha-tocopherol associated protein) (TAP)	Q99J08	32	46301/6.7	MOUSE	Up
Selenium-binding protein 1 (56 KDa selenium-binding protein) (SP56)	P17563	19	52352/6	MOUSE	Up
Selenium-binding protein 2 (56 kDa acetaminophen-binding protein) (AP56)	Q63836	37	52629/5.8	MOUSE	Up
Sepiapterin reductase (SPR)	Q64105	53	27883/5.6	MOUSE	Up
Seryl-tRNA synthetase (Serine--tRNA ligase) (SerRS)	P26638	38	58389/5.9	MOUSE	Up
Stress-70 protein, mitochondrial precursor (75 kDa glucose regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (P66 MOT) (Mortalin)	P38647	21	73529/5.9	MOUSE	Up
Transitional endoplasmic reticulum ATPase (TER ATPase) (15S Mg(2+)-ATPase p97 subunit) (Valosin containing protein) (VCP) [Contains: Valosin]	Q01853	15	89309/5.1	MOUSE	Up
Triosephosphate isomerase (TIM)	P17751	27	26713/6.9	MOUSE	Up
60S acidic ribosomal protein P0 (L10E)	P14869	32	34187/5.9	MOUSE	Down
Actin-like protein 3 (Actin-related protein 3) (Actin-2)	P32391	39	47372/5.6	HUMAN	Down

Protein name	Accession #	Sequence coverage(%)	M.W (Da)//pI	Species	Regulation
S-adenosylmethionine synthetase alpha and beta forms (Methionine adenosyltransferase) (AdoMet synthetase) (MAT-I/III)	Q00266	28	43648/5.9	HUMAN	Down
Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)	P17182	21	47141/6.4	MOUSE	Down
ATP synthase beta chain, mitochondrial precursor	P56480	31	56301/5.2	MOUSE	Down
Dimethylglycine dehydrogenase , mitochondrial precursor (ME2GLYDH)	Q63342	14	96048/6.9	MOUSE	Down
Elongation factor 2 (EF-2)	P05197	8	95285/6.4	RAT	Down
10-formyltetrahydrofolate dehydrogenase (10-FTHFDH)	Q8R0Y6	25	98710/5.6	MOUSE	Down
gi|13384746|ref|NP_079647.1| aminoacylase 1 [Mus musculus]	13384746	53	45795/5.8	UNREADABLE	Down
gi|31982147|ref|NP_775547.2|hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase) [Mus musculus]	31982147	13	89911/6.6	UNREADABLE	Down
Glutathione synthetase (Glutathione synthase) (GSH synthetase) (GSH-S)	P51855	33	52247/5.6	MOUSE	Down
Keratin, type I cytoskeletal 18 (Cytokeratin 18) (Cytokeratin endo B) (Keratin D)	P05784	36	47504/5.2	MOUSE	Down
Keratin, type II cytoskeletal 8 (Cytokeratin 8) (Cytokeratin endo A)	P11679	17	54450/5.5	MOUSE	Down
Methylcrotonyl-CoA carboxylase alpha chain, mitochondrial precursor (3-Methylcrotonyl-CoA carboxylase 1) (MCCase alpha subunit) (3-methylcrotonyl-CoA:carbon dioxide ligase alpha subunit)	Q99MR8	29	79345/7.7	MOUSE	Down
Microsomal triglyceride transfer protein, large subunit precursor	O08601	33	99143/7.8	MOUSE	Down
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor (Complex I-75Kd) (CI-75Kd)	P28331	18	79574/5.8	HUMAN	Down
PCTP-like protein (PCTP-L) (StAR-related lipid transfer protein 10) (StARD10) (START domain-containing protein 10) (Serologically defined colon cancer antigen 28 homolog)	Q9JMD3	31	32952/6.7	MOUSE	Down

Protein name	Accession #	Sequence coverage(%)	M.W (Da)//pI	Species	Regulation
Peroxiredoxin 1(Thioredoxin peroxidase 2) (Thioredoxin-dependent peroxide reductase 2) (Osteoblast specific factor 3) (OSF-3) (Macrophage 23 kDa stress protein)	P35700	47	22177/8.3	MOUSE	Down
Poly(A) polymerase beta (PAP beta) (Polynucleotide adenylyltransferase beta) (Testis-specific poly(A) polymerase)	Q9NRJ5	18	71682/6.0	MOUSE	Down
26S protease regulatory subunit 6B (MIP224) (MB67 interacting protein) (TAT-binding protein-7) (TBP-7)	P43686	29	47367/5.1	HUMAN	Down
26S proteasome non-ATPase regulatory subunit 13 (26S proteasome regulatory subunit S11) (26S proteasome regulatory subunit p40.5)	Q9WVJ2	34	42810/5.5	MOUSE	Down
Pyruvate kinase, isozymes R/L (L-PK)	P53657	22	62309/6.6	MOUSE	Down
Serine/threonine protein phosphatase 2A, 65 KDA regulatory subunit A, alpha isoform (PP2A, subunit A, PR65-alpha isoform) (PP2A, subunit A, R1-alpha isoform) (Medium tumor antigen-associated 61 KDA protein)	P30153	36	65224/5.0	HUMAN	Down
Succinyl-CoA ligase[GDP-forming] beta-chain, mitochondrial precursor(Succinyl-CoA synthetase, betaG chain) (SCS-betaG) (GTP-specific succinyl-CoA synthetase beta subunit)	Q9Z2I8	19	43858/5.9	MOUSE	Down
Thiosulfate sulfurtransferase (Rhodanese)	P52196	48	33466/7.7	MOUSE	Down
Transketolase (TK) (P68)	P40142	29	67631/7.2	MOUSE	Down
Zinc finger protein 189	O75820	16	72979/8.8	HUMAN	Down

Up-regulated : [> 3-fold], Down-regultaed : [< 1/3-fold]

Table 3. Functional annotation of differentially expressed proteins in the liver of HBxTg mice compared to B6 mice

Function	Protein name	Accession #	Species	Regulation
Oxidoreductase activity (metabolism resulting in cell growth)	Aldehyde dehydrogenase family 7 member A1 (Antiquitin 1)	Q9DBF1	MOUSE	Up
	Aldehyde dehydrogenase, mitochondrial precursor (ALDH class 2) (AHD-M1) (ALDHI) (ALDH-E2)	P47738	MOUSE	Up
	Malate dehydrogenase, cytoplasmic *(malate degydrogenase activity)*	P14152	MOUSE	Up
	2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial precursor (Branched-chain alpha-keto acid dehydrogenase E1 component alpha chain) (BCKDH E1-alpha)	P11960	RAT	Up
	Peroxisomal bifunctional enzyme (PBE) (PBFE) [Includes: Enoyl-CoA hydratase ; 3,2-trans-enoyl-CoA isomerase ; 3-hydroxyacyl-CoA dehydrogenase] *(catalytic activity, ion transport : ADP, ATP carrier protein)*	Q9DBM2	MOUSE	Up
	Sepiapterin reductase (SPR)	Q64105	MOUSE	Up
	Dimethylglycine dehydrogenase , mitochondrial precursor (ME2GLYDH) *(electron transport)*	Q63342	MOUSE	Down
	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor (Complex I-75Kd) (CI-75Kd) *(NADH dehydrogenase activity, electron transport activity)*	P28331	HUMAN	Down
	Peroxiredoxin 1(Thioredoxin peroxidase 2) (Thioredoxin-dependent peroxide reductase 2) (Osteoblast specific factor 3) (OSF-3) (Macrophage 23 kDa stress protein) *(antioxidant activity, peroxidase activity)*	P35700	MOUSE	Down
ATP binding and chaperone activity	60 kDa heat shock protein, mitochondrial precursor (Hsp60) (60 kDa chaperonin) (CPN60) (Heat shock protein 60) (HSP-60) (Mitochondrial matrix protein P1) (HSP-65)	P19226	MOUSE	Up
	Seryl-tRNA synthetase (Serine--tRNA ligase) (SerRS) *(serin-tRNA ligase activity)*	P26638	MOUSE	Up

