Differential Proteomic Analysis of Gender-dependent Hepatic Tumorigenesis in Hras12V Transgenic Mice*

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Male prevalence is an outstanding characteristic of hepatocellular carcinoma (HCC), and the underlying mechanisms for this have remained largely unknown. In the present study, Hras12V transgenic mice, in which hepatocyte-specific expression of the ras oncogene induces male-biased hepatic tumorigenesis, were studied, and altered proteins were detected by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE). Protein samples from hepatic tumor tissues (T) and peritumor tissues (P) of transgenic males and females and the corresponding normal liver tissues (Wt) of nontransgenic males and females were subjected to pairwise comparisons based on proteomic analysis. Among 2381 autodetected protein spots, more than 1600 were differentially expressed based on a pairwise comparison (ratio > 1.5, p < 0.05). Of these, 180 spots were randomly selected for matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS) identification; finally, 89 distinct proteins were obtained. Among these 89 proteins, 7 and 50 proteins were further validated by Western blotting and literature investigation, respectively. Intriguingly, compared with Wt, the altered proteins were relatively concentrated in T in transgenic females but in P in transgenic males. Consistently, the levels of p-ERK and p-mTOR were significantly higher in the T of females compared with that of males. The pathway enrichment assay showed that 5 pathways in males but only 1 in females were significantly altered in terms of the upregulated proteins in T compared with Wt. These data indicate that female hepatocytes are disturbed by oncogenes with great difficulty, whereas male hepatocytes readily do so. In addition, 33 proteins were gender-dependently altered in hepatic tumorigenesis. Moreover, 4% DNA packaging and 4% homeostasis-related functional proteins were found in females but not in males, and more nucleus proteins were found in females (8%) than in males (3%). In conclusion, the proteomic data and comparative analysis presented here offer crucial clues for elucidating the mechanisms that underlie the male prevalence in HCC.

Hepatocellular carcinoma (HCC)1 is a heterogeneous cancer for which no promising treatment exists and remains one of the most prevalent and lethal malignancies worldwide (1). Approximately 600,000 new cases are diagnosed each year, of which 55% are identified in China, and increasing incidence is observed in Western countries (2). A unique causative aspect of HCC is related to its prevalence in males (3). Although gender-related differences in HCC are well established, few studies have specifically compared the clinicopathological characteristics of patients with HCC in relation to gender (1, 3–10). Prospective studies on the detailed mechanisms remain lacking.

Ras, as a small signal-transducing guanosine triphosphatase, is the upstream molecule in various pathways, such as MEK/ERK, PI3K/AKT, Raf/EGF/Ral, and plays a central role in controlling cell growth and differentiation (11). Activated mutations in ras have been found in all human tumors, and the frequency of ras mutations is highest among the genes that are associated with occurrence and development of human cancers (12). Although the mutational activation of Ras protein occurs in human hepatic tumors with an incidence of only

1 The abbreviations used are: HCC, hepatocellular carcinoma; 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; 10-F, 10-month-old females; 10-M, 10-month-old males; 15-F, 15-month-old females; 15-M, 15-month-old males; ACN, acetonitrile; DTT, DL-Dithiothreitol; F, females; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; M, males; MALDI-TOF/TOF MS, matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry; MS, mass spectrometry; MW, molecular masses; Non-Tg, C57BL/6J wild-type nontransgenic mice; P, peritumor tissues of Hras12V transgenic mice; pl, isoelectric points; PTM: post-translational modifications; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; T, tumor tissues of Hras12V transgenic mice; TESE, N,N,N',N'-tetramethylethylenediamine; TFA, trifluoroacetate; Tg, Hras12V transgenic mice; Wt, normal liver tissues of nontransgenic mice.

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about 5%, the receptor-mediated hyperactivation of the Ras-dependent signal transduction pathway is a frequent event in human hepatocarcinogenesis (13–16). Recently, application of Ras-pathway inhibitors has become an important therapeutic strategy for clinical cancer treatment including HCC (16, 17).

Liver cancer in mice is characterized by a high frequency of ras activating mutations that can exceed 50% depending on strain and treatment conditions. These mutations appear to represent an early event during the process of hepatocarcinogenesis in susceptible strains of mice and Hras is the most frequently affected isoform in either spontaneous or carcinogen-induced liver tumors (14, 18, 19). In addition, genotoxic agent diethylnitrosamine (DEN)-induced hepatic cancers in Balb c mice showed highly elevated ras isoforms in a dose-dependent manner (20). Above evidences support the view that Ras plays a key role in hepatocarcinogenesis. Many researchers have tried to generate ras-transgenic mice that develop HCC. However, these mice could not be used as a suitable model for investigating HCC associated mechanisms because the mice die early, are unfertile, or have a low incidence of HCC (21–24). We previously reported Hras12V transgenic mouse lineages generated using an Hras12V construct with a mouse albumin enhancer/promoter, which induced hepatocyte-specific expression of the ras oncogene resulting in male-biased hepatic tumorigenesis at the appropriate time and with a high level of reproducibility (25). This transgenic mouse represents a suitable model for investigating the underlying mechanisms of hepatocellular carcinogenesis, not only in relation to the ras oncogene but also in relation to male prevalence.

Proteomics is a key method for advancing our understanding of biological processes and systems. One of the most widely utilized proteomics approaches to quantify and identify differentially expressed proteins is two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) followed by mass spectrometry (MS) identification (26, 27). The obtained proteomics data can be conveniently analyzed by exploring publicly available web-based bioinformatics resources and related software.

In the present study, by using 2D-DIGE and matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS) approaches, differentially expressed proteins were obtained by paired comparisons among hepatic tumor tissues and adjacent nontumorous liver tissues of 10-month-old males or 15 month-old females of Hras12V transgenic mice and corresponding normal control liver tissues of nontransgenic males and females. Further comprehensive bioinformatics analysis provided crucial clues that will prove useful for elucidating the mechanisms underlying ras oncogene-related hepatic tumorigenesis and male prevalence.

