Identification of Proteins Indicating Radiation-induced Hepatic Toxicity in Cirrhotic Rats

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Liver cirrhosis/Radiation/Proteomics/Hepatic injury.

Radiation therapy (RT) has been emerging as one of the palliative treatments for locally advanced hepatocellular carcinoma (HCC). However, hepatic toxicity is a major obstacle in radiotherapy for HCC. The purpose of this study is to identify proteins indicating radiation-induced hepatic toxicity in cirrhotic rats, which can be used as possible biomarkers. Liver cirrhosis was induced in Wistar rats with thioacetamide (TAA) 0.3 g/L in drinking water for 9 weeks. The development of liver cirrhosis was observed histologically. Radiation hepatic injury was induced by treating 1/3 of the liver with 10 Gy single dose radiation. To find out commonly expressed proteins, liver tissue and serum were analyzed using two-dimensional electrophoresis and quadrupole time of flight mass spectrometry. Identified proteins were validated using western blotting. Histological examination showed that the degree of hepatic fibrosis increased by radiation in liver cirrhosis. It was associated with a decrease in the proliferation of cell nuclear antigen and an increase of apoptosis. The proteomic analysis of liver tissue and serum identified 60 proteins which showed significant change in expression between the TAA-alone and TAA-plus-radiation groups. Among these, an increase of heparanase precursor and decrease of hepatocyte growth factor were shown commonly in liver tissue and serum following radiation. Hepatic fibrosis increased following radiation in cirrhotic rats. These proteins might be useful in detecting and monitoring radiation-induced hepatic injury.

INTRODUCTION

Therapeutic success of hepatocellular carcinoma (HCC) is severely limited due to advanced tumor at the time of diagnosis as well as associated liver disease. In this setting, various treatments have been tried but with limited success.[1,2] Radiation therapy (RT) hasn't been actively tried for HCC.[3] This is mainly due to the low radiation tolerance of the whole liver, which is typically lower than the therapeutic dose needed for tumor control.[4–6]

With technical advancement, the application of RT in treating hepatic tumors is rapidly increasing. Then the frequent association of concurrent liver cirrhosis still stands a major challenge in radiotherapy because radiation can induce hepatic toxicity, which might be fatal.[7–9] Two approaches will be possible; development of methods to minimize toxicity or to identify toxicity early using biomarkers.

In our previous study, we set up chemically induced liver cirrhosis in Wistar rats using thioacetamide by histological examination[7] and the protein expression profile has been investigated.[10] This model proved to be quite relevant to clinical setting since the indocyanine green retention test (ICG R15) showed similar results to cirrhotic patients who sent to radiotherapy.

Using this cirrhotic rat model, we attempted to find out protein expression that is significantly related with radiation induced hepatic injury, particularly proteins commonly expressed both in liver tissue and in serum. The purpose of this study is to identify proteins indicating radiation-induced hepatic toxicity in cirrhotic rats, which can be used as possible biomarkers.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200 ± 25 g were used. The rats were housed in a specific pathogen-free (SPF) barrier area

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at the Division of Laboratory Animal Medicine, College of Medicine, Yonsei University. The temperature (22°C) and humidity (55%) were controlled constantly. Water (RO water) and food (PMI) were supplied. The care and use of laboratory animals in this study were based on the Guidelines and Regulations for the Use and Care of Animals at Yonsei University College of Medicine.

Induction of liver cirrhosis

Wistar rats were given 0.3 g/L thioacetamide (TAA, Sigma, St. Louis, MO, USA), which was dissolved in their drinking water for 9 weeks. The rats of TAA-alone group were killed after TAA treatment, and the development of liver cirrhosis was observed histologically.

Experimental groups

Two experimental groups were set: TAA-alone and TAA-plus-radiation groups, with three rats in each group. The rats of TAA-plus-radiation group were treated with partial liver radiation. These rats underwent a simulation, a process to determine the radiation field, under intramuscular anesthesia with mixture of ketamine and xylazine. The partial liver, which was estimated as one-third of the whole liver, was treated with 10 Gy single dose radiation using a linear accelerator (Varian Co., Milpitas, CA, USA). After 3 weeks, liver tissue and blood samples were harvested for analysis.