Function	Protein name	Accession #	Species	Regulation
	Stress-70 protein, mitochondrial precursor (75 kDa glucose regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (P66 MOT) (Mortalin) ***(binding to p53)***	**P38647**	**MOUSE**	**Up**
	26S proteasome non-ATPase regulatory subunit 13 (26S proteasome regulatory subunit S11) (26S proteasome regulatory subunit p40.5)	**Q9WVJ2**	**MOUSE**	**Down**
	26S protease regulatory subunit 6B (MIP224) (MB67 interacting protein) (TAT-binding protein-7) (TBP-7) ***(proteosome ATP binding)***	**P43686**	**HUMAN**	**Down**
	S-adenosylmethionine synthetase alpha and beta forms (Methionine adenosyltransferase) (AdoMet synthetase) (MAT-I/III) ***(amino acid metabolism)***	**Q00266**	**HUMAN**	**Down**
	ATP synthase beta chain, mitochondrial precursor ***(ion transporter activity, neucleotide binding)***	**P56480**	**MOUSE**	**Down**
	Glutathione synthetase (Glutathione synthase) (GSH synthetase) (GSH-S) ***(glutathion biosynthesis)***	**P51855**	**MOUSE**	**Down**
	Methylcrotonyl-CoA carboxylase alpha chain, mitochondrial precursor (3-Methylcrotonyl-CoA carboxylase 1) (MCCase alpha subunit) (3-methylcrotonyl-CoA:carbon dioxide ligase alpha subunit) ***(biotin binding metabolism, ligase activity)***	**Q99MR8**	**MOUSE**	**Down**
Lipid binding & transport	**Apolipoprotein E precursor (Apo-E)**	**P08226**	**MOUSE**	**Up**
	Nonspecific lipid-transfer protein, mitochondrial precursor (NSL-TP) (Sterol carrier protein 2) (SCP-2) (Sterol carrier protein X) (SCP-X) (SCPX) ***(protein peroxisome targeting, fatty acid binding, sterol carrier activity)***	**P32020**	**MOUSE**	**Up**
	SEC14-like protein 2 (Alpha-tocopherol associated protein) (TAP) ***(transcriptional activator activity, peptidase activity)***	**Q99J08**	**MOUSE**	**Up**
	Microsomal triglyceride transfer protein, large subunit precursor	**O08601**	**MOUSE**	**Down**

Function	Protein name	Accession #	Species	Regulation
Lipid binding & transport	**PCTP-like protein (PCTP-L) (StAR-related lipid transfer protein 10) (StARD10) (START domain-containing protein 10) (Serologically defined colon cancer antigen 28 homolog)** ***(may play specific roles in sperm maturation or fertilization)***	**Q9JMD3**	**MOUSE**	**Down**
Hydrolase activity	**Proteasome subunit alpha type 4 (Proteasome component C9) (Macropain subunit C9) (Multicatalytic endopeptidase complex subunit C9) (Proteasome subunit L)** ***(endopeptidase activity, peptidase activity)***	**Q9R1P0**	**MOUSE**	**Up**
	Proteasome subunit alpha type 5 (Proteasome zeta chain) (Macropain zeta chain) (Multicatalytic endopeptidase complex zeta chain) ***(endopeptidase activity, peptidase activity,)***	**Q9Z2U1**	**MOUSE**	**Up**
	Transitional endoplasmic reticulum ATPase (TER ATPase) (15S Mg(2+)-ATPase p97 subunit) (Valosin containing protein) (VCP) [Contains: Valosin] ***(ATP binding, cell growth or maintenace)***	**Q01853**	**MOUSE**	**Up**
Calcium ion binding	**Calreticulin precursor (CRP55) (CALREGULIN) (HACBP) (ERP60)**	**P14211**	**MOUSE**	**Up**
	Transketolase (TK) (P68)	**P40142**	**MOUSE**	**Down**
Cell cycle regulation & signal transduction	**gi|6754472|ref|NP_004847.2| kinesin family member 23 isoform 2; mitotic kinesin-like 1; kinesin-like 5 (mitotic kinesin-like protein 1) [Homo sapiens]**	**6754472**	**UNREADABLE**	**Up**
	Ribonuclease/angiogenin inhibitor 1	**16307569**	**MUS MUSCULUS**	**Up**
	Zinc finger protein 189	**O75820**	**HUMAN**	**Down**
RNA processing	**Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2 / hnRNP B1)**	**O88569**	**MOUSE**	**Up**
	Poly(A) polymerase beta (PAP beta) (Polynucleotide adenylyltransferase beta) (Testis-specific poly(A) polymerase) ***(transferase activity)***	**Q9NRJ5**	**MOUSE**	**Down**
Kinase ctivity	**Guanine nucleotide-binding protein beta subunit-like protein 12.3 (P205) (Receptor of activated protein kinase C 1) (RACK1) (Receptor for activated C kinase)**	**P25388**	**HUMAN**	**Up**
	Pyruvate kinase, isozymes R/L (L-PK)	**P53657**	**MOUSE**	**Down**

Function	Protein name	Accession #	Species	Regulation
Transferase activity	10-formyltetrahydrofolate dehydrogenase (10-FTHFDH) *(biosynthsis)*	Q8R0Y6	MOUSE	Down
	Thiosulfate sulfurtransferase (Rhodanese)	P52196	MOUSE	Down
Cytoskeleton related	Keratin, type I cytoskeletal 18 (Cytokeratin 18) (Cytokeratin endo B) (Keratin D)	P05784	MOUSE	Down
	Keratin, type II cytoskeletal 8 (Cytokeratin 8) (Cytokeratin endo A)	P11679	MOUSE	Down
Aflatoxin biosynthesis	Aflatoxin B1 aldehyde reductase 1	27527042	MUS MUSCULUS	Up
Isomerase activity	Triosephosphate isomerase (TIM) *(glycolysis, fatty acid biosynthesis)*	P17751	MOUSE	Up
Protein bio synthesis	60S acidic ribosomal protein P0 (L10E)	P14869	MOUSE	Down
Cell mobility	Actin-like protein 3 (Actin-related protein 3) (Actin-2)	P32391	HUMAN	Down
Lyase activity & hydratase activity	Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1) *(glycolysis)*	P17182	MOUSE	Down
GTP binding	Elongation factor 2 (EF-2) *(translation elongation factor activity)*	P05197	RAT	Down
Aminoacylase activity	gi|13384746|ref|NP_079647.1| aminoacylase 1 [Mus musculus]	13384746	UNREADABLE	Down
Ligase activity	Succinyl-CoA ligase[GDP-forming] beta-chain, mitochondrial precursor(Succinyl-CoA synthetase, betaG chain) (SCS-betaG) (GTP-specific succinyl-CoA synthetase beta subunit)	Q9Z2I8	MOUSE	Down
Not matched to functional annotation	Serine/threonine protein phosphatase 2A, 65 KDA regulatory subunit A, alpha isoform (PP2A, subunit A, PR65-alpha isoform) (PP2A, subunit A, R1-alpha isoform) (Medium tumor antigen-associated 61 KDA protein)	P30153	HUMAN	Down
	gi|31982147|ref|NP_775547.2|hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase) [Mus musculus]	31982147	UNREADABLE	Down
	Selenium-binding protein 1 (56 KDa selenium-binding protein) (SP56)	P17563	MOUSE	Up
	Selenium-binding protein 2 (56 kDa acetaminophen-binding protein) (AP56)	Q63836	MOUSE	Up

Function	Protein name	Accession #	Species	Regulation
Not matched to functional annotation	**gi|13385656|ref|NP_080428.1| RIKEN cDNA 0610010D20 [Mus musculus]**	**13385656**	**UNREADABLE**	**Up**
	gi|19527178|ref|NP_598721.1| RIKEN cDNA 9130231C15 [Mus musculus]	**19527178**	**UNREADABLE**	**Up**
	gi|21312204|ref|NP_077219.1| RIKEN cDNA 2810435D12 [Mus musculus]	**21312204**	**UNREADABLE**	**Up**

Up-regulated : [> 3-fold], Down-regultaed : [< 1/3-fold]

These results were categories by http://www.ebi.ac.uk/ego/

B. Identification of specifically expressed proteins related with the functional loss of p53 by 2-DE and MALDI-TOF MS

It was 44 proteins changed > 3-fold up-regulation or < 1/3-fold down-regulation in the liver of p53KO compared to B6 mice (Table 4). 44 proteins were found to be affected by p53 knock-out. 44 proteins search for protein function by http://www.ebi.ac.uk/ego/ (Table 5).

Among them, oxidoreductase activity (metabolism resulting in cell Growth) related proteins were 6. For example, aldehyde dehydrogenase 1A1, adehyde dehydrogenase mitochondrial precursor, glyceraldehyde 3-phosphate dehydrogenase, 2-oxoisovalerate dehydrogenase alpha subnuit mitochondrial precursor, short chain 3-hydroxyacyl-CoA dehydrogenase mitochondrial precursor were up-regulated proteins, dimethylglycine dehydrogenase was down-regulated protein. ATP binding and chaperone activity related proteins were 7. For example, ATP synthase alpha chain mitochondrial precursor, stress-70 protein mitochondrial precursor, transitional endoplasmic reticulum ATPase were up-regulated proteins. Heat shock protein 75kDa mitochondrial precursor, methylcrotonyl-CoA carboxylase alpha chain mitochondrial precursor, S-adenosylmethionine synthetase alpha and beta forms, T-complex protein 1 were down-regulated proteins. Lipid binding and transport related proteins were 5. For example, apolipoprotein E precursor, fatty acid–binding protein, nonspecific lipid transfer protein mitochondrial precursor were up-regulated proteins. SEC14-like

protein 2, serum albumin precursor were down-regulated proteins. Down-regulated dihydropyrimidinase was hydrolase activity related protein. Calcium ion binding related proteins were 2. Calreticulin precursor and transketolase were down-regulated proteins. Cell cycle regulation and signal transduction related proteins were 2. They were 14-3-3 protein epsilon and ribosome-binding protein 1. All of them were down-regulated proteins. Up-regulated guanine nucleotide-binding protein beta subunit-like protein 12.3, adenosine kinase and down-regulated ketohexokinase were related with kinase activity. Up-regulated betaine-Homocysteine S-methyltransferase and Down-regulated 10-formyltetrahydrofolate dehydrogenase, ornithine carbamoyltransferase mitochondrial precursor, serine hydroxymethyltransferase, thiosulfate sulfurtransferase were related with transferase activity. There were 3 proteins have Lyase activity and hydratase activity. Alpha enolase, probable urocanate hydratase and cystathionine beta-synthase were down-regulated proteins. Arginase activity related proteins were 3. There were arginase 1, hydrozymethylglutaryl-CoA lyase mitochondrial precursor and phosphoenolpyruvate carboxykinase. All of them were down-regulated proteins. Remaining 5 proteins were aflatoxin biosynthesis, isomerase activity, cell mobility, GTP binding aminoacylase activity related proteins. Function of 2 proteins could not search.