**Experimental Procedures**

**Animals and Sampling**—Procedures for animal handling and tissue sampling were conducted in compliance with protocols approved by the Animal Care and Use Committee of Dalian Medical University. C57BL/6J wild-type nontransgenic mice (Non-Tg) and Hras12V transgenic mice (Tg) were bred and housed in Laboratory Animal Center of Dalian Medical University. Mice were sacrificed and parts of liver tissues of Non-Tg (Wt), hepatic tumors (T) and its paired normal adjacent liver tissues (P) of Tg were removed and immediately flash-frozen in liquid nitrogen. The remaining tissue parts were fixed in 10% formalin and underwent histopathological examination. The paired P and T samples of Tg and Wt samples of Non-Tg (10-month-old males and females; 15-month-old females; n = 9 for each group, total 54 mice) were identified by morphology, pathology, and signaling pathway activities. The morphological diagnosis confirmed Wt, P and T tissues were used for the subsequent experimental procedures.

**Experimental Design and Statistical Rationale**—The proteomic analysis was performed for Wt, P, T in either 15-month-old females or 10-month-old males of Non-Tg and Tg, respectively (3 individuals for each group). The protein samples from the same group were mixed equivalently to generate the composite samples and the paired comparisons among them were performed using a mixed-sample internal standard on 2D DIGE ([supplemental Table S1](#)). Analysis of 2D DIGE was done using DeCyder 5.0 software (GE Healthcare) according to the manufacturer’s recommendations. The paired t test was used for statistical analysis of the data. Protein spots that were differentially expressed in paired comparisons (ratio > 1.5, p < 0.05) were marked. One hundred and eighty marked protein spots were randomly selected for MALDI-TOF/TOF MS identification. Seven of the identified differentially expressed proteins were randomly selected and validated in samples from another set of males and females by Western blot analysis. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for the identified proteins were performed with UniProt and David v. 6.7.

**Reagents**—Cy2, Cy3, and Cy5 were purchased from GE Healthcare. Dimethylformamide was purchased from Aldrich. DTT, urea, agarose, glycerol, bromophenol blue, CHAPS, mineral oil, acrylamide, Bis, Tris base, glycine, SDS, iodoacetamide, ammonium persulfate, TEMED, Immobiline DryStrip gels (24 cm, pH 3–10), and Bio-Lyte solutions (pH 3–10) were purchased from Bio-Rad (Berkeley, CA). Thiourea was purchased from Fluka (Buchs, Switzerland). Protease inhibitor mixture was purchased from Roche Applied Science (Basel, Switzerland). ACN and methanol were purchased from Fisher. TFA was purchased from Merck. Trypsin (sequencing grade) was purchased from Promega (Madison, WI). All buffers were prepared with Milli-Q water (Millipore, Bedford, MA).

**Sample Preparation for 2D DIGE**—Liquid-frozen tissue sample was ground into powder in liquid nitrogen with a precooled mortar and pestle and then was homogenized on ice in 1 ml of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-Cl, pH 8.5, protease inhibitor mixture) using a glass homogenizer. After sonication on ice for 10 s using an ultrasonic processor, the samples were centrifuged for 30 min at 20,000 × g to remove particulate materials. Protein concentrations were determined in duplicate by the Bradford method (Bio-Rad) and confirmed by SDS-PAGE.

2D DIGE and Imaging—In a comparison, 50 μg proteins from each sample were mixed in a tube and labeled with Cy2 minimal dye in which 50 μg proteins were taken and used as the internal standard. In parallel, 50 μg proteins from each sample were labeled with either Cy3 or Cy5, respectively. Labeling was performed for 30 min on ice in the dark. Reactions were then quenched by the addition of 1 ml of lysine (10 mM) for 10 min on ice in the dark.

50 μg Cy3- and Cy5-labeled samples from each group were combined before mixing with 50 μg Cy2-labeled internal standards. Then
an equal volume of 2× sample buffer (7 m urea, 2 m thiourea, 4% CHAPS, 1% Bio-Lyte, pH 3–10, 20 mg/ml DTT) was added to the samples, and the total volume was made up to 410 μl with rehydration buffer (7 m urea, 2 m thiourea, 4% CHAPS, 0.5% Bio-Lyte, 10 mg/ml DTT). Samples were actively rehydrated into 24 cm pH 3–10 IPG strips (Bio-Rad) at 17 °C for 12 h using a Protean IEF cell (Bio-Rad). Isoelectric focusing was performed for a total of 80 kVh (ramped to 250 V in 30 min, held at 1000 V for 1 h, ramped to 10,000 V in 5 h, and held at 10,000 V for 60 kVh). The IPG strips were equilibrated in equilibration buffer (6 m urea, 2% SDS, 50 m thiocyanate, pH 8.8, 30% glycerol) supplemented with 0.5% DTT for 15 min at room temperature followed by 4.5% iodoacetamide in equilibration buffer for another 15 min incubation at room temperature.

IPG strips were placed on the top of 12% homogeneous polyacrylamide gels that had been precast with low fluorescence glass plates using an Ettan DALT twelve gel caster. The second dimension SDS-PAGE was carried out using the Protean Plus system (Bio-Rad). After 2D-DIGE, gels were scanned on the Typhoon 9410 scanner with Ettan DALT gel alignment guides using excitation/emissions wavelengths specific for Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm). The intensity was adjusted to ensure that the maximum volume of each image was within 60,000–90,000.

**In-gel Digestion—** Spot picking was carried out with preparative gels. Two-dimensional electrophoresis was performed as described under “2D DIGE and Imaging” except that the IPG strips were loaded with 500–1000 μg of protein, and gels were stained with Coomassie Brilliant Blue. Protein spots of interest were excised and destained with 25 mM ammonium bicarbonate, 50% ACN. Gels were then dried completely by centrifugal lyophilization. In-gel digestion was performed with 0.01 mg/ml trypsin (Promega) in 25 mM ammonium bicarbonate for 15 h at 37 °C. The supernatants were collected, and the trypic peptides were extracted from the gel sequentially with 5% TFA at 40 °C for 1 h and with 2.5% TFA, 50% ACN at 30 °C for 1 h. The extracts were pooled and dried completely by centrifugal lyophilization.