Histological analysis

Liver tissues were embedded in paraffin. 4-μm liver sections were stained with either Masson's trichrome or picrosirius red, and development of liver fibrosis was examined. The liver fibrosis area was quantified using a microscope (Olympus, Tokyo, Japan) equipped with a CCD camera. Briefly, the red area of picrosirius red staining, considered fibrotic, was assessed by computer-assisted image analysis using the Meta-Morph software (Universal Imaging Corporation, Downingtown, PA, USA). Apoptosis was assessed in liver sections using hematoxylin and eosin (H&E) staining. Ten fields of slide sections were selected randomly at a magnification of x400 and in each field apoptotic bodies were expressed as a percentage of 1000 nuclei.

Immunohistochemistry

4-μm liver sections were immunohistochemically stained. To determine hepatocyte proliferation, the primary antibody was used a mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA; PC10; Dako A/S, Glostrup, Denmark; 1:100).

The quantitative PCNA index was determined by counting PCNA-positive cells among at least 1000 hepatocytes in randomly selected fields of each slide section, and was indicated as a percentage.

Proteomic analysis

A proteomic analysis was conducted both in liver tissue and serum. The extracted proteins from frozen liver tissue and serum were applied to immobilized pH gradient (IPG) strips (pH 3–10 NL; nonlinear, 18 cm long; Bio-Rad), then rehydration and isoelectric focusing (IEF) were performed. These proteins were separated on a 9%–17% gradient gel. After SDS-PAGE, the gels were fixed, stained with Coomasie blue G 250 (Bio-Rad) and scanned. The scanned gel images were normalized and comparatively analyzed using the PD QUEST program (v6.2, Bio-Rad).

For mass spectrometry, protein spots were selected and excised from gels. After enzymatic digestion, proteins were changed into peptides. Peptides were ionized and analyzed using quadrupole time of flight (Q-TOF) mass spectrometer (Waters). The acquired data were identified using the Mascot search program and then compared with the Swiss-Prot and NCBInr database.

Western blot analysis

Proteins were extracted from liver tissue and serum. Equal amounts of total protein (10 μg) were fractionated by SDS-PAGE. Antibodies against heparanase (HPA 2/3; H-100; Santa Cruz, CA, USA) and hepatocyte growth factor receptor (c-Met; C-28; Santa Cruz, CA, USA), diluted both 1:500, were used. The blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The specific bands were detected using the enhanced chemiluminescent (ECL) western blot detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Coomassie blue staining was performed on the gel as loading controls of serum proteins.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). For statistical comparison of values, a Student's *t*-test was used. *P* values less than 0.05 were deemed to indicate statistical significance.

RESULTS

Measurement of liver weight

For TAA-alone and TAA-plus-radiation groups, body and

Table 1. Comparison of body weight, liver weight, and the ratio of liver weight/body weight between the TAA-alone and TAA-plus-radiation groups

	TAA alone	TAA + radiation	*P* value*
Body weight (g)	386 ± 17.7	378 ± 14.7	0.043
Liver weight (g)	17.2 ± 1.59	20 ± 2.29	0.115
Liver weight/body weight (%)	0.04 ± 0.003	0.05 ± 0.004	0.100