Table 4. Lists of up and down-regulated proteins in the liver of p53KO mice compared to B6 mice

B6 vs p53KO					
Protein name	**Accession #**	**Sequence coverage(%)**	**M.W (Da)//pI**	**Species**	**Regulation**
Actin-like protein 3 (Actin-related protein 3) (Actin-2)	P32391	53	47372/5.6	HUMAN	Up
Aldehyde dehydrogenase 1A1 (Aldehyde dehydrogenase, cytosolic) (ALDH class 1) (ALHDII) (ALDH-E1)	P24549	36	54450/7.9	MOUSE	Up
Aldehyde dehydrogenase, mitochondrial precursor (ALDH class 2) (AHD-M1) (ALDHI) (ALDH-E2)	P47738	30	56538/7.5	MOUSE	Up
Apolipoprotein E precursor (Apo-E)	P08226	24	35867/5.6	MOUSE	Up
ATP synthase alpha chain, mitochondrial precursor	Q03265	28	59753/9.2	MOUSE	Up
Betaine—Homocysteine S-methyltransferase	O35490	40	45021/8.0	MOUSE	Up
Fatty acid-binding protein, liver (L-FABP) (14 kDa selenium-binding protein)	P12710	54	14246/8.6	MOUSE	Up
gi|19527306|ref|NP_598840.1| adenosine kinase [Mus musculus]	19527306 M	27	40149/5.8	UNREADABLE	Up
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	P16858	28	35810/8.4	MOUSE	Up
Guanine nucleotide-binding protein beta subunit-like protein 12.3 (P205) (Receptor of activated protein kinase C 1) (RACK1) (Receptor for activated C kinase)	P25388	70	35077/7.6	HUMAN	Up
Nonspecific lipid-transfer protein, mitochondrial precursor (NSL-TP) (Sterol carrier protein 2) (SCP-2) (Sterol carrier protein X) (SCP-X) (SCPX)	P32020	23	59126/7.2	MOUSE	Up
2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial precursor (Branched-chain alpha-keto acid dehydrogenase E1 component alpha chain) (BCKDH E1-alpha)	P11960	53	50165/7.7	RAT	Up
Short chain 3-hydroxyacyl-CoA dehydrogenase, mitochondrial precursor (HCDH) (Medium and short chain L-3-hydroxyacyl-coenzyme A dehydrogenase)	Q61425	36	34464/8.8	MOUSE	Up

Protein name	Accession #	Sequence coverage(%)	M.W (Da)//pI	Species	Regulation
Stress-70 protein, mitochondrial precursor (75 kDa glucose regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (P66 MOT) (Mortalin)	P38647	27	73529/5.9	MOUSE	Up
Transitional endoplasmic reticulum ATPase (TER ATPase) (15S Mg(2+)-ATPase p97 subunit) (Valosin containing protein) (VCP) [Contains: Valosin]	Q01853	36	89309/5.1	MOUSE	Up
Aflatoxin B1 aldehyde reductase 1	27527042	40	37677/6.4	MOUSE	Down
Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)	P17182	23	47141/6.4	MOUSE	Down
Arginase 1 (Liver-type arginase)	Q61176	24	34808/6.5	MOUSE	Down
Calreticulin precursor (CRP55) (CALREGULIN) (HACBP) (ERP60)	P14211	35	47995/4.3	MOUSE	Down
Cystathionine beta-synthase (Serine sulfhydrase) (Beta-thionase) (Hemoprotein H-450)	P32232	17	61455/6.1	RAT	Down
Dihydropyrimidinase (DHPase) (Hydantoinase) (DHP)	Q9EQF5	46	56725/6.7	MOUSE	Down
Dimethylglycine dehydrogenase, mitochondrial precursor (ME2GLYDH)	Q63342	17	96048/6.9	RAT	Down
Elongation factor 2 (EF-2)	P05197	39	95285/6.4	RAT	Down
10-formyltetrahydrofolate dehydrogenase (10-FTHFDH)	Q8R0Y6	28	98710/5.6	MOUSE	Down
gi|13384746|ref|NP_079647.1| aminoacylase 1 [Mus musculus]	13384746	53	45795/5.8	UNREADABLE	Down
gi|20149748|ref|NP_619606.1| sarcosine dehydrogenase [Mus musculus]	20149748 M	37	101683/6.3	UNREADABLE	Down
Heat shock protein 75 kDa, mitochondrial precursor (HSP 75) (Tumor necrosis factor type 1 receptor associated protein) (TRAP-1) (TNFR-associated protein 1)	Q9CQN1	35	80210/6.2	MOUSE	Down
Hydroxymethylglutaryl-coA lyase, mitochondrial precursor (HMG-COA LYASE) (HL) (3-hydroxy-3-methylglutarate-coA lyase)	P38060	28	34161/8.6	MOUSE	Down
Ketohexokinase (Hepatic fructokinase)	P97328	28	32751/5.8	MOUSE	Down

Protein name	Accession #	Sequence coverage(%)	M.W (Da)//pI	Species	Regulation
Methylcrotonyl-CoA carboxylase alpha chain, mitochondrial precursor (3-Methylcrotonyl-CoA carboxylase 1) (MCCase alpha subunit) (3-methylcrotonyl-CoA:carbon dioxide ligase alpha subunit)	Q99MR8	17	79345/7.7	MOUSE	Down
Methylmalonyl-CoA mutase, mitochondrial precursor (MCM)	P16332	32	82966/6.7	MOUSE	Down
Ornithine carbamoyltransferase, mitochondrial precursor (OTCase) (Ornithine transcarbamylase)	P11725	20	39765/8.8	MOUSE	Down
Phosphoenolpyruvate carboxykinase, cytosolic [GTP] (Phosphoenolpyruvate carboxylase) (PEPCK-C)	Q9Z2V4	28	69355/6.2	MOUSE	Down
Probable urocanate hydratase (Urocanase) (Imidazolonepropionate hydrolase)	Q8VC12	34	74591/7.3	MOUSE	Down
14-3-3 protein epsilon (Mitochondrial import stimulation factor L subunit) (Protein kinase C inhibitor protein-1) (KCIP-1) (14-3-3E)	P42655	27	29174/4.6	HUMAN	Down
Ribosome-binding protein 1 (Ribosome receptor protein) (mRRp)	Q99PL5	26	172881/9.4	MOUSE	Down
S-adenosylmethionine synthetase alpha and beta forms (Methionine adenosyltransferase) (AdoMet synthetase) (MAT-I/III)	Q00266	29	43648/5.9	HUMAN	Down
SEC14-like protein 2 (Alpha-tocopherol associated protein) (TAP)	Q99J08	41	46301/6.7	MOUSE	Down
Serine hydroxymethyltransferase, cytosolic (Serine methylase) (Glycine hydroxymethyltransferase) (SHMT)	P50431	23	52585/6.5	MOUSE	Down
Serine/threonine protein phosphatase 2A, 65 KDA regulatory subunit A, alpha isoform (PP2A, subunit A, PR65-alpha isoform) (PP2A, subunit A, R1-alpha isoform) (Medium tumor antigen-associated 61 KDA protein)	P30153	32	65224/5.0	HUMAN	Down
Serum albumin precursor	P07724	45	68693/5.7	MOUSE	Down
T-complex protein 1, zeta subunit (TCP-1-zeta) (CCT-zeta) (CCT-zeta-1)	P80317	34	58005/6.6	MOUSE	Down
Thiosulfate sulfurtransferase (Rhodanese)	P52196	47	33466/7.7	MOUSE	Down

Protein name	Accession #	Sequence coverage(%)	M.W (Da)//pI	Species	Regulation
Transketolase (TK) (P68)	**P40142**	**27**	**67631/7.2**	**MOUSE**	**Down**

Up-regulated : [> 3-fold], Down-regultaed : [< 1/3-fold]

Table 5. Functional annotation of differentially expressed proteins in the liver of p53KO mice compared to B6 mice