**Protein Identification—** Peptide mixtures were redissolved in 0.5% TFA, and 1 μl of peptide solution was mixed with 1 μl of matrix (4-hydroxy-α-cyanocinnamic acid in 30% ACN, 0.1% TFA) before spotting on the target plate. MALDI-TOF mass spectrometry and tandem TOF/TOF mass spectrometry were carried out on a 4800 Proteomics Analyzer (Applied Biosystems, Waltham, MA). Peptide mass maps were acquired in positive reflection mode, averaging 1500 laser shots per MALDI-TOF spectrum and 3000 shots per TOF/TOF spectrum (the resolution was 20,000). The 4700 calibration mixtures (Applied Biosystems) were used to calibrate the spectrum to a mass accuracy of 0.1 Da. Parent mass peaks with a mass range of 600–4000 Da and minimum signal to noise ratio of 15 were picked out for tandem TOF/TOF analysis. MS spectra were processed by using the 4700 GPS Explorer™ software. The spectra were recorded in a mass range from 600–4000 Da with a focus mass of 1600 Da. For one main spectrum, 20 subspectra with 100 shots/subspectrum with the first 10 shots discarded were accumulated using a random and uniform search pattern. The five peaks with the strongest intensity in the MS spectra (setting for laser intensity, 3600; minimum signal/noise of 15 within the window of 200 Da; cluster area signal/noise of 20) were picked automatically and used to produce the tandem TOF/TOF mass spectrometry were carried out on a 4800 Proteomics Analyzer (Applied Biosystems). 4700 GPS Explorer software was used to provide identification at a level of 95% confidence. The proteins with a confidence level of more than 95% and that Mascot ion scores > 60 (f test p < 0.01 identity threshold) were considered a significant identification.

To remove redundancy because of the assignment multiprotein group, only the first default matching for each protein group was retained except that when the first default matching was an unnamed product, a protein with clearly annotated name would be retained. The confident identification had a statistically significant (f test p < 0.05) protein score (based on combined mass and mass/mass spectra) and best ion score (based on mass/mass spectra). Keratin, and redundancy of proteins that appeared in the database under different names and accession numbers were eliminated. If more than one protein was identified in one spot, the single protein member with the highest protein score (top rank) was singled out from the multiprotein family. Proteins not satisfying the significance criteria (ANOVA p value ≤ 0.05 and q-value ≤ 0.05) were filtered out. Finally, proteins showing less than 1.5-fold change of expression were discarded as well. The molecular weight and pI values of most proteins were consistent with the gel regions from which the spots were excised.

**Western Blot Analysis—** Liver issues were homogenized on ice in 10 mM Tris buffer (pH 7.4) with 1 mM EDTA and Roche complete protease inhibitor mixture (PhosSTOP EASYpack, REF: 04906845001), centrifuged at 10,000 × g for 30 min and then the concentration was determined by Bradford assay (BioRad). Protein samples were separated on 12% polyacrylamide gels and transferred to PVDF membranes (Amersham Biosciences, Uppsala, Sweden). After blocking with 5% skim milk, primary and secondary antibodies were used to incubate the members. Primary and secondary antibodies: anti-phospho-MEK1/2 polyclonal antibody (diluted 1:1000, Elabscience, Wuhan, China), anti-phospho-ERK1/2 monoclonal antibody (diluted 1:2000, Cell Signaling Technology, MA), anti-phospho-Pi3 Kinase polyclonal antibody (diluted 1:1000, Cell Signaling Technology), anti-phospho-Akt monoclonal antibody (diluted 1:2000, Cell Signaling Technology), anti-phospho-mTOR monoclonal antibody (diluted 1:1000, Cell Signaling Technology), anti-APO1 monoclonal antibody (diluted 1:1000, Proteintech, Wuhan, China), anti-β-actin polyclonal antibody (diluted 1:5000, Bioworld Technology Co., Ltd., MN), anti-HSP90B1 polyclonal antibody (diluted 1:1000, Elabscience), anti-albumin polyclonal antibody (diluted 1:1000, Abclonal, Boston, MA), anti-calreticulin polyclonal antibody (diluted 1:1000, Abclonal, Shang-hai, China), anti-fatty acid binding protein 5 polyclonal antibody (diluted 1:500, Proteintech), anti-peroxiredoxin VI monoclonal antibody (diluted 1:2000, Abfrontier, Santiago, CA), anti-mouse-IgG antibody (diluted 1:5000, Bioworld), anti-rabbit-IgG antibody (diluted 1:5000, Bioworld). Immunoreactive complexes were visualized using ECL reagents (Biotool, Shanghai, China).

**Raw Data Repository—** All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (28) via the MassIVE partner repository with the dataset identifiers: MSV00080343 (http://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=b3d7367932bd3430fa466e0f3519077a1).
with SPSS 10.0 software. Differences with p values of < 0.05 were considered significant.

RESULTS

Gender-dependent hepatic tumorigenesis and activation of the MEK/ERK and PI3K/AKT/mTOR pathways in Hras12V transgenic mice—Because of the complex etiological factors, multiple-stage progression, and limitations regarding the availability of clinical samples, it is difficult to elucidate the complex mechanisms underlying hepatocellular carcinogenesis and the characteristic male prevalence. However, transgenic hepatic tumor animal models offer opportunities to dissect and elucidate the related mechanisms. We previously reported gender-dependent hepatic alterations in a transgenic mouse model in which the Hras12V oncogene was specifically expressed in hepatocytes (25). This hepatic tumor model provides the opportunity to understand the contributions of the activated Ras/MAPK pathway and gender factors to hepatic tumorigenesis.

To select representative samples for proteomic analysis, 10-month-old males (10-M), 10- and 15-month-old females (10-F and 15-F) transgenic mice, and nontransgenic control mice of the same age and gender were sampled (n = 9 for each group). Multiple and massive hepatic tumors were only present in transgenic 10-M and 15-F, but not in transgenic 10-F or any nontransgenic controls (Fig. 1A). H&E staining showed similar histopathological characteristics between the hepatic tumors of transgenic 10-M and 15-F (Fig. 1B). Further incidence analysis of the dependence of hepatic alteration on diameter (according to the histopathological diagnosis criteria described by Frith and Ward (29)) showed that although the incidence of nodules (< 2 mm) did not significantly differ between transgenic 15-F and 10-M, significant lower incidences of adenoma (2–5 mm), carcinoma (> 5 mm), and total hepatic alterations were found in 15-F compared with 10-M transgenic mice. In addition, few nodules were found in transgenic 10-F (Fig. 1C). Consistently, in addition to a significant increase in the liver/body ratio in transgenic mice compared with the control groups, the liver/body ratio of transgenic 10-M was significantly higher than those of transgenic 10-F and 15-F (Fig. 1D). These findings indicate a significant male prevalence for hepatic tumorigenesis in the transgenic mice, consistent with our previous report (25).

