Cancer Science (2015.8) 106(8):972–981.

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Differential reactivation of fetal/neonatal genes in mouse liver tumors induced in cirrhotic and non-cirrhotic conditions

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Key words
Hepatocellular carcinoma, insulin-like growth factor 2, liver cirrhosis, mRNA expression, trefoil factor 3

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Funding Information
Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Received January 16, 2015; Revised May 6, 2015; Accepted May 17, 2015

Cancer Sci 106 (2015) 972–981
doi: 10.1111/cas.12700

Hepatocellular carcinoma develops in either chronically injured or seemingly intact livers. To explore the tumorigenic mechanisms underlying these different conditions, we compared the mRNA expression profiles of mouse hepatocellular tumors induced by the repeated injection of CCl₄ or a single diethylnitrosamine (DEN) injection using a cDNA microarray. We identified tumor-associated genes that were expressed differentially in the cirrhotic CCl₄ model (H19, Igf2, Cbr3, and Krz20) and the non-cirrhotic DEN model (Tff3, Akr1c18, Gpc3, Afp, and Apc2d) as well as genes that were expressed comparably in both models (Ly6d, Slpi, Spink3, Sca2, and Cpe). The levels and patterns of mRNA expression of these genes were validated by quantitative RT-PCR analyses. Most of these genes were highly expressed in mouse livers during the fetal/neonatal periods. We also examined the mRNA expression of these genes in mouse tumors induced by thioacetamide, another cirrhotic inducer, and those that developed spontaneously in non-cirrhotic livers and found that they shared a similar expression profile as that observed in CCl₄-induced and DEN-induced tumors, respectively. There was a close relationship between the expression levels of Igf2 and H19 mRNA, which were activated in the cirrhotic models. Our results show that mouse liver tumors reactivated fetal/neonatal genes, some of which are specific to cirrhotic or non-cirrhotic modes of pathogenesis.

Various risk factors for hepatocellular carcinoma (HCC) exist, including infection with hepatitis B virus (HBV) and hepatitis C virus, alcoholic and non-alcoholic fatty liver disease, and several hereditary metabolic diseases.¹ However, chronic liver injury, typically cirrhosis, is the most important and common setting for the development of HCC. Although recent studies have revealed critical roles of the interleukin-6 (IL-6)/STAT pathway and the nuclear factor-κB pathway, the exact mechanisms underlying the development of HCC in chronic liver disease remain obscure.² Furthermore, a small fraction of HCC has been known to occur in patients with a seemingly intact liver. Such non-cirrhotic HCC may share several characteristics with hepatocellular adenoma,³ which has been shown to undergo malignant transformation with an overall frequency of 4.2%.⁴ There might be different tumorigenic mechanisms between tumors associated with chronic injury or cirrhosis and those that develop in seemingly intact livers.

A variety of mouse models of hepatocarcinogenesis have been used to elucidate the mechanisms underlying the development of HCC. The most widely used is the diethylnitrosamine (DEN)-induced model,⁵ in which hepatocellular adenoma and HCC develop in an intact, non-cirrhotic liver. Several liver tumor-prone transgenic mouse lines have also been generated by the introduction of HBsAg and HBx⁶,⁷ SV40 large T antigen, a secretable form of epidermal growth factor (EGF),⁸ or oncogenes such as E2F-1, c-Myc, and transforming growth factor-β.⁹ In these models, liver tumors also developed in non-cirrhotic livers; thus, the results that were obtained need to be interpreted cautiously when they are extrapolated for understanding the pathogenesis of human HCC that develops in fibrotic or cirrhotic backgrounds. Conversely, long-standing centrilobular injury inflicted by the chronic administration of CCl₄ or thioacetamide (TAA) in adult mice can induce liver tumors in a fully established cirrhotic background.⁹,¹⁰

In the present study, to gain insights into the tumorigenic mechanisms in cirrhotic and non-cirrhotic conditions, we compared the mRNA expression profiles of mouse liver tumors induced by the repeated injection of CCl₄ (cirrhotic protocol) or a single DEN injection when the mice were 2 weeks old (non-cirrhotic protocol) using a cDNA microarray and quantitative RT-PCR (qRT-PCR). We identified several genes whose mRNA expression was increased predominantly in either CCl₄-induced or DEN-induced liver tumors as well as genes that were increased comparably in both. We further examined the mRNA expression of the identified genes, most of which were also highly expressed in the fetal/neonatal liver, in other mouse liver tumors induced under cirrhotic and non-cirrhotic conditions.
Materials and Methods

Animals. C3H/HeNCrlCrj (C3H) and C57BL/6J (C57) mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). C3H × C57 F1 offspring were generated by breeding male C3H and female C57 mice. The mice were killed under deep anesthesia, and the livers were removed for further examination. The protocols used for animal experimentation were approved by the Animal Research Committee, Asahikawa Medical University (Asahikawa, Japan), and all animal experiments adhered to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences (8th edn, 2011).