Values are expressed as the mean ± SD. *Student's *t* test.
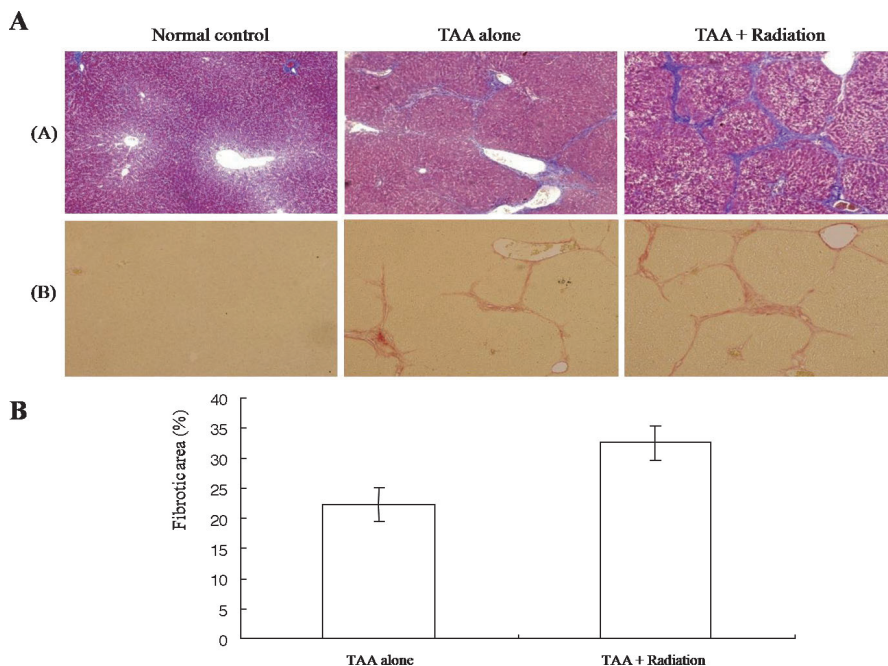Abbreviations: TAA, thioacetamide.

Fig. 1. A. Histological analysis of liver tissue sections using Masson's trichrome (A) or picrosirius red stain (B) (original magnification, x100). B. Quantitative analysis of liver fibrosis assessed by computer-assisted image analysis in the thioacetamide (TAA)-alone and TAA-plus-radiation groups. Liver fibrotic area was calculated using picrosirius red staining, as described in the Materials and Methods. Percent area of liver fibrosis increased almost 1.4-fold following radiation exposure. The data are shown as the mean ± SD.
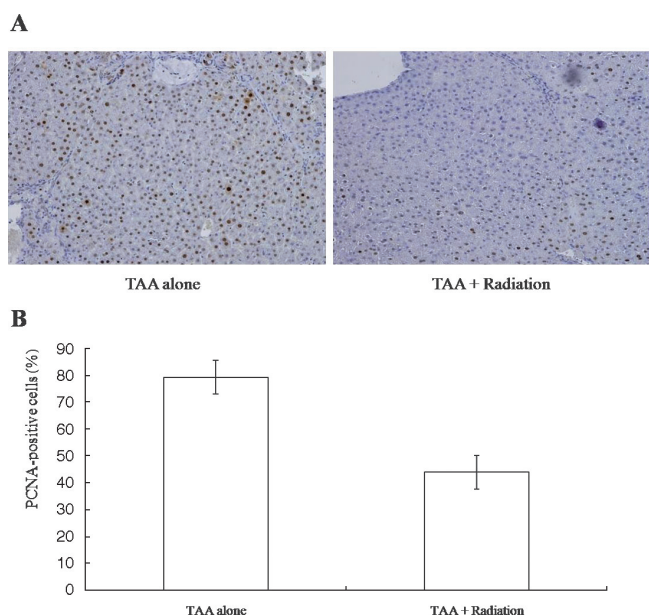

Fig. 2. A. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) in liver tissue sections. PCNA-positive cells decreased markedly in the thioacetamide (TAA)-plus-radiation group compared to the TAA-alone group (original magnification, x200). B. The percentage of PCNA-positive cells decreased markedly, about 1.8-fold, in the TAA-plus-radiation group compared to the TAA-alone group. The data are shown as the mean ± SD.
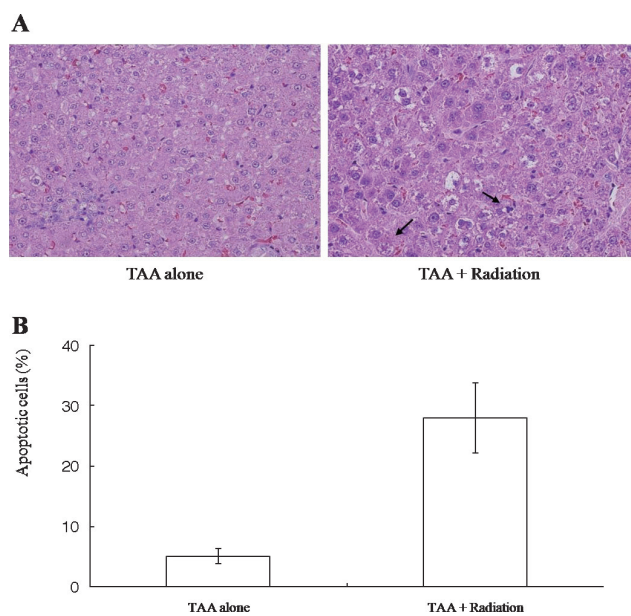