To investigate if differences exist between males and females regarding the activities of signaling pathways that are related to hepatic tumorigenesis, proteins in the two predominately activated signaling pathways, the MEK/ERK and PI3K/AKT/mTOR pathways, were detected in transgenic 10-M and 15-F by Western blot assay. Intriguingly, although p-MEK levels did not obviously differ between the hepatic tumor tissues of transgenic 10-M and 15-F, p-ERK levels were obviously decreased in the hepatic tumor tissues of transgenic 10-M compared with transgenic 15-F (Fig. 1E). Moreover, although the p-PI3K levels were significantly higher, the p-AKT and p-mTOR levels were significantly lower in the hepatic tumor tissues of transgenic 10-M compared with transgenic 15-F (Fig. 1E). In addition, the levels of p-mTOR and p-ERK were also obviously lower in the livers of nontransgenic 10-M compared with those of nontransgenic 15-F. However, the phosphorylated signal protein levels of the two pathways did not obviously differ between the peritumor tissues of transgenic 10-M and 15-F (Fig. 1E). These findings imply a significant gender-dependent difference in the molecular mechanisms underlying hepatic tumorigenesis. Together, these data indicate that the samples obtained from transgenic and nontransgenic 10-M and 15-F mice were suitable for further proteomic analysis.

2D-DIGE Analysis—To detect differentially expressed proteins related to the Hras12V oncogene and male prevalence in hepatic tumors, hepatic tumor tissues (T) and the paired adjacent normal liver tissues (P) of transgenic 10-M and 15-F as well as the normal liver tissues of nontransgenic 10-M and 15-F (Wt) were collected. Depending on the appearance and size of the liver and hepatic tumor in terms of gross anatomy, histopathological diagnosis, and signaling pathway analysis, 3 representative individuals were selected in each group (18 protein samples were prepared from 12 individuals). Because of the consistently inbred genetic background (C57BL/6J), single Hras12V transgene location, and definite etiology (Hras12V oncogene) regarding hepatic tumor development, the 3 protein samples obtained from each group were mixed equivalently to generate the following 6 composite protein samples: normal liver tissues of nontransgenic 10-M and 15-F; normal peritumor tissues of transgenic 10-M and 15-F; and hepatic tumor tissues of transgenic 10-M and 15-F.

The 2D-DIGE images of 9 gels for paired comparison among the 6 composite protein samples labeled with different cyanine dyes were obtained by fluorescence scanning (Fig. 2A, supplemental Table S1). The 2-D DIGE images were analyzed using DeCyder 5.0 software to objectively estimate the abundance of proteins in each sample and to generate quantitative data. Briefly, the DeCyder biological variation analysis module was used to detect spots. After setting the appropriate filter threshold in the software, 2381 protein spots were autodetected. The relative abundances of protein spots among Wt, P and T were calculated using the normalized spot volume (the ratio of each spot volume relative to IS (Cy3:Cy2 /H11021/H11005)), and the Student paired t test (p < 0.05) was used to identify protein spots that were differentially expressed among Wt, P, and T. Protein spots that were differentially expressed in paired comparisons (|ratio| > 1.5, p < = 0.05) were marked. The relative abundance volume ratios of protein spots in each group are shown in supplemental Table S2. The numbers of upregulated and downregulated protein spots among Wt, P, and T in females or males are summarized in Fig. 2B. Interestingly, significant differences were found between males and females regarding the numbers of protein spots that were differentially expressed.
Fig. 1. Gender-dependent hepatic tumorigenesis and progression in Hras12V transgenic mice. A, Representative liver stereogram images of 10- and 15-month-old wild-type nontransgenic (Non-Tg) and Hras12V transgenic (Tg) females and 10-month-old Wt and Tg males are shown. Red arrows indicate hepatic alterations. B, Relative histopathological H&E staining images (magnifications: 40×) of liver and hepatic alterations of Wt and Tg mice, respectively. C, The dependence of the incidence of hepatic alterations in the transgenic mice on diameter was analyzed. D, Liver/body weight ratio of the mice. E, Activation of the MEK/ERK and PI3K/AKT/MTOR signal pathways in the liver and hepatic tumor tissues of 10-month-old males and 15-month-old females was detected using Western blot assay. Bradford reagent was used as a loading control. Wt, liver tissues of Non-Tg; P: peritumor tissue of Tg; T: tumor tissue of Tg; F-10: 10-month-old females; F-15: 15-month-old females; M-10: 10-month-old males; Statistical analysis was performed using Student’s t test. The numbers indicate different individuals. Data are expressed as the means ± S.E. (n = 9). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
among Wt, P, and T, regardless of whether the spots represented up- or downregulated proteins. Specifically, in males, more spots were detected when comparing P and T versus Wt than when comparing P versus T. However, in females, more spots were detected when comparing T versus P and Wt, and fewer than 100 spots were differentially expressed between P and Wt. This finding indicates that ras oncogene expression in normal hepatocytes induces more changes in gene expression in males than in females, and this phenomenon reflects the significant male prevalence in hepatic tumorigenesis.

MALDI-TOF/TOF MS Analysis—After scanning, the gels were stained with SYPRO-Ruby and were matched with the
DIGE image by the software. Protein spots exhibiting significant changes were selected using a SpotCutter and were subjected to in-gel digestion for MALDI-TOF/TOF MS analysis. In total, 180 randomly selected protein spots were identified. Information on the identified proteins is presented in supplemental Table S3 and supplemental Fig. S1. Many proteins were resolved as two or more spots on the 2D-DIGE gels, possibly because of post-translational modification, protein cleavage or the presence of different isoforms; thus, the 180 spots corresponded to 89 distinct proteins (supplemental Table S4). The abundance of an identified multi-spot protein was measured as the total volume obtained by integrating the intensities of each of the related spots (30). Fold-changes of the identified differentially expressed proteins ranged from 1.5 to 118.2 with an approximate average of 5.0. A summary of the numbers of upregulated and downregulated identified proteins and their comparisons among Wt, P, and T in females or males is presented in Fig. 3A (ratio \( r \geq 1.5, p < 0.05 \)).

The same trend was observed as that for the summarized total protein spots (Fig. 2B), indicating once again the underlying molecular changes for male prevalence.