Function	Protein name	Accession #	Species	Regulation
Oxidoreductase activity (metabolism resulting in cell growth)	Aldehyde dehydrogenase 1A1 (Aldehyde dehydrogenase, cytosolic) (ALDH class 1) (ALHDII) (ALDH-E1) *(aldehyde degydrogenase activity)*	P24549	MOUSE	Up
	Aldehyde dehydrogenase, mitochondrial precursor (ALDH class 2) (AHD-M1) (ALDHI) (ALDH-E2) *(aldehyde degydrogenase activity)*	P47738	MOUSE	Up
	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) *(glycolysis)*	P16858	MOUSE	Up
	2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial precursor (Branched-chain alpha-keto acid dehydrogenase E1 component alpha chain) (BCKDH E1-alpha)	P11960	RAT	Up
	Short chain 3-hydroxyacyl-CoA dehydrogenase, mitochondrial precursor (HCDH) (Medium and short chain L-3-hydroxyacyl-coenzyme A dehydrogenase) *(fatty acid metabolism)*	Q61425	MOUSE	Up
	Dimethylglycine dehydrogenase, mitochondrial precursor (ME2GLYDH) *(electron transport)*	Q63342	RAT	Down
ATP binding and chaperone activity	ATP synthase alpha chain, mitochondrial precursor	Q03265	MOUSE	Up
	Stress-70 protein, mitochondrial precursor (75 kDa glucose regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (P66 MOT) (Mortalin) *(binding to p53)*	P38647	MOUSE	Up
	Transitional endoplasmic reticulum ATPase (TER ATPase) (15S Mg(2+)-ATPase p97 subunit) (Valosin containing protein) (VCP) [Contains: Valosin] *(cell growth or maintenace)*	Q01853	MOUSE	Up
	Heat shock protein 75 kDa, mitochondrial precursor (HSP 75) (Tumor necrosis factor type 1 receptor associated protein) (TRAP-1) (TNFR-associated protein 1)	Q9CQN1	MOUSE	Down

Function	Protein name	Accession #	Species	Regulation
	Methylcrotonyl-CoA carboxylase alpha chain, mitochondrial precursor (3-Methylcrotonyl-CoA carboxylase 1) (MCCase alpha subunit) (3-methylcrotonyl-CoA:carbon dioxide ligase alpha subunit) *(biotin binding metabolism, ligase activity)*	Q99MR8	MOUSE	Down
	S-adenosylmethionine synthetase alpha and beta forms (Methionine adenosyltransferase) (AdoMet synthetase) (MAT-I/III) *(amino acid metabolism)*	Q00266	HUMAN	Down
	T-complex protein 1, zeta subunit (TCP-1-zeta) (CCT-zeta) (CCT-zeta-1)	P80317	MOUSE	Down
Lipid binding & transport	**Apolipoprotein E precursor (Apo-E)**	P08226	MOUSE	Up
	Fatty acid-binding protein, liver (L-FABP) (14 kDa selenium-binding protein)	P12710	MOUSE	Up
	Nonspecific lipid-transfer protein, mitochondrial precursor (NSL-TP) (Sterol carrier protein 2) (SCP-2) (Sterol carrier protein X) (SCP-X) (SCPX) *(protein peroxisome targeting, fatty acid binding, sterol carrier activity)*	P32020	MOUSE	Up
	SEC14-like protein 2 (Alpha-tocopherol associated protein) (TAP) *(transcriptional activator activity, peptidase activity)*	Q99J08	MOUSE	Down
	Serum albumin precursor	P07724	MOUSE	Down
Hydrolase activity	**Dihydropyrimidinase (DHPase) (Hydantoinase) (DHP)**	Q9EQF5	MOUSE	Down
Calcium ion binding	**Calreticulin precursor (CRP55) (CALREGULIN) (HACBP) (ERP60)**	P14211	MOUSE	Down
	Transketolase (TK) (P68)	P40142	MOUSE	Down
Cell cycle regulation & signal transduction	**14-3-3 protein epsilon (Mitochondrial import stimulation factor L subunit) (Protein kinase C inhibitor protein-1) (KCIP-1) (14-3-3E)** *(protein folding, signal cascade)*	P42655	HUMAN	Down
	Ribosome-binding protein 1 (Ribosome receptor protein) (mRRp)	Q99PL5	MOUSE	Down

Function	Protein name	Accession #	Species	Regulation
Kinase activity	Guanine nucleotide-binding protein beta subunit-like protein 12.3 (P205) (Receptor of activated protein kinase C 1) (RACK1) (Receptor for activated C kinase) *(signal transduction)*	P25388	HUMAN	Up
	gi|19527306|ref|NP_598840.1| adenosine kinase [Mus musculus]	19527306_M	UNREADABLE	Up
	Ketohexokinase (Hepatic fructokinase) *(ketohexokinase activity, transferase activity)*	P97328	MOUSE	Down
Transferase activity	Betaine—Homocysteine S-methyltransferase	O35490	MOUSE	Up
	10-formyltetrahydrofolate dehydrogenase (10-FTHFDH) *(biosynthesis)*	Q8R0Y6	MOUSE	Down
	Ornithine carbamoyltransferase, mitochondrial precursor (OTCase) (Ornithine transcarbamylase) *(amino acid metabolism, arginine biosynthesis)*	P11725	MOUSE	Down
	Serine hydroxymethyltransferase, cytosolic (Serine methylase) (Glycine hydroxymethyltransferase) (SHMT)	P50431	MOUSE	Down
	Thiosulfate sulfurtransferase (Rhodanese)	P52196	MOUSE	Down
Aflatoxin biosynthesis	aflatoxin B1 aldehyde reductase 1	27527042	MOUSE	Down
Isomerase activity	Methylmalonyl-CoA mutase, mitochondrial precursor (MCM)	P16332	MOUSE	Down
Cell mobility	Actin-like protein 3 (Actin-related protein 3) (Actin-2)	P32391	HUMAN	Up
Lyase activity & hydratase activity	Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)	P17182	MOUSE	Down
	Probable urocanate hydratase (Urocanase) (Imidazolonepropionate hydrolase) *(histidine, catabolism)*	Q8VC12	MOUSE	Down
	Cystathionine beta-synthase (Serine sulfhydrase) (Beta-thionase) (Hemoprotein H-450) *(amino acid metabolism, cystein biosynthesis)*	P32232	RAT	Down
Aminoacylase activity	gi|13384746|ref|NP_079647.1| aminoacylase 1 [Mus musculus]	13384746	UNREADABLE	Down

Function	Protein name	Accession #	Species	Regulation
Arginase activity	**Arginase 1 (Liver-type arginase)**	**Q61176**	**MOUSE**	**Down**
	Hydroxymethylglutaryl-coA lyase, mitochondrial precursor (HMG-COA LYASE) (HL) (3-hydroxy-3-methylglutarate-coA lyase) ***(catalytic activity)***	**P38060**	**MOUSE**	**Down**
	Phosphoenolpyruvate carboxykinase, cytosolic [GTP] (Phosphoenolpyruvate carboxylase) (PEPCK-C) ***(lyase activity, lipid metabolism)***	**Q9Z2V4**	**MOUSE**	**Down**
GTP binding	**Elongation factor 2 (EF-2)** ***(translation elongation factor activity)***	**P05197**	**RAT**	**Down**
Not matched to functional annotation	**Serine/threonine protein phosphatase 2A, 65 KDA regulatory subunit A, alpha isoform (PP2A, subunit A, PR65-alpha isoform) (PP2A, subunit A, R1-alpha isoform) (Medium tumor antigen-associated 61 KDA protein)**	**P30153**	**HUMAN**	**Down**
	gi|20149748|ref|NP_619606.1| sarcosine dehydrogenase [Mus musculus]	**20149748 M**	**UNREADABLE**	**Down**

Up-regulated : [> 3-fold], Down-regultaed : [< 1/3-fold]

These results were categories by http://www.ebi.ac.uk/ego/

C. Identification of specifically expressed proteins in the liver HBxTg/p53KO double mutant mice by 2-DE and MALDI-TOF MS.

It was 31 proteins changed > 3-fold up-regulation or < 1/3-fold down-regulation in the liver of HBxTg/p53KO compared to B6 mice (Table 6). 31 proteins were found to be affected by HBx transgene and p53 knock-out. 31 proteins search for protein function by http://www.ebi.ac.uk/ego/ (Table 7).

Among them, Oxidoreductase activity (metabolism resulting in cell Growth) related proteins were 8. For example, acyl-CoA dehydrogenase long-chain specific mitochondrial precursor, acyl-CoA dehydrogenase medium-chain specific mitochondrial precursor, acyl-CoA dehydrogenase very long-chain specific mitochondrial precursor, aldehyde dehydrogenase 1A1, 2-oxoisovalerate dehydrogenase alpha subnuit mitochondrial precursor and phenylalanine-4-hydroxylase were up-regulated proteins. Dimethylglycine dehydrogenase and sulfite oxidase mitochondrial precursor were down-regulated proteins. ATP binding and chaperone activity related proteins were 4. For example, transitional endoplasmic reticulum ATPase is up-regulated protein. Heat shock protein 75KDa mitochondrial precursor, pyruvate carboxylase mitochondrial precursor and S-adenosylmethionine synthetase alpha and beta forms were down-regulated proteins. Transferase activity related proteins were 7. 4-aminobutyrate aminotransferase mitochondrial precursor, aspartate amonotransferase, hydrozymethylglutaryl-CoA synthase mitochondrial precursor and serine hydroxymethyltrasnferase were up-regulated proteins. 10-

formyltetrahydrofolate dehydrogenase, guanidinoacetate N-methyltransferase and thiosulfate sulfurtransferase were down-regulated proteins. There were 3 proteins have Lyase activity and hydratase activity. Lactoylflutathione lyase and probable urocanate hydratase were up-regulated proteins. Alpha enolase is down-regulated protein. Remaining 8 proteins were lipid binding and transport, hydrolase activity, calcium ion binding, aflatoxin biosynthesis, cell cycle regulation and signal transduction, ligase activity, malate metabolism and actin binding related proteins. Function of 1 protein could not search.