Fig. 3. A. Hematoxylin and eosin -stained liver sections of the thioacetamide (TAA)-alone and TAA-plus-radiation groups. Apoptotic cells (arrows), identified by histological criteria, increased in the TAA-plus-radiation group (original magnification, x400). B. Quantitative analysis of hematoxylin and eosin staining at a magnification of x400. The percentage of apoptotic cells increased almost 5-fold following radiation treatment. The data are shown as the mean ± SD.

liver weights were measured at the time of sampling (Table 1). The liver weight of the TAA-plus-radiation group showed a mild increase compared to the TAA-alone group and the ratio of liver to body weight was not statistically significant between the 2 groups ($p > 0.05$).

Histological assay and measurement of fibrotic area

Liver sections were stained with either Masson's trichrome or picrosirius red, which was stained specifically for collagen fibers. These staining showed a normal distribution of fibrous tissues around vascular structures in the normal control group. The livers of normal control rats had no specific alterations. However, abnormal deposition of collagen was seen in the TAA-alone group. After irradiation, more fibrosis was seen than in the TAA-alone group (Fig. 1A).

In quantitative analysis of liver fibrosis, the percent area of liver fibrosis was approximately 23% in the TAA-alone group (Fig. 1B); in the TAA-plus-radiation group, it was 32%, corresponding to mildly enhance liver fibrosis (Fig. 1B).

The PCNA and apoptotic indices were determined by counting the number of positive stained cells among at least 1000 cells, and are indicated as a percentage. The percentage of PCNA-positive cells decreased markedly, about 1.8-fold, in the TAA-plus-radiation group compared to the TAA-alone group (79.27 ± 6.25 vs. 43.97 ± 6.28, $p < 0.05$) (Fig. 2). The percentage of apoptotic cells significantly increased, by almost 5-fold following radiation treatment (5.13 ± 1.28 vs. 27.97 ± 5.77, $p < 0.05$) (Fig. 3).