Mouse liver tumor models. Male C3H × C57 F1 or C57 mice (8–10-weeks old) were treated with CCl4 (Kanto Chemical, Tokyo, Japan) three times per week (1 mL/kg, s.c.; 1:5 dilution in olive oil) for 24 weeks to induce liver cirrhosis and subsequent tumor formation. Male C3H × C57 F1 mice were also treated with TAA (Sigma-Aldrich, St. Louis, MO, USA) (0.03% in drinking water) for 30 weeks to generate another cirrhotic model of liver tumors.

To induce liver tumors in a non-cirrhotic background, C3H × C57 F1 mice were treated with a necrotizing dose of DEN (5 mg/kg, i.p.) at 2 weeks after birth and killed after 44 weeks. As another non-cirrhotic model, liver tumors that had spontaneously developed in C3H mice aged 13–15 months were analyzed.11

cDNA microarray analysis. Total RNA was prepared from snap-frozen liver tissues using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). Samples of CCl4-induced liver tumors (a mixture of five independent large tumors), DEN-induced liver tumors (a mixture of five independent large tumors), CCl4-induced cirrhotic liver tissues (non-tumorous tissues of livers harboring tumors, a mixture of five tissues from five mice), and control liver tissue (olive oil-treated; a mixture of two tissues from two mice) were analyzed and compared by one-color microarrays (3D-Gene Microarray; Toray, Tokyo, Japan). After background subtraction, the raw microarray data were normalized using a standard global normalization technique, and the signal intensities were calculated as the fold changes of expression values. The data of differentially expressed genes that were significantly changed (>4-fold, compared with control) were subjected to Z-score transformation and loaded in a centroid-linkage hierarchical clustering assay using a Pearson correlation (uncentered) similarity metric with Cluster 3.0. Then, the Java TreeView software (http://jtreeview.sourceforge.net/) was applied to reveal the hierarchical gene groups.
Hierarchical clustering of tumor-associated genes was carried out using Z-score-normalized data. The Z-score was cropped to -2.0 to +2.0 when generating a two-color heat map.

**Microscopic examination, immunohistochemistry, and in situ hybridization.** The livers were fixed in phosphate-buffered 10% formalin for 24 h, and paraffin sections were then prepared. Immunohistochemical staining was carried out with the EnVision®-HRP system (Dako, Carpinteria, CA, USA) on deparaffinized sections treated with Target Retrieval Solution (Dako). The antibodies used were as follows: anti-insulin-like growth factor 2 (IGF2) (ab9574; Abcam, Cambridge, UK), anti-α-fetoprotein (AFP) (14550-1-AP, for mouse tissues; Proteintech Group, Chicago, IL, USA), anti-AFP (A0008, for human tissues; Dako), and anti-trefoil factor 3 (TFF3) (Abcam, San Diego, CA, USA). Staining was assessed by two skilled pathologists.

**Human liver samples.** The retrospective analysis of surgical specimens was approved by the internal review board of Asahikawa Medical University. A total of 33 HCC samples from patients who had curative hepatectomy and five intact liver tissues surrounding the resected cavernous hemangiomas were collected. Among the HCC samples, nine cases were devoid of any detectable fibrosis or inflammation in the non-tumorous liver parenchyma, whereas the rest showed various degrees of liver fibrosis (fibrosis expansion of the portal tract, bridging fibrosis, and cirrhosis).