Further classification of the identified proteins was performed according to their subcellular locations and biological processes according to the categories described by the Gene Ontology Consortium (http://www.geneontology.org/index.shtml) (supplemental Table S4). It is generally considered that proteins with extremely high or low pI and hydrophobic proteins are detected with difficulty by 2D-DIGE. Consistent with this notion, most of the identified proteins are in the cytoplasm (Fig. 3C), indicating that the identified proteins presented here were biased in favor of cytoplasmic and soluble proteins. In addition, approximately half of the identified proteins were related to metabolic processes (Fig. 3C). Interestingly, more nuclear proteins were found in females (8%) than in males (3%). Moreover, 4% DNA packaging and 4% homeostasis-related functional proteins were found in females but not in males. These findings provide another perspective that might aid in elucidating the male prevalence of hepatic tumors.

**Protein Variants**—Protein variants are commonly detected in proteomic analysis and include post-translational modifications such as phosphorylation (31), glycosylation (32), and acetylation (33). Consistently, in the present study, variants were detected for \( \sim 40\% \) of proteins, and most of the variants had the same isoelectric points (pI) and molecular masses (MW); a similar variation trend was found while comparing Wt, P, and T, indicating that the expression levels of most of these proteins were mainly regulated at the transcriptional or post-transcriptional stage or at the degradation stage, with similar mechanisms. Interestingly, the variants of the proteins Acoc1, Rpsa, Otc, and Hbb-b1 showed different pls or MWs when comparing Wt, P, and T (supplemental Table S3). This finding indicates that different forms of post-translational or post-transcriptional regulation may be involved, such as charge isomerization, protein backbone cleavage and cross-linking. These phenomena indicate that the protein variants may play important roles in hepatic tumorigenesis and development.

**Validation by Western Blot Analysis**—To verify the authenticity and reproducibility of the 2D-DIGE proteomic results, seven of the identified differentially expressed proteins were randomly selected and were confirmed in samples from another set of males and females by Western blot analysis. Coomassie staining was used as a control to normalize the results for the amount of protein loaded. The expression changes of the selected proteins were consistent with the obtained 2-DIGE results in both males and females, excepting PRDX6 in females that showed decreased protein levels in T compared with P and Wt, indicating that there were undetected PRDX6 isoforms (Fig. 4). These results demonstrate that the proteomic data analyzed in this study are convincing.

**Proteins Involved in Hepatic Tumorigenesis**—Among the identified 89 proteins, 77 proteins were significantly altered in the hepatic tumors of transgenic males, and 68 proteins were significantly altered in the hepatic tumors of transgenic females compared with the normal liver tissues of nontransgenic males and females. Among these proteins, 55 were upregulated and 22 were downregulated in transgenic males, and 40 were upregulated and 28 were downregulated in transgenic females (supplemental Table S5). Bioinformatics analysis of the functional GO term enrichment via DAVID is reported for the up- and downregulated proteins in supplemental Table S6. The observed proteins were primarily grouped into extracellular exosome (GO:0005788), pigment granule (GO:0048770), and cytoplasmic vesicle (GO:0031410) compartments. Pathway annotation of these proteins via DAVID indicated that the upregulated proteins were mainly enriched in 5 KEGG pathways for males and in 1 KEGG pathway for females. The downregulated proteins were mainly enriched in 6 KEGG pathways for males and females, respectively (Fig. 5; supplemental Table S7).

Fifty-four proteins were commonly altered in the hepatic tumors of both transgenic males and females. Of those, 36 were upregulated and 18 were downregulated (supplemental Table S5). Among the 36 upregulated proteins, 7 significantly participated in 3 metabolic pathways: lipid metabolism (CES2E and EPHX2); cellular amino acid metabolism (FABP5, P4HB and GSTP1); and magnesium ion binding (MAT1a and PPA1). Seven proteins belonged to the heat shock proteins or chaperones: HSPA4, HSPA5, HYOU1, HSP90B1, Calr, Cct5, and P4hb. Two proteins were associated with cell motility: ACTB1 (\( \beta \)-actin) and ACTG1. Other proteins were associated with transport, transcription, translation, and the cytoskeleton. Subcellular location analysis indicated that 28% of the proteins were in the cytoplasm, 22% were in the endoplasmic reticulum, and 14% were in the extracellular region. Among the 18 downregulated proteins, 10 significantly participated in 3 metabolic pathways: the monosaccharide metabolic process (FBP1, GALK1, LDHA, and GNMT); the nitrogen com-
pound biosynthetic process (ATP5A1, ASS1, CPS1, and OTC); and the carboxylic acid catabolic process (HPD and SARDH). Others were associated with transcription, RNA processing, and homeostasis. Subcellular location analysis indicated that 42% of the proteins were in the mitochondrion, 21% were in the cytoplasm, 11% were in the endoplasmic reticulum, and 11% were in the extracellular region (supplemental Table S4, S5).

Nineteen proteins in males and 10 in females were found to be differentially expressed in a gender-dependent manner.

**Fig. 3.** Identification of differentially expressed protein spots by MALDI-TOF/TOF-MS, relative function classification, and subcellular location analysis. Detailed information related to the identified proteins that were differentially expressed among wild-type normal liver tissues (Wt), the peritumor tissues of transgenic mice (P), and the tumor tissues of transgenic mice (T) is shown in supplemental Table S3, S4. The number of upregulated (A) and downregulated (B) proteins in paired comparisons is shown. C, Subcellular location (top panel) and functional classification (bottom panel) of the proteins that were differentially expressed between tumor tissues and normal liver tissues in transgenic males (left panel) and females (right panel).
during hepatic tumorigenesis. Intriguingly, most of the 19 proteins with altered expression in male hepatic tumors were upregulated, whereas most of the 10 proteins with altered expression in females were downregulated (supplemental Table S5). Female-dependent downregulated proteins were mainly classified into metabolic (ACOX1, AHCY, and BHMT2), anti-oxidant (GSTA3), transcription (BNC2), cell cycle (CALM1), and development (PFN1) categories. The 3 proteins that were upregulated in females were APOA4 (transport), Eef2 (translation), and HSPA8 (protein folding). Male-dependent upregulated proteins were mainly involved in signal transduction (ARHGDIA and FABP1), transport (NPM1), translation (RPLP2), oxidation reduction (DHDH), metabolism (ATP5D and FDPS), response to hypoxia (UBQLN1), DNA packing (Histone H4, Histone H2AA and Histone H2AB), DNA repair (SLF2), homeostasis (PDIA6), and development (TPM1). The 3 proteins that were downregulated proteins in males were all classified into the metabolic category (ALDH1L1, PGK1 and SCP2). Four proteins that were reversely regulated in males and females were GPX1 and PRDX6 (anti-oxidation); CYB5A (transport); and HSPE1 (protein folding) (supplemental Table S4, S5).