Proteomic analysis and validation

In the proteomic analysis of liver tissue and serum, the expression pattern of proteins differed between the TAA-alone and TAA-plus-radiation groups (Fig. 4). Using two-dimensional electrophoresis (2DE), protein spots were separated: about 800 in serum and about 1700 in liver tissue. Among these protein spots, 60 proteins differed significantly in expression between the two groups. Screening of serum showed that 6 protein spots (spot numbers from 1 to 6) were up-regulated and 9 protein spots (spot numbers from 7 to 15) were down-regulated in the TAA-plus-radiation group com-
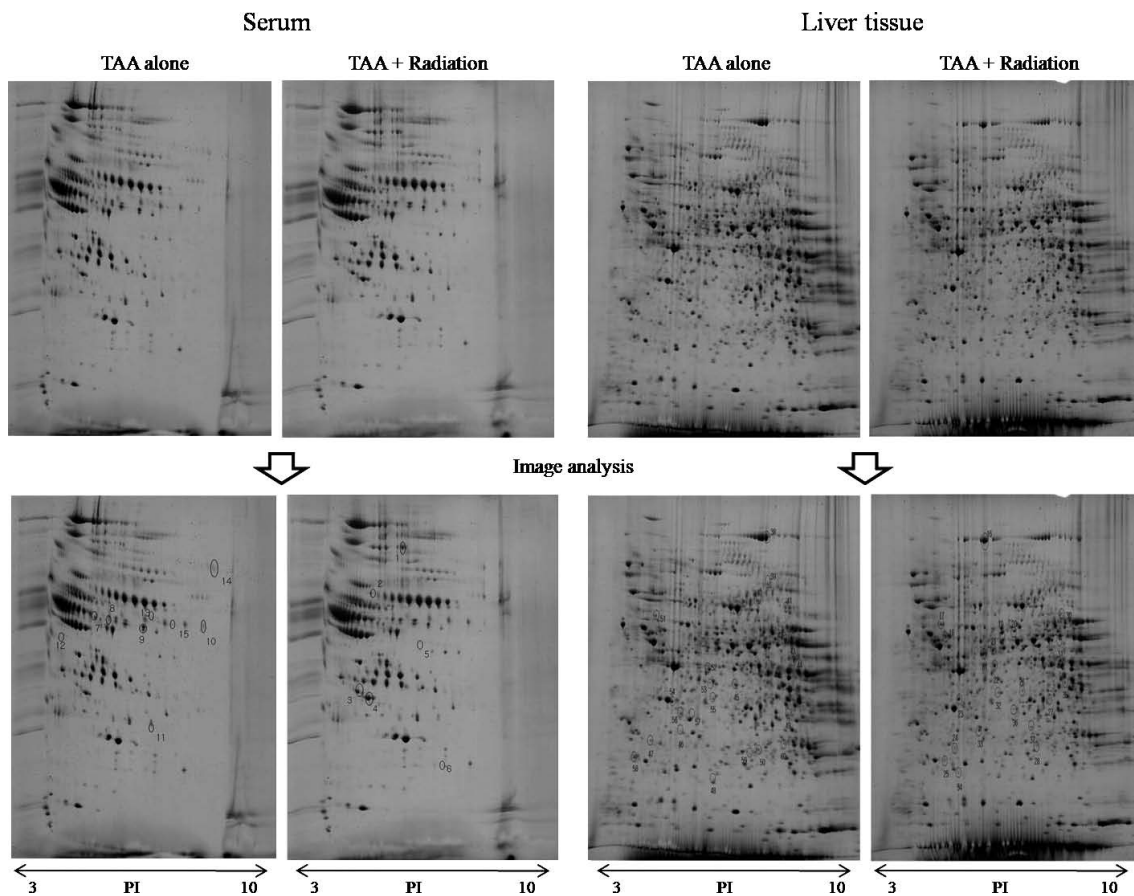

Fig. 4. Two-dimensional electrophoresis (2DE) images of protein expression between the thioacetamide (TAA)-alone and TAA-plus-radiation groups. The labeled circles on the TAA-alone images from serum or liver tissue indicated down-regulated spots following radiation. Also, the labeled circles on the TAA-plus-radiation images indicated up-regulated spots following radiation.

Table 2. Proteins that were altered by radiation

Proteins in serum	Accession #	Proteins in liver tissue	Accession #
Up-regulated			
Ceruloplasmin precursor (Ferroxidase)	P13635	Tumor necrosis factor, alpha-induced protein3	XP_001060980
Collagen alpha-1 (XI) chain Precursor	P20909	Thioredoxin reductase 2, mitochondrial-precursor	
Heparanase precursor[†]	Q71RP1		Q9Z0J5
Apolipoprotein E precursor	P02650	Catenin alpha-1	NP_001007146
Proteasome subunit alpha type 4	P21670	Stress-70 protein, mitochondrial precursor	P48721
		Cytochrome P450 2C12	NP_113760
		Heparanase precursor[†]	Q71RP1
		Interleukin-4 receptor alpha chain precursor	Q63257
		Tumor necrosis factor receptor superfamily-member 6 precursor	Q63199
		Transforming growth factor beta-1 precursor	P17246
		SNF-related serine/threonine-protein kinase	Q63553
		Slit homolog 3 protein precursor	O88280
		Tuberin	P49816
Down-regulated			
T-cell surface glycoprotein CD5 precursor	P51882	Adenomatous polyposis coli protein	P70478
Activation signal cointegrator 1 complex -subunit 1		Cytochrome P450, subfamily IIB	NP_001128316
	NP_001007633	Hepatocyte growth factor receptor[†]	P97523
Retinoblastoma-like protein 1	XP_001067833	Coagulation factor XII precursor	NP_001014028
Hepatocyte growth factor receptor[†]	P97523	Metallothionein-1	P02803
Ras GTPase-activating protein 2	Q63713	Transformation/transcription domain-associated protein	
Plasminogen precursor	Q01177		NP_001099377
		Platelet endothelial cell adhesion molecule-precursor	Q3SWT0
		Calpastatin	P27321
		Collagen alpha-1(III) chain precursor	P13941