### Table 1. Top 10 highly expressed genes in CCl4-induced tumors in genetic cluster C

| Gene                         | Cirrhosis (CCl4-NT) | CCl4 tumor (CCl4-T) | DEN tumor (DEN-T) |
|------------------------------|---------------------|---------------------|-------------------|
| Insulin-like growth factor 2 | 6.97                | 9.70                | 3.06              |
| Lymphocyte antigen 6 complex | 6.46                | 8.33                | 6.55              |
| H19 fetal liver mRNA (H19)   | 5.48                | 8.13                | 6.47              |
| Secretory leukocyte peptidase inhibitor (Slep) | 3.52 | 6.92 | 6.35 |
| Keratin 20 (Krt20)           | 4.70                | 6.83                | 4.41              |
| Stearyl-Coenzyme A           | 4.86                | 6.51                | 5.65              |
| Desaturase 2 (Scd2)          | 5.03                | 6.18                | 3.89              |
| Calcium and integrin binding family member 3 (Cib3) | 3.76 | 6.15 | 3.58 |
| Carbonyl reductase 3 (Cbr3)  | 2.55                | 6.04                | 2.35              |

### Table 2. Top 10 highly expressed genes in diethylnitrosamine (DEN)-induced tumors in genetic cluster D

| Gene                         | Cirrhosis (CCl4-NT) | CCl4 tumor (CCl4-T) | DEN tumor (DEN-T) |
|------------------------------|---------------------|---------------------|-------------------|
| Glypican 3 (Gpc3)            | 2.63                | 6.06                | 8.62              |
| Aldo-keto reductase family 1, member C18 (Akr1c18) | 3.26 | 6.44 | 8.57 |
| Flavin containing monooxygenase 3 (Fmo3) | 0.60 | 1.90 | 7.33 |
| ATP-binding cassette, subfamily D, member 2 (Abcd2) | 3.89 | 4.90 | 7.32 |
| Tetraspanin 8 (Tspan8)       | 3.92                | 6.29                | 7.23              |
| Alpha fetoprotein (AfP)      | 4.19                | 4.47                | 7.11              |
| Trefoil factor 3, intestinal (Tff3) | 0.91 | 6.30 | 6.98 |
| Serine peptide inhibitor, Kazal type 3 (Spink3) | 6.68 | 6.22 | 6.92 |
| Patatin-like phospholipase domain containing 5 (Pnpla5) | 1.94 | 3.88 | 6.53 |
| Cyclin-dependent kinase inhibitor 2B (Cdkn2b) | 1.84 | 4.73 | 5.96 |

DEN-T, diethylnitrosamine-induced tumor; CCl4-NT, CCl4-induced cirrhotic tissue (non-tumor); CCl4-T, CCl4-induced tumor.

The Z-score was cropped to -2.0 to +2.0 when generating a two-color heat map.
expressed in either CCl₄- or DEN-induced tumors (Tables 1, 2).

Validation of mRNA expression profiles by RT-qPCR and in situ detection of IGF2, H19 mRNA, TFF3, and AFP in tumors. The levels and patterns of mRNA expression of the identified genes were validated by RT-qPCR analyses. The mRNA expression of H19, Igf2, Cbr3, and Krt20 was predominantly increased in CCl₄-induced tumors (more than 4-fold that observed in DEN-induced tumors; referred to as CCl₄-associated); that of Tff3, Akr1c18, Gpc3, Afp, and Abcd2 was predominantly increased in DEN-induced tumors (more than 4-fold over that in CCl₄-induced tumors; referred to as DEN-associated), and that of Ly6d, Sipi, Spink 3, Scd2, and Cpe was increased at comparable levels in CCl₄- and DEN-induced tumors (referred to as Common) (Fig. 2a). The changes in mRNA expression observed in the remaining genes (Cib3, Top2a, Cdkn2b, Pnpla5, and Tspan8) in the tumors were not statistically significant (Fig. S1).

Among the changes in mRNA expression associated with CCl₄-induced tumors, an increase in Igf2 and H19 mRNA was the most specific and was found in approximately half of the tumors. In addition, the magnitude of the induction of Tff3 mRNA, especially in DEN-tumors, was very impressive. To confirm the protein expression of IGF2 and TFF3 and the mRNA expression of H19 in liver tumors, we carried out immunohistochemistry for IGF2 and TFF3 and in situ hybridization for H19. The expression of AFP, a prototype oncofetal marker for HCC, was also examined. Although all of these were negative in adult liver tissues, Igf2 was positive in approximately half of CCl₄-induced tumors but completely negative in DEN-induced tumors (Fig. 2b). H19 mRNA was strongly expressed in some CCl₄-induced tumors (Fig. 2b). Diethylnitrosamine-induced tumors also expressed H19 mRNA, but its levels were generally low (Fig. 2b). Although TFF3 was detected in both types of tumors, DEN-induced tumors tended to show stronger staining (Fig. 2b). α-Fetoprotein was strongly positive in DEN-induced tumors, whereas CCl₄-induced tumors were negative or contained scattered positive cells (Fig. 2b).