Table 6. Lists of up and down-regulated proteins in the liver of HBxTg/p53KO double mutant mice compared to B6 mice

B6 vs HBxTg/p53KO					
Protein name	**Accession #**	**Sequence coverage(%)**	**M.W (Da)//pI**	**Species**	**Regulation**
4-aminobutyrate aminotransferase, mitochondrial precursor (Gamma-Amino-N-Butyrate Transaminase) (GABA transaminase) (GABA aminotransferase) (GABA-AT)	P50554	36	56530/9.0	RAT	Up
Acyl-CoA dehydrogenase, long-chain specific, mitochondrial precursor (LCAD)	P51174	21	47908/8.5	MOUSE	Up
Acyl-CoA dehydrogenase, medium-chain specific, mitochondrial precursor (MCAD)	P45952	24	46482/8.6	MOUSE	Up
Acyl-CoA dehydrogenase, very-long-chain specific, mitochondrial precursor (VLCAD) (MVLCAD)	P50544	35	70876/8.9	MOUSE	Up
Aldehyde dehydrogenase 1A1 (Aldehyde dehydrogenase, cytosolic) (ALDH class 1) (ALHDII) (ALDH-E1)	P24549	34	54450/7.9	MOUSE	Up
Aspartate aminotransferase, cytoplasmic (Transaminase A) (Glutamate oxaloacetate transaminase-1)	P05201	49	46232/6.7	MOUSE	Up
Calreticulin precursor (CRP55) (CALREGULIN) (HACBP) (ERP60)	P14211	40	47995/4.3	MOUSE	Up
Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor (HMG-CoA synthase) (3-hydroxy-3-methylglutaryl coenzyme A synthase)	P54869	26	53787/8.0	MOUSE	Up
Lactoylglutathione lyase (Methylglyoxalase) (Aldoketomutase) (Glyoxalase I) (Glx I) (Ketone-aldehyde mutase) (S-D-lactoylglutathione methylglyoxal lyase)	Q9CPU0	53	20810/5.2	MOUSE	Up
NADP-dependent malic enzyme (NADP-ME) (Malic enzyme 1)	P06801	38	63999/7.2	MOUSE	Up
2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial precursor (Branched-chain alpha-keto acid dehydrogenase E1 component alpha chain) (BCKDH E1-alpha)	P11960	53	50165/7.7	RAT	Up

Protein name	Accession #	Sequence coverage(%)	M.W (Da)//pI	Species	Regulation
Phenylalanine-4-hydroxylase (PAH) (Phe-4-monooxygenase)	P16331	43	51929/6.0	MOUSE	Up
Probable urocanate hydratase (Urocanase) (Imidazolonepropionate hydrolase)	Q8VC12	28	74591/7.3	MOUSE	Up
Serine hydroxymethyltransferase, cytosolic (Serine methylase) (Glycine hydroxymethyltransferase) (SHMT)	P50431	44	52585/6.5	MOUSE	Up
Transitional endoplasmic reticulum ATPase (TER ATPase) (15S Mg(2+)-ATPase p97 subunit) (Valosin containing protein) (VCP) [Contains: Valosin]	Q01853	29	89309/5.1	MOUSE	Up
Aflatoxin B1 aldehyde reductase 1	27527042	40	37677/6.4	MOUSE	Down
Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)	P17182	50	47141/6.4	MOUSE	Down
D-lactate dehydrogenase	33585872	24	51848/6.2	MUS MUSCULUS	Down
Dimethylglycine dehydrogenase, mitochondrial precursor (ME2GLYDH)	Q63342	17	96048/6.9	RAT	Down
Eukaryotic translation initiation factor 3 subunit 2 (eIF-3 beta) (eIF3 p36) (eIF3i) (TGF-beta receptor interacting protein 1) (TRIP-1)	Q9QZD9	20	36461/5.4	MOUSE	Down
10-formyltetrahydrofolate dehydrogenase (10-FTHFDH)	Q8R0Y6	45	98710/5.6	MOUSE	Down
Fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase) (FBPase)	Q9QXD6	31	36913/6.1	MOUSE	Down
Glutamine synthetase (Glutamate--ammonia ligase)	P15105	33	42146/6.5	MOUSE	Down
Guanidinoacetate N-methyltransferase	O35969	45	26336/5.4	MOUSE	Down
Heat shock protein 75 kDa, mitochondrial precursor (HSP 75) (Tumor necrosis factor type 1 receptor associated protein) (TRAP-1) (TNFR-associated protein 1)	Q9CQN1	35	80210/6.2	MOUSE	Down
Pyruvate carboxylase, mitochondrial precursor (Pyruvic carboxylase) (PCB)	Q05920	28	129686/6.3	MOUSE	Down

Protein name	Accession #	Sequence coverage(%)	M.W (Da)//pI	Species	Regulation
S-adenosylmethionine synthetase alpha and beta forms (Methionine adenosyltransferase) (AdoMet synthetase) (MAT-I/III)	Q00266	29	43648/5.9	HUMAN	Down
Serum albumin precursor	P07724	35	68693/5.7	MOUSE	Down
Sulfite oxidase, mitochondrial precursor	Q8R086	19	54049/5.7	MOUSE	Down
Thiosulfate sulfurtransferase (Rhodanese)	P52196	47	33466/7.7	MOUSE	Down
Vitamin D-binding protein precursor (DBP) (Group-specific component) (GC-globulin) (VDB)	P21614	29	53086/5.3	MOUSE	Down

Up-regulated : [> 3-fold], Down-regultaed : [< 1/3-fold]

Table 7. Functional annotation of differentially expressed proteins in the liver of HBxTg/p53KO double mutant mice compared to B6 mice

Function	Protein name	Accession #	Species	Regulation
Oxidoreductase activity (metabolism resulting in cell growth)	Acyl-CoA dehydrogenase, long-chain specific, mitochondrial precursor (LCAD) *(acyl-coA dehydrogenase activity, electron transport, fatty acid metabolism)*	P51174	MOUSE	Up
	Acyl-CoA dehydrogenase, medium-chain specific, mitochondrial precursor (MCAD) *(acyl-coA dehydrogenase activity, electron transport, fatty acid metabolism)*	P45952	MOUSE	Up
	Acyl-CoA dehydrogenase, very-long-chain specific, mitochondrial precursor (VLCAD) (MVLCAD) *(acyl-coA dehydrogenase activity, electron transport, fatty acid metabolism)*	P50544	MOUSE	Up
	Aldehyde dehydrogenase 1A1 (Aldehyde dehydrogenase, cytosolic) (ALDH class 1) (ALHDII) (ALDH-E1) *(aldehyde degydrogenase activity)*	P24549	MOUSE	Up
	2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial precursor (Branched-chain alpha-keto acid dehydrogenase E1 component alpha chain) (BCKDH E1-alpha)	P11960	RAT	Up
	Phenylalanine-4-hydroxylase (PAH) (Phe-4-monooxygenase) *(iron ion binding, amino acid binding)*	P16331	MOUSE	Up
	Dimethylglycine dehydrogenase, mitochondrial precursor (ME2GLYDH) *(electron transport)*	Q63342	RAT	Down
	Sulfite oxidase, mitochondrial precursor *(electron transport)*	Q8R086	MOUSE	Down
ATP binding and chaperone activity	Transitional endoplasmic reticulum ATPase (TER ATPase) (15S Mg(2+)-ATPase p97 subunit) (Valosin containing protein) (VCP) [Contains: Valosin] *(cell growth or maintenace)*	Q01853	MOUSE	Up
	Heat shock protein 75 kDa, mitochondrial precursor (HSP 75) (Tumor necrosis factor type 1 receptor associated protein) (TRAP-1) (TNFR-associated protein 1)	Q9CQN1	MOUSE	Down