Up and down: approximate twofold different change of protein expression
[†] Proteins commonly seen in both liver tissue and serum following radiation.

pared to the TAA-alone group by a factor of at least two in each case. Additionally, screening of liver tissue showed that 22 protein spots (spot numbers from 16 to 37) were up-regulated and 23 protein spots (spot numbers from 38 to 60) were down-regulated in the TAA-plus-radiation group compared to the TAA-alone group by a factor of at least two in each case. These protein spots were processed using in gel enzymatic digestion and Q-TOF, allowing their identification.

The identified proteins had functions in the immune response, signal transduction, apoptosis, proliferation and metabolism. Proteins seen in both liver tissue and serum following radiation included the heparanase precursor and hepatocyte growth factor receptor (HGFR). The expression of heparanase precursor increased, while the expression of HGFR decreased more than twofold in the TAA-plus-radiation group compared to the TAA-alone group (Table 2).

These proteins were validated using western blotting. The expression of heparanase precursor increased in the TAA-plus-radiation group compared to the TAA-alone group, while the expression of HGFR decreased following radiation (Fig. 5).

DISCUSSION

Radiation of normal tissue results in fibrosis, which is perhaps the most universal late effect of radiation. In liver, radiation-induced late injury is histologically characterized by a loss of parenchymal hepatocytes and the distortion of the lobular architecture which is accompanied by both peri-
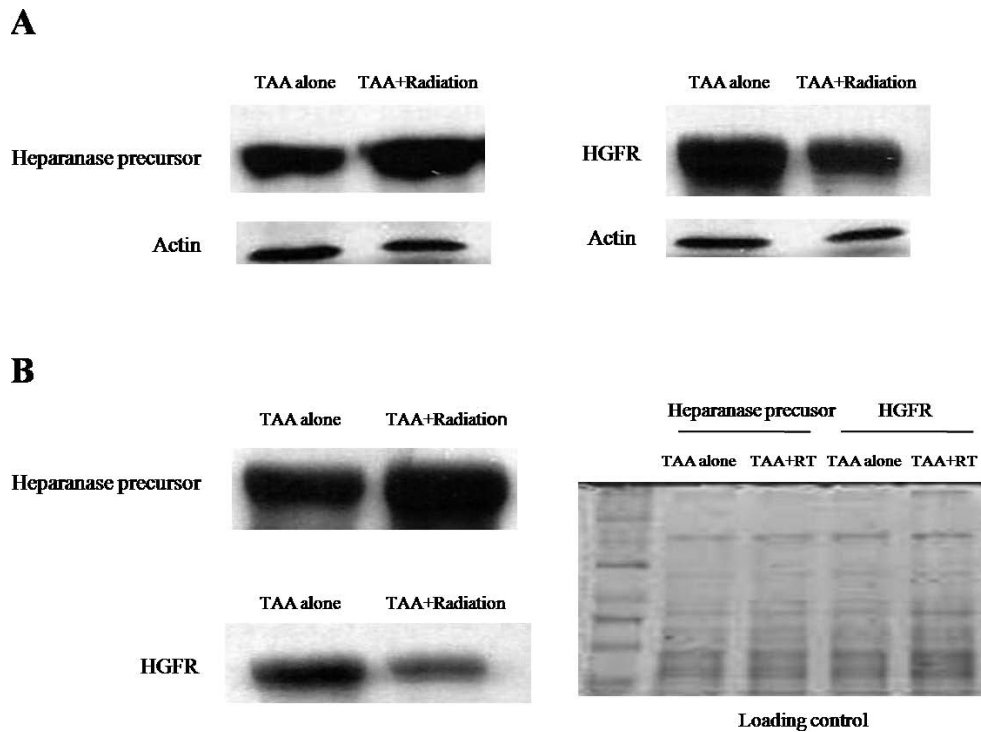
A

B

Loading control

Fig. 5.   Heparanase precursor and hepatocyte growth factor receptor (HGFR) were shown as common marker proteins. These were validated using western blotting in liver tissue (A) and serum (B). Loading controls in serum were revealed by coomassie blue staining (B).