Relationship between proliferative activity of tumor cells and mRNA expression levels of tumor-associated genes. We next examined whether the mRNA expression of the tumor-associated genes correlated with tumor cell proliferation, as estimated by Ki-67 immunohistochemistry. In the CCl₄ model, the expression levels of Cbr3 and Tff3 were correlated with the proliferative activity of the tumor cells (Fig. 3). Although Igf2

Fig. 2. Differential expression of mRNA and their products in CCl₄-induced and diethylnitrosamine (DEN)-induced liver tumors. (a) Quantitative RT-PCR analyses of mRNA expression of 15 tumor-associated genes. The genes preferentially expressed in CCl₄-induced tumors and DEN-induced tumors are designated “CCl₄-associated” and “DEN-associated,” respectively, and those comparatively expressed in CCl₄-induced and DEN-induced tumors are designated “Common.” Each value is expressed as the mean ± SEM. The ages of the mice at analyses were 32–34 weeks and 46 weeks in the CCl₄-induced and DEN-induced models, respectively. The number of samples in each group was 5, 12, 15, 6, and 13 for CCl₄ control (olive oil, C), CCl₄-induced cirrhosis (NT), CCl₄-induced tumors (T), DEN control (NT), and DEN-induced tumors (T), respectively. *P < 0.05, **P < 0.01, ***P < 0.001 versus control; one-way factorial ANOVA. (b) In situ detection of insulin-like growth factor 2 (IGF2), H19 mRNA, trefoil factor 3 (TFF3), and α-fetoprotein (AFP) in CCl₄-induced and DEN-induced tumors. Immunohistochemistry for IGF2, TFF3, and AFP and in situ hybridization for H19 mRNA. NT, non-tumor; T, tumor. Scale bar = 20 μm.
encodes IGF2, which has been shown to play important roles in cell growth. IGF2 mRNA expression was not significantly correlated with tumor cell proliferation, similar to other genes (Figs 3, S2). In the DEN model, the expression of none of the genes analyzed was related to the proliferative activity of the tumor cells (Figs 3, S2).

**Fetal or neonatal expression of tumor-associated genes.** Because the identified tumor-associated genes included well-known oncofetal genes, such as IGF2, H19, Gpc3, and Afp, we examined their mRNA expression during fetal and neonatal periods. Our results clearly showed that all of these genes, with the exceptions of Cbr3 and Cpe, were highly expressed in either the fetal or neonatal periods (Fig. 4a), indicating that the differential activation of fetal/neonatal gene expression occurs in CCl4- and DEN-induced liver tumors. As expected, the protein expression of IGF2 and TFF3 and the mRNA expression of H19 were detected in hepatoblasts/hepatocytes during the fetal or neonatal period (Fig. 4b).

**Comparison with other cirrhotic and non-cirrhotic liver tumor models.** Next, we examined whether similar differential activation could also be observed in other liver tumor models. Thioacetamide-induced selective centrifugal injuries and chronic TAA administration resulted in multiple liver tumors (hepatocellular adenoma or well differentiated HCC), which were associated with marked cirrhosis in the surrounding liver (Fig. 5a). In TAA-induced tumors, there were increases in the expression of IGF2 mRNA and Krt20 mRNA, with a tendency for increased mRNA expression of H19 and Igf2bp3 (Fig. 5b). In contrast, the mRNA expression of Akr1c18, Gpc3, and Afp, which was highly characteristic of DEN-induced tumors, was not observed in TAA-induced tumors, although there was an increase in Abcd2 mRNA (Fig. 5b). In spontaneously formed liver tumors (well to moderately differentiated HCC) in the intact livers of aged C3H mice, there was no increase in the mRNA expression of the genes that were selectively increased in CCl4-induced tumors, but the mRNA expression of Tff3, Akr1c18, Afp, and Abcd2 was significantly increased (Fig. 5b). The mRNA expression of all the "common" genes was increased in TAA-induced tumors, whereas that of Spink3, Sca2, and Cpe was lacking in the spontaneously formed tumors (Fig. 5b).