Gender-dependent Protein Expression Styles in Hepatic Tumorigenesis—In Hras12V transgenic mice, the ras oncogene is expressed in all hepatocytes. However, a limited number of hepatic tumors occur. These findings indicate that the ras oncogene alone is insufficient to induce hepatic tumorigenesis and that intracellular defense responses contribute to protect hepatocytes. In addition, the gender dependence of hepatic tumorigenesis indicates that different defense responses occur between males and females. Thus, it is very interesting to investigate related clues. Therefore, the proteins that were significantly differentially expressed in the comparison among Wt, P, and T in males and females were comprehensively analyzed. To clearly demonstrate the variation trends in protein expression, the altered proteins were classified into four categories: HCC positive-related proteins; HCC negative-related proteins; Ras positive-related proteins; and Ras negative-related proteins (Fig. 6). The symbols used in following paragraphs indicate the following: “>,” proteins were significantly upregulated in the former tissues compared with the latter; “<,” proteins were significantly downregulated in the former tissues compared with the latter; “=,” no significant difference in protein expression levels were observed between the former and the latter.

The classification of proteins as HCC positive-related proteins indicated that these proteins were significantly upregulated in T compared with P and/or Wt. In this category, the enrolled proteins could be categorized into 3 types: (1) T>P=Wt, these proteins were significantly upregulated in T compared with P and/or Wt. Most proteins were classified into this type, which indicated that the proteins were HCC-related; (2) T=Wt>P, and (3) T>Wt>P, these proteins were significantly downregulated in P compared with Wt and T. The classification of proteins into this type indicates their negative correlation to oncogene expression in normal hepatocytes and the possibility that they are regulated by the tumor defense system (Fig. 6A).

The classification of proteins as HCC negative-related proteins indicated that these proteins were significantly down-

![Fig. 4. Validation of the differentially expressed proteins that were identified by MALDI-TOF/TOF-MS. Seven proteins (HSP90B1, beta-actin, albumin, APOA1, CALR, FABP5, and PRDX6) were randomly selected from the differently expressed proteins that were identified by MALDI-TOF/TOF-MS and were validated in transgenic and nontransgenic males (A) and females (B) by Western blot assay. Wt: the liver tissue of wild-type mice; P: the peritumors tissue of transgenic mice; T: the tumor tissue of transgenic mice; Non-Tg, C57BL/6J wild-type nontransgenic mice; Tg: transgenic mice. The numbers indicate different individuals. Bradford reagent was used as the loading control.](image)
regulated in T compared with P and/or Wt. In this category, the enrolled proteins could be categorized into 3 types: (1) T>P=Wt, these proteins were significantly downregulated in T compared with P and Wt. Most proteins were classified into this type, which indicated that the proteins were HCC-related; (2) T=Wt>P and (3) T<Wt<P, these proteins were significantly upregulated in P compared with Wt and T. The classification of proteins into this type indicates their positive correlation to oncogene expression in normal hepatocytes and the possibility that they are regulated by the tumor defense system (Fig. 6B).

The classification of proteins as the ras oncogene positive-related proteins indicated that these proteins were significantly upregulated in P and T compared with Wt. In this category, the enrolled proteins could be categorized into 2 types: (1) T=P=Wt, these proteins were equally and significantly upregulated in P and T compared with Wt. The classification of proteins into this type indicate their positive correlation to the ras oncogene regardless of whether they occurred in normal hepatocytes or hepatic tumor cells and regardless of the expression levels of the ras oncogene; (2) T>P=Wt, these proteins were gradually and significantly upregulated in P and T compared with Wt. The classification of proteins into this type indicates their positive correlation to the expression levels of the ras oncogene (Fig. 6C).

The classification of proteins as the ras oncogene negative-related proteins indicated that these proteins were significantly downregulated in P and T compared with Wt. The classification of proteins into this type indicate their negative correlation to the ras oncogene regardless of whether they occurred in normal hepatocytes or hepatic tumor cells and regardless of the expression levels of the ras oncogene; (2) T<P=Wt, proteins were gradually and significantly downregulated in P and T compared with Wt. The classification of proteins into this type indicate their negative correlation to the expression levels of the ras oncogene (Fig. 6D).

Interestingly, this analysis also clearly showed gender-dependent trends in protein expression. Most of the proteins that were expressed differentially and were classified into the...
Differentially expressed proteins were divided into expected regulation patterns that depended on hepatic tumorigenesis or the ras oncogene. This study aimed to discover proteins that are related to hepatic tumorigenesis or the ras oncogene by a comprehensive analysis of the liver tissue proteins of wild-type mice (Wt), the peritumor tissue proteins of transgenic mice (P), and the tumor tissue proteins of transgenic mice (T). The identity of HCC-positive-related (A) or -negative-related (B) proteins indicated that these proteins were significantly up- or downregulated in T as compared with P and/or Wt, respectively. Analysis of ras oncogene positive-related (C) or negative-related (D) proteins indicated that these proteins were significantly up- or downregulated in P and T when compared with Wt, respectively. The relative expression levels of proteins are represented as 1, 2, and 3 graded models, and the different grades indicate significant differences in protein levels. Red colors indicate that the proteins are present in both male and females.
HCC-positive or -negative related categories were biased toward females. However, most of the proteins that were expressed differentially and were classified into the ras oncogene-positive or -negative related categories were biased toward males. These findings indicate that the ras oncogene more easily induces the molecular changes contributing to hepatic tumorigenesis in the hepatocytes of males than in those of females, and this finding may play a crucial role in understanding the mechanism underlying the male prevalence of HCC.