Function	Protein name	Accession #	Species	Regulation
ATP binding and chaperone activity	Pyruvate carboxylase, mitochondrial precursor (Pyruvic carboxylase) (PCB) (lipid biosynthesis, ligase activity)	Q05920	MOUSE	Down
	S-adenosylmethionine synthetase alpha and beta forms (Methionine adenosyltransferase) (AdoMet synthetase) (MAT-I/III) (amino acid metabolism)	Q00266	HUMAN	Down
Lipid binding & transport	Serum albumin precursor	P07724	MOUSE	Down
Hydrolase activity	Fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase) (FBPase)	Q9QXD6	MOUSE	Down
Calcium ion binding	Calreticulin precursor (CRP55) (CALREGULIN) (HACBP) (ERP60)	P14211	MOUSE	Up
Transferase activity	4-aminobutyrate aminotransferase, mitochondrial precursor (Gamma-Amino-N-Butyrate Transaminase) (GABA transaminase) (GABA aminotransferase) (GABA-AT)	P50554	RAT	Up
	Aspartate aminotransferase, cytoplasmic (Transaminase A) (Glutamate oxaloacetate transaminase-1) (amino acid metabolism, biosynthesis)	P05201	MOUSE	Up
	Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor (HMG-CoA synthase) (3-hydroxy-3-methylglutaryl coenzyme A synthase) (acetyl coA metabolism, cholesterol biosynthesis)	P54869	MOUSE	Up
	Serine hydroxymethyltransferase, cytosolic (Serine methylase) (Glycine hydroxymethyltransferase) (SHMT)	P50431	MOUSE	Up
	10-formyltetrahydrofolate dehydrogenase (10-FTHFDH) (biosynthesis)	Q8R0Y6	MOUSE	Down
	Guanidinoacetate N-methyltransferase	O35969	MOUSE	Down
	Thiosulfate sulfurtransferase (Rhodanese)	P52196	MOUSE	Down
Aflatoxin biosynthesis	Aflatoxin B1 aldehyde reductase 1	27527042	MOUSE	Down

Function	Protein name	Accession #	Species	Regulation
Cell cycle regulation & signal transduction	**Eukaryotic translation initiation factor 3 subunit 2 (eIF-3 beta) (eIF3 p36) (eIF3i) (TGF-beta receptor interacting protein 1) (TRIP-1)**	**Q9QZD9**	**MOUSE**	**Down**
Lyase activity & hydratase activity	**Lactoylglutathione lyase (Methylglyoxalase) (Aldoketomutase) (Glyoxalase I) (Glx I) (Ketone-aldehyde mutase) (S-D-lactoylglutathione methylglyoxal lyase)** ***(carbohydrate metabolism)***	**Q9CPU0**	**MOUSE**	**Up**
	Probable urocanate hydratase (Urocanase) (Imidazolonepropionate hydrolase) ***(histidine, catabolism)***	**Q8VC12**	**MOUSE**	**Up**
	Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1) ***(glycolysis)***	**P17182**	**MOUSE**	**Down**
Ligase activity	**Glutamine synthetase (Glutamate--ammonia ligase)** ***(nitrogen fixation)***	**P15105**	**MOUSE**	**Down**
Malate metabolism	**NADP-dependent malic enzyme (NADP-ME) (Malic enzyme 1) (malate degydrogense activity – NAD dependent)**	**P06801**	**MOUSE**	**Up**
Actin binding	**Vitamin D-binding protein precursor (DBP) (Group-specific component) (GC-globulin) (VDB)**	**P21614**	**MOUSE**	**Down**
Not matched to functional annotation	**D-lactate dehydrogenase**	**33585872**	**MUS MUSCULUS**	**Down**

Up-regulated : [> 3-fold], Down-regultaed : [< 1/3-fold]

These results were categories by http://www.ebi.ac.uk/ego/

D. Summary of specifically expressed proteins profiles among groups.

After we identified up- and down- regulated proteins in each pair (Table 2, 4 and 6), all of the proteins profiles were compared with each other. Expressed proteins profiling were divided into three groups according to their expressed pattern in different groups (Table 8).

Group 1 proteins were differentially expressed proteins in the liver of both HBxTg mice and p53KO mice compared to B6 mice. These 12 proteins group included oxidoreductase activity (metabolism resulting in cell growth), lipid binding and transport, kinase activity, ATP binding and chaperone activity, GTP binding, calcium ion binding, aminoacylase activity and cell mobility related protein.

Group 2 proteins were differentially expressed proteins in the liver of both p53KO mice and HBxTg/p53KO mice compared to B6 mice. These 3 proteins were aldehyde dehydrogenase 1A1 related with oxidoreductase activity, probable urocanate hydratase related with lyase activity and hydratase activity, heat shock protein 75 kDa mitochondrial precursor (HSP 75) related with ATP binding and chaperone activity. 2-D gel images of HSP 75 be shown in Fig 7A. This protein was dramatically down-regulated by 4-fold in pair 2 and by > 5-fold in pair 3.

Group 3 proteins were regulated proteins in all groups, HBxTg mice, p53KO mice and HBxTg/p53KO double mutant mice compared to B6 mice. 2-D gel images of group 3 proteins be shown total 9 proteins (Figs. 7B~J). 2-oxoisovalerate dehydrogenase alpha subunit related with oxidoreductase activity. This protein was

up-regulated by 3-fold in pair 1 and pair 2 and up-regulated by > 4-fold in pair 3 (Fig. 7B). Transitional endoplasmic reticulum ATPase related with ATP binding and chaperone activity. This protein was up-regulated by 3-fold in pair 1 and pair 2, and this protein up- regulated by 4-fold in pair 3 (Fig. 7C). S-adenosylmethionine synthetase alpha and beta forms were amino acid metabolism related protein. This protein was down-regulated by > 5-fold in pair 1 and by 4-fold in pair 2, but, down-regulated only by 3-fold in pair 3 (Fig. 7D). Alpha enolase (2-phospho-D-glycerate hydro-lyase) related with lyase activity and hydratase activity. This protein was down-regulated by 3-fold in pair 1 and pair 3. But, alpha enolase was down-regulated by > 4-fold in pair 2 (Fig. 7E). Dimethylglycine dehydrogenase mitochondrial precursor (ME2GLYDH) related with oxidoreductase activity. This protein was down-regulated by > 5-fold in pair 1 and pair 2 but, ME2GLYDH was a little down-regulated in pair 3 (Fig 7F). 10-formyltetrahydrofolate dehydrogenase (10-FTHFDH) related with transferase activity. This protein was down-regulated by 4-fold in pair 1 and pair 2. But, in pair 3, 10-FTHFDH disappeared almost (Fig. 7G). Thiosulfate sulfurtransferase (Rhodanese) related with transferase activity. This protein was down-regulated by 4-fold in pair 1 and pair 2. But, thiosulfate sulfurtransferase was little down-regulated in pair 3 (Fig. 7H). Aflatoxin B1 aldehyde reductase related with aflatoxin biosynthesis. This protein was up-regulated by 4-fold in pair 1, down-regulated by 3-fold in pair 2 and up-regulated by 5-fold in pair 3 (Fig. 7I). Calreticulin precursor (CRP55) related with calcium ion binding. In particular, this protein dramatically changed in all pairs. This protein shifted their position as well as

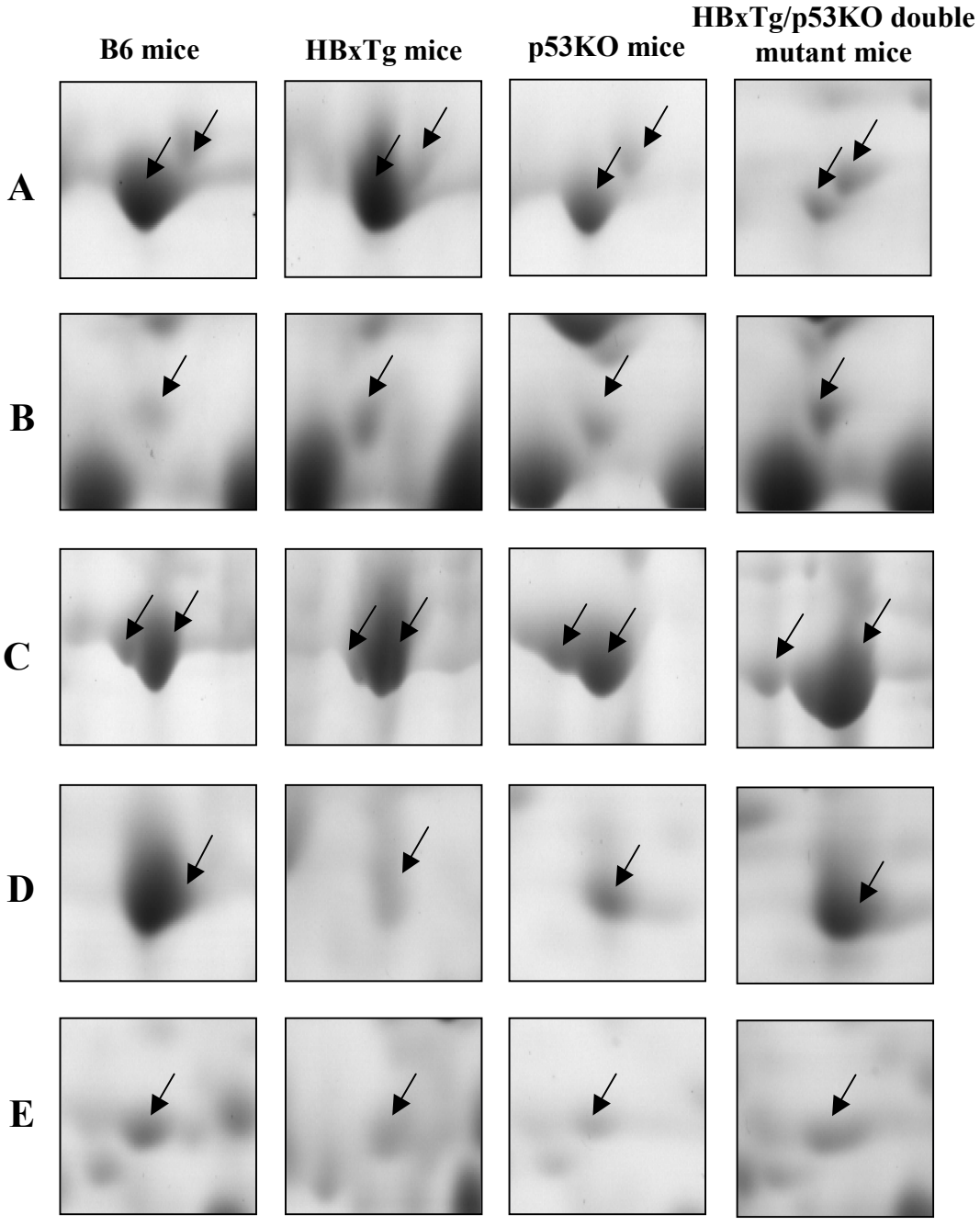
changed their amount. This protein was up-regulated by > 4-fold in pair 1 and was down-regulated by 3-fold as well as changed one spot to two spots in pair 2. Moreover, in pair 3, this protein was up-regulated a litter as well as changed one spots to three spots (Fig. 7J).