**Two-dimensional hierarchical cluster analysis of tumor-associated genes in cirrhotic and non-cirrhotic models.** The mRNA expression data of the 15 tumor-associated genes in the four different liver tumor models (CCl4-induced, DEN-induced, TAA-induced, spontaneous) were subjected to unsupervised 2-D hierarchical cluster analysis (Fig. 6). Interestingly, the mRNA expression profiles of the 15 genes almost clearly segregated control liver tissues, cirrhotic tissues, and tumors that had developed in the cirrhotic and non-cirrhotic backgrounds. Furthermore, there were several clusters of between two and four transcripts, which characterized tumors that had developed in the cirrhotic background (H19, IGF2, and Igf2bp3), in the non-cirrhotic background (Tff3, Akr1c18, Abcd2, and Gpc3), and in either background (Sca2 and Slpi; Cpe and Ly6d).

**Selective activation of IGF2 and its related genes in CCl4- or TAA-induced tumors.** There was a significant positive correlation between the mRNA expression levels of IGF2 and H19 (Fig. 7a), which are known to be adjacenty located in the genome and are regulated through reciprocal imprinting and a common enhancer.\(^ {12,13} \) The mRNA expression of Igf2bp3, whose product is functionally correlated with Igf2 mRNA and H19 mRNA,\(^ {14} \) was also increased in CCl4-induced tumors (Fig. 2). The expression of Igf2bp3 mRNA was significantly higher in tumors with substantial levels of Igf2 mRNA expression (Igf2/Gapdh > 0.01) compared with those that showed very low or no Igf2 mRNA expression (Igf2/Gapdh < 0.01) (Fig. 7b). Similar findings were obtained with TAA-induced tumors (Fig. 7).

**Expression of IGF2, TFF3, and AFP in human HCC with or without liver fibrosis.** The above experiments indicated that IGF2

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### CCl4-induced Tumors

**IGF2**

![IGF2](image1)

**Cbr3**

![Cbr3](image2)

**TFF3**

![TFF3](image3)

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### DEN-induced Tumors

**TFF3**

![TFF3](image4)

**Gpc3**

![Gpc3](image5)

**Afp**

![Afp](image6)

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**Fig. 3.** Relationship between mRNA expression of tumor-associated genes and tumor cell proliferation. Scatter plots of mRNA expression levels of selected tumor-associated genes and Ki-67 labeling index (%). Afp and IGF2 were significantly correlated with tumor cell proliferation.
expression showed significant discrimination ability for mouse liver tumors developed in cirrhotic and non-cirrhotic backgrounds. We examined IGF2 expression in human HCC developed with seemingly intact (non-fibrotic) livers or fibrotic livers. Immunohistochemically, tumor cells stained positive for either IGF2, TFF3, or AFP were found in several HCC cases, whereas these were negative in the surrounding liver parenchyma (Fig. 8a). Clusters of IGF2-positive tumor cells were present in 11.1% and 54.2% of HCC developed with non-fibrotic livers (n = 9) and those developed with fibrotic livers (n = 24), respectively (Fig. 8b). This difference was statistically significant (P = 0.0466, Fisher’s exact test). In contrast, there were no significant differences in the expression of TFF3 or AFP in HCC developed under these different conditions (Fig. 8b).

Discussion

We identified genes whose mRNA expression was increased in mouse liver tumors induced in cirrhotic and non-cirrhotic conditions. Based on gene expression clustering of these genes, we could distinguish tumors arising in the two different backgrounds, indicating the presence of genes that are differentially expressed during the tumorigenic course. Interestingly, most of the tumor-associated genes were also activated in the fetal/neonatal periods. The activation of these genes, including well-established oncofetal genes (e.g., Afp, Igf2, H19, and Gpc3),15–17 occurred in the late fetal period (E16.5) and persisted during the postnatal period. This finding indicates that the mRNA expression of these genes in the liver tumors might not reflect the simple dedifferentiation of transformed hepatocytes into immature hepatoblasts. Although the significance of the reactivation of fetal/neonatal genes in mouse liver tumors is unclear, our study shows that transformed hepatocytes might use the cellular system that is active during the fetal/neonatal periods, when the most robust physiological proliferation of hepatocytes occur.18