DISCUSSION

A marked male predominance regarding HCC incidence is observed worldwide, with reported male-to-female ratios ranging from 2:1 to 8:1 in most series, regardless of risk (high or low) area status, ethnic and geographic diversity, and the causative agent (34–37). However, the gender disparity in the survival and prognosis of HCC patients is controversial. It was previously thought that environmental factors, such as a higher prevalence of persistent HBV or HCV infection, alcohol abuse, and smoking as well as more unfavorable pathological features of HCC at initial diagnosis, such as significantly higher mean serum bilirubin levels, larger mean tumor size, less frequent nodular type but more frequent massive and diffuse types of HCC, more advanced Okuda’s stage, and more frequent association with venous invasion (and therefore less likely to undergo curative therapy) may contribute to the worse survival and prognosis in male patients as compared with female patients (38). However, recent findings in a large (1138 HCC cases) study provide compelling evidence that female gender represents a distinct survival advantage over male gender in patients with unresectable HCC that present with similar tumor characteristics, liver function, and coexisting liver disease (39). In addition, the results of animal studies have indicated that male rodents are more susceptible to spontaneous or chemically induced hepatocarcinogenesis than females, and this finding may play a crucial role in understanding the mechanism underlying the male prevalence of HCC.

Sex hormones, the main factors controlling the differences in sex characteristics and biological progresses between males and females, have been logically recognized as contributors to gender-dependent hepatocarcinogenesis. It is generally thought that androgens have stimulatory effects whereas estrogen plays a protective role in HCC. However, the biological activity of natural progesterones in HCC is controversial and lacks clear results (43). Notably, the hormone-dependent protection against HCC in women is impressively demonstrated by the finding that a marked increase in female liver cancer incidence occurs following menopause, but HCC resistance is restored after hormone replacement therapy (HRT) in postmenopausal women (44, 45). Genetically, the higher DNA synthesis found in the cirrhotic livers of men may be a contributory factor to the higher rate of cell turnover and, therefore, to the gender discrepancy of HCC (10, 46). Molecular studies in animal models have suggested that testosterone plays a role in enhancing transforming growth factor (TGF) alpha-related hepatocarcinogenesis and hepatocyte proliferation (47). Estrogen has been suggested to contribute directly to liver tumor inhibition through its effects on nonparenchymal hepatic immune cells (41, 48). However, in contrast to the most common view, complex findings have shown that HCC occurs more often in males with chronic liver disease, which in turn leads to a hyperestrogenic state that has been implicated in the pathogenesis of HCC (49–52). Experimental and clinical data have shown that both estrogens and androgens have important effects in controlling the replication rate of hepatic cells (53, 54). Both estrogens and androgens may also have an effect on inducing or at least promoting the growth of HCC. Evidence obtained from clinical trials utilizing anti-androgen and antiestrogen therapies suggests that once the tumor has developed, these therapies have no clinically significant effect on disease progression (49, 52, 54). Therefore, more advanced technologies and disease models should be explored to uncover the mechanisms that underlie the controversial phenomena related to gender bias in HCC.

Proteomics is a key method for advancing our understanding of biological processes and systems. Although the proteomic analysis of HCC samples obtained from human beings or experimental animals has been widely investigated, comparative proteomics studies of HCC between males and females are rare. In the present study, we report for the first time proteomics data that were obtained by comparing hepatic tumorigenesis induced by the Hras12V oncogene between transgenic males and females. To our surprise, we found that significantly higher numbers of altered protein spots were detected in the comparison between the hepatic tumor tissues of transgenic mice and the normal liver tissues of nontransgenic mice in males than in females, regardless of whether the spots represented up- or downregulated proteins (524 versus 411 for upregulated and 515 versus 381 for downregulated protein spots, respectively) (Fig. 2B, 2C). This trend was extremely clear when comparing the peritumor tissues (normal hepatocytes expressing the Hras12V oncogene) and normal liver tissues of nontransgenic mice in males than in females, regardless of whether the spots represented up- or downregulated proteins (475 versus 95 for upregulated and 330 versus 67 for downregulated protein spots, respectively) (Fig. 2B, 2C). However, the numbers of altered protein spots were significantly lower when comparing hepatic tumor tissues and peritumor tissues in transgenic males than when making the corresponding comparison in females (289 versus 423 for upregulated and 301 versus 412 for downregulated protein spots, respectively) (Fig. 2B, 2C).
Further comparison among randomly identified proteins also showed similar trends (Fig. 3A, 3B). These findings indicate that not only do gender-dependent biological processes occur in hepatic tumorigenesis but also gender-dependent molecular responses to oncogenes occur in normal hepatocytes. We therefore hypothesized that female hepatocytes are disturbed by oncogenes with great difficulty, whereas male hepatocytes are readily disturbed. Further comprehensive analysis among Wt, P, and T in males and females provided the following supplemental evidence: the altered proteins responding to the ras oncogene were biased toward males but not toward females (Fig. 6). Moreover, this finding also indicates that the resistance to hepatic tumorigenesis in females might result from the poor induction of HCC-related proteins in female normal hepatocytes because these HCC-related proteins were mainly concentrated in the hepatic tumor tissues of females but not in those of males (Fig. 6). Supporting this evidence, the activation levels of p-ERK and p-Akt/p-mTOR were much higher in the hepatic tumors of females than in those of males (Fig. 1E). In addition, our previous report showed that, compared with males, female could effectively activate the p53/p21 pathways to protect hepatocytes from tumorigenesis caused by the ras oncogene discussed here (25). Taken together, these findings show that gender-dependent molecular responses to the stress of oncogenes play crucial roles in hepatic tumorigenesis disparity, and these offer valuable clues for elucidating the mechanisms that contribute to male-biased hepatocarcinogenesis.

To further validate our proteomic data, a literature investigation was performed. Because few researchers have examined the gender disparity of HCC, we focused on differentially expressed proteins that were identified in the hepatic tumor tissues of transgenic males. Among the identified 89 proteins, 77 were significantly altered in the hepatic tumors of transgenic males. Among the 77 significantly altered proteins, 50 have been described in previous proteomics or transcriptomics analysis or in specific studies on hepatic tumor tissues or on human and/or mouse cells (supplemental Table S5). Among the 77 significantly altered proteins, 50 have been described in previous proteomics or transcriptomics analysis or in specific studies on hepatic tumor tissues or on human and/or mouse cells (supplemental Table S8). The trend in the variations of 48 out of the 50 proteins identified in our proteomic data was consistent with the findings in published studies, except for CYB5A and LDHA. This literature investigation further validated the reliability of our proteomic data and the validity of the hepatic tumor model of Hras12V transgenic mice. Therefore, although subsequent cellular and molecular biology investigations are needed, the significantly altered proteins that were identified for the first time in the present study may offer valuable clues for uncovering the mechanisms related to hepatocarcinogenesis and HCC gender disparity.