central and periportal fibrosis.[11]

Several studies showed the changes in the proteome profile of liver following radiation. Lin *et al.* investigated proteomic analysis of mouse liver after radiation. They found that radiation altered the proteins associated with antioxidant response, energy metabolism, molecular chaperones, protein and amino acid metabolism, and skeletal protein.[12] An *et al.* also reported similar results. Particularly, the expression of proteins related to ROS metabolism, including cytochrome c, glutathione S-transferase Pi, NADH dehydrogenase, and peroxiredoxin VI, were increased after radiation.[13] These results were confirmed in a subsequent study of proteomic analysis comparing tissue with different radiosensitivity.[14] Park *et al.* also studied the oxidative stress caused by radiation and the changes in the proteome profile in irradiated rat liver tissue. They showed that radiation induces alteration in expression of proteins with antioxidant and related oxidoreductase functions, regulatory cytokines, signal transduction, metabolism, protein synthesis and degradation.[15]

In our previous study on TAA-induced rat liver cirrhosis model, proteomic analysis showed significant difference between the normal and the cirrhotic livers. Identified proteins were those related to extracellular matrix/cellular skeleton, cell proliferation/death signal, metabolism, DNA damage/stress and immune response. Well known profibrotic molecules, TGF-β1, TIMP-1 and MMP-9, were significantly

elevated in liver cirrhosis. The level of these proteins correlated with the severity of liver cirrhosis.[10]

In the present study, we established liver cirrhosis by TAA administration, and identified proteins that were commonly expressed both in liver tissue and serum following radiation using a proteomic analysis. Histological examination of liver tissues showed that the degree of hepatic fibrosis increased following radiation in the TAA-induced rat cirrhotic model. Proteomic analysis of liver tissue and serum showed that the expression pattern of proteins following radiation in liver cirrhosis differed significantly. Common proteins in both liver tissue and serum following radiation were found to be the heparanase precursor and HGFR.

The expression of heparanase precursor increased in both liver tissue and serum following radiation in TAA-induced liver cirrhosis. Heparanase is an endoglycosidase enzyme that acts on cell surface and extracellular matrix. Heparanase partially or completely degrades polymeric heparin sulfate molecules of heparan sulfate proteoglycans, which are essential constituents of extracellular matrix around cells.[16–20] Heparanase is important in various biological processes, including inflammation and tissue remodeling. Heparanase is detected in activated platelets, macrophages, granulocytes and lymphocytes.[21–23] Increased heparanase in this study suggests that it may play a role in inflammation and tissue remodeling, which frequently associated in progression of

liver cirrhosis.

Several studies showed that heparanase is increasing in cancer and that its activity directly contributes to invasion, angiogenesis and metastasis of cancer cells.[24–27] These actions have not been investigated in this study. However, there are several studies showing that a significant proportion of HCC patients undergoing radiotherapy subsequently develop intrahepatic and extrahepatic metastasis. Increased heparanase following radiation in cirrhotic livers might support such clinical finding.[28–30]

This study also showed that the expression of HGFR decreased following radiation as compared to the TAA-alone group. Hepatocyte growth factor (HGF), a polypeptide growth factor, contributes to various biological processes in the liver, including tissue regeneration, cell proliferation, and cytoprotective activities. HGF plays major roles in hepatocytes proliferation through its binding to a specific receptor HGFR. It is observed that a reduction of hepatic proliferation caused by decreased HGF and HGFR is associated with liver diseases.[31–33] HGFR, which is essential for wound healing and liver regeneration, promotes growth and motility of HGF and inhibits apoptotic cell death in process of liver regeneration.[34–36] This study showed decrease of HGFR following radiation, suggesting that it may suppress liver regeneration in TAA-induced liver cirrhosis.

The present study identified common proteins that were detected in both liver tissue and serum following radiation in a rat cirrhosis model. The expression of heparanase precursor increased and the expression of HGFR decreased following radiation. These proteins may be used as possible biomarkers for predicting and monitoring of hepatic injury caused by radiation. Further study is required to test sensitivity and specificity of these proteins as biomarkers for clinical setting.

ACKNOWLEDGEMENTS

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