Tff3 mRNA and its product, an intestinal polypeptide, TFF3, were highly expressed in mouse liver tumors, particularly in those induced by DEN in C3H × C57 Fl and those that developed spontaneously in C57Fl mice. The TFF3 polypeptide was also detected in human HCC arising in either fibrotic or non-fibrotic liver. It has been reported that Tff3 mRNA expression is increased in spontaneous liver tumors in tumor-prone PWK mice and those that develop in SV40 T antigen transgenic mice, as well as in human HCC.19 Marked increases in Tff3

Fig. 4. Fetal/neonatal activation of tumor-associated genes and their products. (a) Quantitative RT-PCR analyses of mRNA expression of tumor-associated genes during fetal/neonatal periods. Each value is expressed as the mean ± SEM. The number of samples in each group was 3, 5, 7, 10, 4, 4, 4, and 7 for E13.5, E16.5, P0 (immediately after birth), P1 (1 day after birth), P3 (3 days after birth), P6 (6 days after birth), 1 m (1 month old), and 5 m (5 months old), respectively. *P < 0.05, **P < 0.01, ***P < 0.001 versus control (5 m); one-way factorial ANOVA. (b) In situ detection of insulin-like growth factor 2 (IGF2), H19 mRNA, trefoil factor 3 (TFF3), and α-fetoprotein (AFP) in developing livers. Immunohistochemistry for IGF2, TFF3, and AFP and in situ hybridization for H19 mRNA in fetal (E16.5) and neonatal (P0) livers. Scale bar = 20 μm.
mRNA have also been found in liver tumors that developed in HBx transgenic mice(20) and those that developed in secretable EGF-expressing transgenic mice. The expression of Tff3 mRNA is governed by several transcription factors, including hepatocyte nuclear factor 3 and nuclear factor-κB, and by DNA methylation of its promoter region. Specifically, promoter hypomethylation has been found in mouse liver tumors and human HCC.(19) Although TFF3 is highly expressed in goblet cells in the intestinal mucosa and has been suggested to be important in the processes of mucosal repair,(21) its role in hepatocarcinogenesis is currently obscure. However, Tff3 mRNA or TFF3 polypeptide may serve as useful biomarkers due to their high specificity to liver tumors.

Our study revealed that Igf2 and its related genes, H19 and Igf2bp3, were selectively activated in approximately half of the mouse liver tumors induced under cirrhotic, but not non-cirrhotic, conditions. The expression of Igf2bp3 mRNA was significantly higher in the Igf2-expressing tumors induced by CCl4, suggesting their functional correlation. Igf2 and H19, which is located immediately downstream of Igf2, are reciprocally imprinted through the hypomethylation and methylation of the differentially methylated region on the maternal allele and paternal allele, respectively, and their expression is dependent on the two endoderm-specific enhancers that lie 3' of H19.(12) Although we do not know whether the loss of imprinting or altered methylation states of this gene cluster might contribute to their activation, a highly significant correlation between the Igf2 mRNA and H19 mRNA levels in the tumors suggests that their transcriptional activation is mediated by common enhancers.(13) Increased mRNA expression of IGFL and H19, as well as a significant correlation of their mRNA expression levels, have been shown in human HCC.(22–25) However, following a partial hepatectomy in rodents, whereas H19 mRNA is markedly induced, Igf2 mRNA expression is

Fig. 5. Differential gene expression in thioacetamide (TAA)-induced liver tumors and spontaneously developed liver tumors in mice. (a) Histology of liver tissues from TAA-induced and spontaneous tumors. The ages of mice at analyses were 38–40 weeks and 13–15 months in the TAA-induced model and spontaneous model, respectively. HE staining. NT, non-tumor; T, tumor. Scale bar = 50 μm. (b) Quantitative RT-PCR analyses of mRNA expression of 15 tumor-associated genes in TAA-induced and spontaneous (Spont.) tumors. Each value is expressed as the mean ± SEM. The number of samples in each group was 7, 6, 9, 4, and 3 for the TAA control (C), TAA-induced cirrhotis (NT), TAA-induced tumors (T), control C3H mouse liver (NT), and spontaneous tumors (T) in C3H mice, respectively. *P < 0.05, **P < 0.01, ***P < 0.001 versus control; one-way factorial ANOVA.
not activated, indicating a striking contrast between non-transformed and transformed hepatocytes. Our analysis of human liver samples showed the lower frequency of IGF2 positivity in tumor cells in cases of HCC arising in almost intact liver, compared with those with various degrees of fibrosis. Interestingly, in an attempt of transcriptional classification of human HCC into six subgroups, an identified subgroup (G1) was characterized by the reactivation of IGF2 gene expression, as well as the association with HBV infection. Although further confirmation is needed in view of the small number of cases analyzed, our data suggest the possible involvement of the IGF2 signaling in human hepatocarcinogenesis that is associated with chronic liver injury and fibrosis.