Table 8. Differentially expressed protein profiles of the liver in HBxTg, p53KO and HBxTg/p53KO double mutant mice compared to B6 mice

	Protein name	Regulation			Function
		HBxTg	p53KO	HBxTg/ p53KO	
Group 1	Aldehyde dehydrogenase, mitochondrial precursor (ALDH class 2) (AHD-M1) (ALDHI) (ALDH-E2)	↑	↑	—	Oxidoreductase activity (metabolism resulting in cell growth)
	Apolipoprotein E precursor (Apo-E)	↑	↑	—	Lipid binding & transport
	Guanine nucleotide-binding protein beta subunit-like protein 12.3 (P205) (Receptor of activated protein kinase C 1) (RACK1) (Receptor for activated C kinase)	↑	↑	—	Kinase activity
	Nonspecific lipid-transfer protein, mitochondrial precursor (NSL-TP) (Sterol carrier protein 2) (SCP-2) (Sterol carrier protein X) (SCP-X) (SCPX)	↑	↑	—	Lipid binding & transport
	Stress-70 protein, mitochondrial precursor (75 kDa glucose regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (P66 MOT) (Mortalin)	↑	↑	—	ATP binding and chaperone activity
	Elongation factor 2 (EF-2)	↓↓	↓↓	—	GTP binding
	Transketolase (TK) (P68)	↓↓	↓↓	—	Calcium ion binding
	gi|13384746|ref|NP_079647.1| aminoacylase 1 [Mus musculus]	↓	↓	—	Aminoacylase activity
	Methylcrotonyl-CoA carboxylase alpha chain, mitochondrial precursor (3-Methylcrotonyl-CoA carboxylase 1) (MCCase alpha subunit) (3-methylcrotonyl-CoA:carbon dioxide ligase alpha subunit)	↓	↓↓	—	ATP binding and chaperone activity
	Serine/threonine protein phosphatase 2A, 65 KDA regulatory subunit A, alpha isoform (PP2A, subunit A, PR65-alpha isoform) (PP2A, subunit A, R1-alpha isoform) (Medium tumor antigen-associated 61 KDA protein)	↓	↓↓	—	Not matched to functional annotation
	SEC14-like protein 2 (Alpha-tocopherol associated protein) (TAP)	↑	↓	—	Lipid binding & transport
	Actin-like protein 3 (Actin-related protein 3) (Actin-2)	↓	↑	—	Cell mobility

	Protein name	Regulation			Function
		HBxTg	p53KO	HBxTg/ p53KO	
Group 2	Aldehyde dehydrogenase 1A1 (Aldehyde dehydrogenase, cytosolic) (ALDH class 1) (ALHDII) (ALDH-E1)		↑	↑	Oxidoreductase activity (metabolism resulting in cell growth)
	Heat shock protein 75 kDa, mitochondrial precursor (HSP 75) (Tumor necrosis factor type 1 receptor associated protein) (TRAP-1) (TNFR-associated protein 1)		↓↓	↓↓↓	ATP binding and chaperone activity
	Probable urocanate hydratase (Urocanase) (Imidazolonepropionate hydrolase)		↓	↑↑	Lyase activity & hydratase activity
Group 3	2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial precursor (Branched-chain alpha-keto acid dehydrogenase E1 component alpha chain) (BCKDH E1-alpha)	↑	↑	↑↑	Oxidoreductase activity (metabolism resulting in cell growth)
	Transitional endoplasmic reticulum ATPase (TER ATPase) (15S Mg(2+)-ATPase p97 subunit) (Valosin containing protein) (VCP) [Contains: Valosin]	↑	↑	↑↑	ATP binding and chaperone activity
	S-adenosylmethionine synthetase alpha and beta forms (Methionine adenosyltransferase) (AdoMet synthetase) (MAT-I/III)	↓↓↓	↓↓	↓	ATP binding and chaperone activity
	Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)	↓	↓↓	↓	Lyase activity & hydratase activity
	Dimethylglycine dehydrogenase, mitochondrial precursor (ME2GLYDH)	↓↓↓	↓↓	↓	Oxidoreductase activity (metabolism resulting in cell growth)
	10-formyltetrahydrofolate dehydrogenase (10-FTHFDH)	↓↓	↓↓	↓↓↓	Transferase activity
	Thiosulfate sulfurtransferase (Rhodanese)	↓↓	↓↓	↓	Sulfate transport
	Aflatoxin B1 aldehyde reductase 1	↑↑	↓	↓↓↓	Aflatoxin biosynthesis
	Calreticulin precursor (CRP55) (CALREGULIN) (HACBP) (ERP60)	↑↑	↓	↑	Calcium ion binding

* ↓ : 3-fold change, ↓↓ : 4-fold change, ↓↓↓ : > 5-fold change


B6 mice
HBxTg mice
p53KO mice
HBxTg/p53KO double mutant mice
A
B
C
D
E

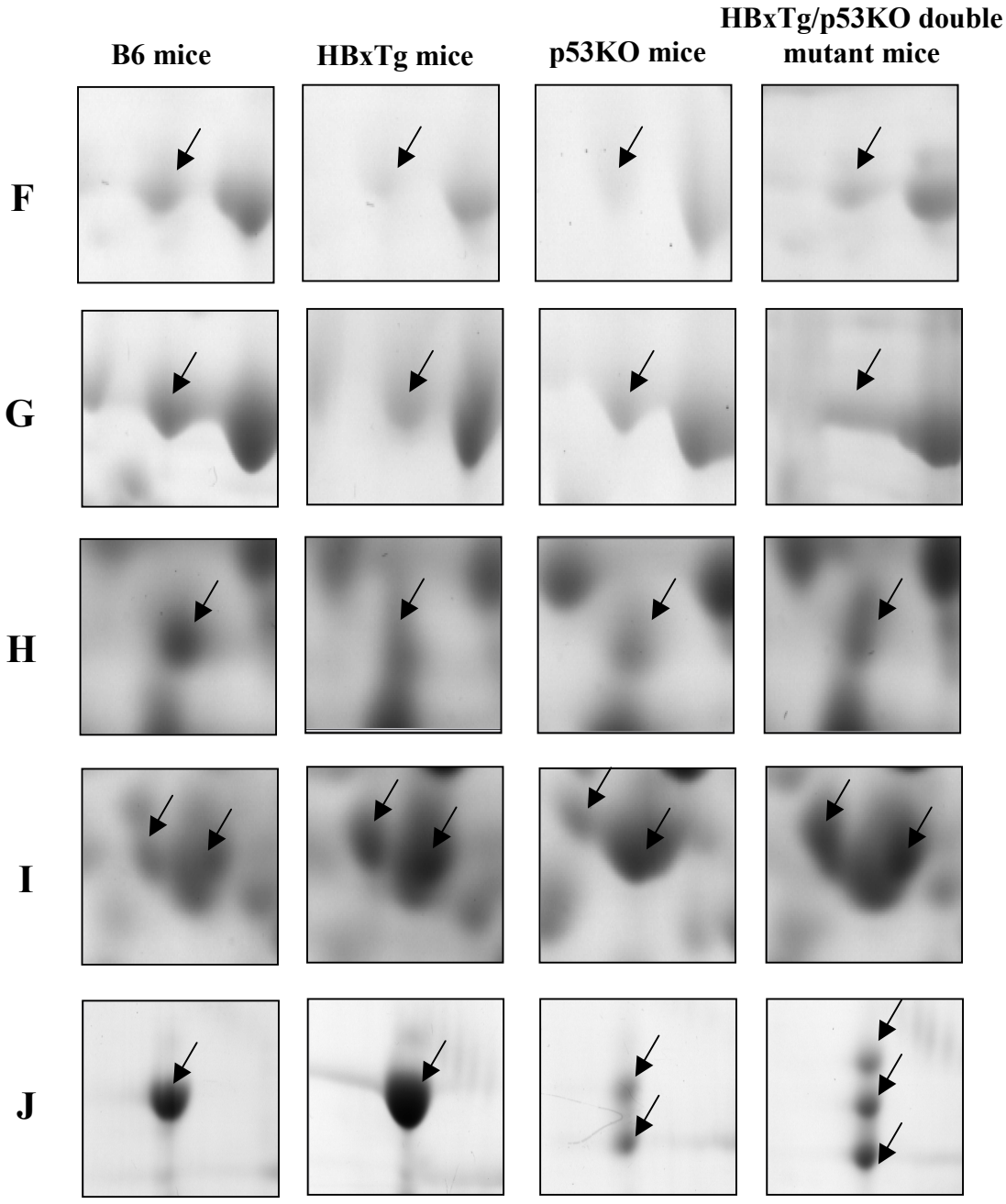

B6 mice
HBxTg mice
p53KO mice
HBxTg/p53KO double mutant mice
F
G
H
I
J

Figure 7. 2-DE images of differentially expressed proteins in the liver of B6, HBxTg, p53KO and HBxTg/p53KO double mutant mice.