Apolipoprotein A-I (ApoA1) has been suggested to contribute to liver steatosis based on several lines of evidence (55, 56). In the present study, the yellow-white appearance of hepatic tumors in the gross anatomy examination and the abundance of vesicles observed in the hepatic tumor tissues under histopathological examination indicate the presence of lipid metabolism disorders (Fig. 1A, 1B). Consistently, ApoA1 was found to be significantly elevated in the hepatic tumors of transgenic mice, and these results were further validated by Western blot assays (Fig. 4; supplemental Table S5). The elevation in ApoA1 protein levels and its accumulation around fatty vesicles has been previously reported by us (57), and the present data further confirmed these molecular events. Similarly, we previously reported the significant development of steatosis in transgenic mice expressing hepatitis C virus non-structural 5A (NS5A) protein, and this was also primarily because of the accumulation of apolipoprotein A-I around fatty droplets (58). Therefore, we suggest that the accumulation of ApoA1 may indicate its dysfunction. From different points of view, other investigators have proposed that ApoA1 plays a protective function in reducing the already elevated hepatic lipid content (59). Intriguingly, in the present study, we identified at least eight spots as ApoA1, and these spots were all significantly upregulated, not only in tumor tissues but also in peritumor tissues (supplemental Table S9). These proteomic data indicate that ApoA1 is subject to multiple modifications and has complex functions. In addition, the disorder involving the secreted ApoA1 protein in the hepatocytes and hepatic tumor cells of the transgenic mice indicated that it plays a dominant role in Ras-related signaling pathways and therefore provides a possible biomarker for predicting Ras/ERK-activated HCC and guiding clinical treatment.

Fatty-acid-binding protein 5 (FABP5) is a member of the FABP family of intracellular lipid-binding proteins; these proteins are involved in the binding and storing of hydrophobic ligands, such as long-chain fatty acids, and transporting them to the appropriate compartments in the cell, where they are involved in various biological processes, including cell growth and differentiation (60). Although FABP5 has been recognized as a cancer-promoting gene that is upregulated in various cancers and is associated with cell growth, metastasis, and poor survival (61–63), its roles in HCC are largely unknown. It has been reported that FABP5 is expressed in normal endothelial cells of the microvasculature of the placenta, heart, skeletal muscle, small intestine, lung, and renal medulla and is not detectable in liver tissues (64). Consistently, we also did not observe the expression of FABP5 in normal liver tissues (Fig. 4). Intriguingly, FABP5 was upregulated in the liver of Hras12V transgenic mice and was found at extremely high levels in hepatic tumor tissues (Fig. 4). Consistently, the overexpression of FABP5 was also found in human HCC using proteomics (65). Although the detailed mechanisms remained to be elucidated, these findings indicate that FABP5 may be not only a potential biomarker for HCC but also an important HCC promoter. In addition, FABP5 transcription has been suggested to be upregulated because of the hypomethylation of the FABP5 promoter and to be dependent on the transcription factors Sp1 (specificity protein 1) and c-Myc in prostate cancer (60) or NF-κB in breast cancer (66). Interestingly, in the
Hras12V transgenic mice, consistent with the observed protein overexpression, the mRNA levels of FABP5 were also significantly upregulated not only in hepatic tumor tissues but also in normal peritumor tissues, as observed by next generation sequencing (NGS) and RT-qPCR assays (supplemental Table S10, supplemental Fig. S2). Because Sp1, c-Myc, and NF-κB are critical downstream effectors of Ras/ERK, we suggest that the upregulation of FABP5 in hepatocytes or HCC might be because of activation of the Ras/ERK pathway, which is an important molecular characteristic of HCC.

Peroxiredoxin 6 (PRDX6) is a member of the peroxiredoxin family of antioxidant proteins that exhibit peroxidase activity. Although PRDX6 is highly expressed in many human organs, including liver (67), the functions of PRDX6 are complex, and little is known regarding its role in human cancer. PRDX6 has been shown to protect cells from oxidative damage and promote cell maintenance and tumor-supportive adaptation in cancerous states; therefore, this protein contributes to many types of tumors, such as breast cancer and lung cancer (68, 69). However, in HCC patients, PRDX6 was significantly decreased in HCC tissues compared with peritumor tissues, and this decrease is an independent risk factor indicating a poor prognosis of HCC (70). This finding demonstrates the tumor-suppressing potential of PRDX6, and a new perspective is required for studying this protein. Unlike other members in the PRDX family, PRDX6 exhibits Ca²⁺-independent phospholipase A2 (iPLA2) activity in addition to peroxidase activity. A recent study has suggested that PRDX6 might inhibit the carcinogenesis of HCC and the iPLA2 activity of PRDX6-promoted cancer cell death, which is induced by TNF-α (70).

Consistent with this clinical report, the present study also found that PRDX6 was significantly downregulated in T compared with P and WT (Fig. 4, supplemental Table S5). These findings indicate that PRDX6 plays crucial roles in suppressing hepatocellular carcinogenesis and that the Hras12V transgenic mice offer a valuable in vivo model that will prove useful in further investigations.

The unique properties of 2D gels are the efficient separation and characterization of different protein isoforms resulting from transcript splicing or dynamic post-translational modifications (PTM) such as acetylation, hydroxylation, glycosylation, and functional cleavage (31, 71). Therefore, the number of spots (protein isoforms) detected is much larger than the number of genes identified, as the data showed in the present study (supplemental Table S3, S4). Moreover, depending on our validation of 2D-DIGE data by Western blot, although the variation tendencies of majority protein spots could reflect the variation tendencies of identified genes, a small part of protein spots just represent the variation tendencies of the protein isoforms per se, such as PRDX6 in females (Fig. 4), and these offer valuable clues for further revealing their roles in biological processes and pathogenic conditions.

In conclusion, the present study presents proteomics data related to the gender disparity in ras oncogene-induced hepatic tumorigenesis for the first time. The findings of gender-dependence of the biological and molecular responses that occur during hepatic tumorigenesis offer valuable clues that may prove useful in elucidating the mechanisms that contribute to male-biased hepatocarcinogenesis. In addition, the differentially expressed proteins that were identified in the hepatic tumors will add important biological information to tumor-related bioinformatics databases.

DATA AVAILABILITY

All mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the MassIVE partner repository with the dataset identifiers: MSV000080343 (http://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task_bd7367932bd3430fa466e0f5319077a1).

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