Although the exact mechanisms of HCC development in chronically injured and fibrotic liver are not clear, it is probable that the continued stimuli for hepatocyte regeneration following liver injury may play important roles in this process. Repeated hepatocyte injury promotes hepatic tumorigenesis in hepatitis C virus transgenic mice, possibly through excessive EGF receptor and/or c-Met signaling activities. While our study elucidated the close association between the activation of Igf2 gene expression and the cirrhotic hepatocarcinogenesis, reactivation of Igf2 gene expression in liver tumors has been shown in the absence of liver injury in various experimental models with enhanced hepatocyte proliferation in whole livers, such as HBx and SV40 T Ag transgenic mice. In SV40 T Ag transgenic mice, as well as in HBV presurface gene (preS1 and preS2) transgenic mice, in which benign and malignant hepatocytic nodules appear following perpetual hepatocyte apoptosis and regeneration, Igf2 reactivation has been found to be associated with late progression steps toward HCC. Furthermore, in secretable EGF-expressing transgenic mice, there is a switch from the initial
have shown that the mouse liver tumors induced in cirrhotic genes might serve as signatures of distinctive modes of liver carcinogenesis. Thus, the reactivation of EGF-dependent state to an EGF-independent, IGF2-dependent selections, which render some of the altered hepatocytes more proliferative than the surrounding intact hepatocytes. Because epigenetic alterations, including losses and gains of DNA methylation, have been increasingly recognized to occur during carcinogenesis, we speculate that epigenetically regulated genes might serve as signatures of distinctive modes of liver tumor formation. In conclusion, by studying transcriptome characteristics, we showed that the mouse liver tumors induced in cirrhotic and non-cirrhotic conditions differentially reactivate various fetal/neonatal genes. In particular, our data raise the possibility that the IGF2 axis could be selectively activated in liver tumors induced by excessive proliferative stimuli following chronic liver injury.

Acknowledgments

We would like to thank Mr. Yoshiyasu Satake for animal care and Ms. Eina Yamatomi and Ms. Hiroko Chiba for secretarial assistance. We are also grateful to the staff of the Department of Pathology, Ashikawa Medical University Hospital for their generous help. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#18590362, #21590426, and #24390092) to Yuji Nishikawa.

Disclosure Statement

The authors have no conflict of interest.