A. Heat shock protein 75 kDa, mitochondrial precursor (HSP 75) (Tumor necrosis factor type 1 receptor associated protein) (TRAP-1) (TNFR-associated protein 1)

B. 2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial precursor (Branched-chain alpha-keto acid dehydrogenase E1 component alpha chain) (BCKDH E1-alpha)

C. Transitional endoplasmic reticulum ATPase (TER ATPase) (15S Mg(2+)-ATPase p97 subunit) (Valosin containing protein) (VCP) [Contains: Valosin]

D. S-adenosylmethionine synthetase alpha and beta forms (Methionine adenosyltransferase) (AdoMet synthetase) (MAT-I/III)

E. Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)

F. Dimethylglycine dehydrogenase, mitochondrial precursor (ME2GLYDH)

G. 10-formyltetrahydrofolate dehydrogenase (10-FTHFDH)

H. Thiosulfate sulfurtransferase (Rhodanese)

I. Aflatoxin B1 aldehyde reductase 1

J. Calreticulin precursor (CRP55) (Calregulin) (HACBP) (ERP60)

V. Discussion

We aimed to elucidate the molecular mechanism of HCC and to discover the effect of functional loss of p53 in the early HCC by HBx. In human, HCC incidence is consistently higher in man than in woman. This result has been reported to be similar in other transgenic mice which developed HCC. [9,29]The liver weight in HBxTg, p53KO and HBxTg/p53KO double mutant mice were significantly increased compared to B6 mice. ALT level of HBxTg/p53KO double mutant mice was significantly higher than that of B6, HBxTg and p53KO mice. In previous study (not published), HBxTg/p53KO double mutant mice, HCC was developed in 5 month old HBxTg/p53KO double mutant mice, however, HCC was formed in 9 month old HBxTg mice. The development of HCC was accerelated in HBxTg/p53KO double mutant mice compared to HBxTg mice. Therefore, we indentified HBxTg/p53KO double mutant mice in 1 month old, when it was clinically normal, but genetically affected by HBx and p53.

We identified several proteins, which were differentially expressed in each pair. We found cell cycle and several metabolism related protein in pair 1 (B6 vs HBxTg). Also signal transduction pathway and ATP binding and chaperone activity related proteins were found. These proteins have function of cellular signal pathway. In previous reports, these protein profiles of cancers were deduced using 2-DE and MALDI-TOF MS, and from these informations, cancer classifications, the

establishments of diagnostic madders, and selection of therapeutic target candidateds were sought [30,31,32]. There were no examples of HCC protein profiles obtained by 2-DE and MALDI-TOF MS the proteome changes have not been studied from HBxTg/p53KO double mutant mice liver tissue. Therefore, in this study, 2-DE and MALDI-TOF MS were employed to obtain the protein profile of HCC together with gain of HBx function and loss of p53 function. We analyzed proteome in mouse liver tissue when oncogenic HBx expressed and tumor suppress p53 repressed by using HBxTg/p53KO mice

Comparing the liver with each other, a significant change in expression level was found in 24 proteins. Some of the representative proteins annotated in 2D were proteins related with lipid metabolism, fatty acid metabolism, cell cycle proteins and ATP binding and chaperone activity.

In particular calreticulin precursor (CRP55) was considered HBx and p53 related protein in HCC. Calreticulin precursor (CRP55) was calcium ion binding related protein. This protein dramatically changed in all pairs. This protein shifted their position as well as changed their amount. This protein was considered amount of this protein was regulated by gain of HBx function and position of protein was shifted by loss of p53 function. In conclusion, the identified proteins, both up-regulated and down-regulated, may participate in the progression of HCC caused by the HBx with the functional loss of p53.

VI. Conclusion

To elucidate factors involved in HCC caused by HBx under the functional loss of p53, we employed two dimensional polyacrylamide gel electrophoresis (2DE) and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and determined profiles of differentially expressed proteins in the liver of 1-month-old male HBxTg, p53KO and HBxTg/p53KO mice compared to its age-matched normal mice (B6).

Cell cycle, cytoskeleton and several metabolisms related proteins were mainly differentially expressed in the liver of HBxTg mice compared to B6 mice. Signal transduction pathway, ATP binding and chaperone activity and lipid binding related proteins were differentially expressed in the liver of p53KO mice compared to B6 mice. Cell cycle, several metabolisms, ATP binding and chaperone activity, transferase activity related proteins were differentially expressed in the liver of HBxTg/p53KO double mutant mice compared to B6 mice.

We identified 24 liver proteins, which were differentially regulated in each of HBxTg, p53KO and HBxTg/p53KO double mutant mice compared with B6 mice. They were proteins related with lipid metabolism, fatty acid metabolism, ATP synthesis and several metabolism related proteins. Some of identified proteins, Calreticulin precursor and Aflatoxin B1 aldehyde reductase 1 were significantly differentially regulated in the HBxTg mice under the functional loss of p53 compared

to B6 mice These proteins may be involved in the acceleration of HCC in HBxTg/p53KO double mutant mice.

Our results may provide useful information for the understanding of molecular pathogenesis of HCC by HBx related with the functional loss of p53.

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Abstract in Korea

HBxTg/p53KO 이중형질전환 마우스의 간에서 프로테오믹스를 이용한 단백질 발현 양상 분석

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B 형간염 바이러스는 간세포 암의 병인 요소로 알려져 있다. B 형간염 바이러스는 잠재적으로 발암 단백질인 HBx 를 암호화 하고 있다. 그러나 간세포 암 발생에 있어서의 정확한 기전은 아직까지 잘 알려져 있지 않다. 다만 HBX 가 p53 유전자의 발현을 억제 또는 다른 유전자와 결합하는 것을 억제함으로써 간세포의 증식을 유도하고 이러한 비정상적인 세포의 증식이 간세포 암을 유발하는 것으로 알려져 있다. 이와 반대로 HBx 가 간

세포의 세포사멸을 유도한다는 보고도 있으나 그 과정에서 p53 의 관련성에 대해서는 아직까지 불확실한 실정이다. 최근까지 많은 연구들이 있지만 HBx 에 의한 간세포 암 발생에 있어서 p53 의 기능에 대해서는 아직까지 불확실한 실정이다. 이에 본 연구에서는 HBx 에 의한 간세포 암의 발생에 관여하는 단백질들을 탐색하고 p53 유전자의 기능적 상실이 어떠한 영향을 미치는지 확인하기 위해 다음과 같은 실험을 하였다. HBx 형질전환 마우스와 p53 knock-out 마우스를 교배하여 HBxTg/p53KO 이중형질전환 마우스를 생산한 후, 간 기능 검사를 수행하고 H & E 염색을 통해 간의 조직변화를 관찰하였다. HBxTg/p53KO 이중형질전환 마우스에서는 ALT 수치가 B6 마우스, HBx 형질전환 마우스, p53 knock-out 마우스와 비교해 현저하게 높았다. 또한 HBxTg/p53KO 이중형질전환 마우스는 간세포의 팽창과 불규칙적인 hepatic cord 를 보였다. 2-DE 와 MALDI-TOF MS 를 이용하여 HBx 형질전환 마우스, p53 knock-out 마우스, HBxTg/p53KO 이중형질전환 마우스의 1 개월령 간에서 다르게 발현되는 단백질을 분석하였다. 단백질 발현 분석결과 HBx 형질전환 마우스와 p53 knock-out 마우스, HBxTg/p53KO 이중형질전환 마우스 간 조직에서 정상 마우스인 B6 마우스 간 조직과 비교해 다르게 발현하는 단백질을 발견했고 중요하게 발현이 변화하는 24 개의 단백질을 발견했다. 이러한 단백질 중에는 cell cycle regulation, lipid metabolism, fatty acid metabolism, ATP synthesis 등과 관련된 단백질들이었다. 이러한 단백질들

의 발현 변화는 HBx 에 의한 간암발생이 p53 의 기능적 손실에 의해서 가속되는데 관련이 있는 것으로 사료된다.

핵심되는 말 : **HBx, p53, HBxTg/p53KO 형질전환마우스, 간**