References

1 El-Serag HB. Hepatocellular carcinoma. N Engl J Med 2011; 365: 1118–27.
2 Ramakrishna G, Rastogi A, Trehanpati N, Sen B, Khosla R, Sarin SK. From cirrhosis to hepatocarcinoma: new molecular insights on inflammation and cellular senescence. Liver Cancer 2013; 2: 367–83.
3 Liu TC, Vachharajani N, Chapman WC, Brunt EM. Noncirrhotic hepatocellular carcinoma: derivation from hepatocellular adenoma? Clinopathologic analysis. Mod Pathol 2014; 27: 420–32.
4 Stoot JH, Coelen RJ, De Jong MC, Dejong CH. Malignant transformation of hepatocellular adenomas into hepatocellular carcinomas: a systematic review including more than 1600 adenoma cases. HPB (Oxford) 2010; 12: 509–22.
5 Vesselovitch SD. Infant mouse as a sensitive bioassay system for carcinogenicity of N-nitroso compounds. JARC Sci Publ 1980; 31: 645–55.
6 Wang Y, Cui F, Lv Y et al. HBsAg and HBx knocked into the p21 locus causes hepatocellular carcinoma in mice. Hepatology 2004; 39: 318–24.
7 Borlak J, Meier T, Halter R, Spanel R, Spanel-Borowski K. Epidermal growth factor-induced hepatocellular carcinoma: gene expression profiles in precursor lesions, early stage and solitary tumours. Oncogene 2005; 24: 1809–19.
8 Calvisi DF, Factor VM, Luda S, Conner EA, Thorpeirsson SS. Disruption of beta-catenin pathway or genomic instability define two distinct categories of liver cancer in transgenic mice. Gastroenterology 2004; 126: 1374–86.
9 Edwards JE. Hepatomas in mice induced with carbon tetrachloride. J Natl Cancer Inst 1941; 2: 197–9.
10 Gothoskar SV, Talwalkar GV, Bhide SV. Tumorigenic effect of thioacetamide in Swiss strain mice. Br J Cancer 1970; 24: 498–503.
11 Andervont HB. Studies on the occurrence of spontaneous hepatomas in mice of strains C3H and CBA. J Natl Cancer Inst 1950; 11: 581–92.
12 Leighton PA, Saam JR, Ingram RS, Stewart CL, Tilghman SM. An enhancer deletion affects both H19 and Igf2 expression. Genes Dev 1995; 9: 2079–89.
13 Vernucci M, Cerrato F, Bestard N et al. The H19 endodermal enhancer is required for Igf2 activation and tumor formation in experimental liver carcinogenesis. Oncogene 2000; 19: 6376–85.
14 Lederer M, Bley N, Schleifer C, Huttelmaier S. The role of the oncofetal IGF2 mRNA-binding protein 3 (IGF2BP3) in cancer. Semin Cancer Biol 2014; 29C: 3–12.
15 Sell S, Becker FF, Leffert HL, Watabe L. Expression of an oncodevelopmental gene product (alpha-fetoprotein) during fetal development and adult oncogenesis. Cancer Res 1976; 36: 4239–49.
16 Nordin M, Bergman D, Halje M, Engstrom W, Ward A. Epigenetic regulation of the Igf2/H19 gene cluster. Cell Prolif 2014; 47: 189–99.
levels of the tumor-associated genes by RT-qPCR and Ki-67 labeling index (%) in CCl4-induced tumors (Fig. S2). The relationship between the mRNA expression of tumor-associated genes and tumor cell proliferation. Scatter plots of mRNA expression and promoter methylation in mouse and human hepatocellular carcinomas. Int J Oncol 2005; 26: 369–77.

Sun Q, Zhang Y, Liu F, Zhao X, Yang X. Identification of candidate biomarkers for hepatocellular carcinoma through pre-cancerous expression analysis in an HBx transgenic mouse. Cancer Biol Ther 2007; 6: 1532–8.

Sohda T, Yun K, Iwata K, Soejima H, Okumura M. Increased expression of insulin-like growth factor 2 in hepatocellular carcinoma is primarily regulated at the transcriptional level. Lab Invest 1996; 75: 307–11.

Sohda T, Iwata K, Soejima H, Kamimura S, Shijo H, Yun K. In situ detection of insulin-like growth factor II (IGF2) and H19 gene expression in hepatocellular carcinoma. J Hum Genet 1998; 43: 49–53.

Arieli I, Mao H-Q, Ji XR et al. Imprinted H19 oncofetal RNA is a candidate tumour marker for hepatocellular carcinoma. Mol Pathol 1998; 51: 21–5.

Boyault S, Rickman DS, de Reynies A et al. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. Hepatology 2007; 45: 42–52.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. RT-qPCR analyses of mRNA expression of Cib3, Top2a, Cdkn2b, Pnpla5, and Tspan8 in CCl4-induced and DEN-induced liver tumors. Each value is expressed as the mean ± SEM. The numbers of samples in each group was 5, 12, 15, 6, and 13 for the CCl4 control (olive oil), CCl4-induced cirrhosis, CCl4-induced tumors, DEN control, and DEN-induced tumors, respectively.

Fig. S2. The relationship between the mRNA expression of tumor-associated genes and tumor cell proliferation. Scatter plots of mRNA expression levels of the tumor-associated genes by RT-qPCR and Ki-67 labeling index (%) in CCl4-induced tumors (n = 16) and DEN-induced tumors (n = 15). The data of the genes that were not included in Figure 4 are shown here. Spearman correlation coefficients were used to test the association between mRNA expression and tumor cell proliferation.

Table S1. Primer sequences.

Table S2. Top 10 highly-expressed genes in CCl4-induced cirrhosis in Cluster B.