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Integrative Genomics: Liver Regeneration and Hepatocellular Carcinoma

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ABSTRACT

Numerous genome wide profiles of gene expression changes in human hepatocellular carcinoma (HCC), compared to normal liver tissue, have been reported. Hierarchical clustering of these data reveal distinct patterns, which underscore conservation between human disease and mouse models of HCC, as well as suggest specific classification of subtypes within the heterogeneous disease of HCC. Global profiling of gene expression in mouse liver, challenged by partial hepatectomy to regenerate, reveals alterations in gene expression that occur in response to acute injury, inflammation, and re-entry into cell cycle. When we integrated datasets of gene expression changes in mouse models of HCC and those that are altered at specific times of liver regeneration, we saw shared, conserved alterations in gene expression within specific biological pathways, both up-regulated, for example, cell cycle, cell death, and cellular development, or down-regulated, for example, vitamin and mineral metabolism, lipid metabolism, and molecular transport. Additional molecular mechanisms shared by liver regeneration and HCC, as yet undiscovered, may have important implications in tumor development and recurrence. These comparisons may offer a way to judge how liver resection, in the treatment of HCC, introduces challenges to care of the disease. Further, uncovering the pathways conserved in inflammatory response, hypertrophy, proliferation, and architectural remodeling of the liver, which are shared in liver regeneration and HCC, versus those specific to tumor development and progression in HCC, may reveal new biomarkers or potential therapeutic targets in HCC. J. Cell. Biochem. 113: 2179–2184, 2012.

KEY WORDS: LIVER; TUMORIGENESIS; MICROARRAY; GENOMICS; BIOINFORMATICS

Hepatocellular carcinoma (HCC) is the most common type of human liver cancer, and the third leading cause of cancer deaths worldwide [Farazi and DePinho, 2006; Ferlay et al., 2010]. The development of cancers of the liver, or hepatocarcinogenesis, is a multistep process that involves numerous and diverse genetic alterations, including telomere shortening and loss of tumor suppressor p53 functions [Farazi and DePinho, 2006]. Treatment of this disease offers a major challenge and relatively few options. Liver resection is commonly used to remove diseased and surrounding tissue, but is linked to a tumor recurrence rate of 75–100% within 5 years [Llovet, 2005; Schwartz et al., 2007]. The remarkable ability of the liver to regenerate after resection may actually enhance tumor recurrence due to the effects of acute injury, inflammation, and induced growth of previously undetected intrahepatic lesions, which combine in malignant transformation. Understanding the commonalities between liver regeneration and HCC may aid in development of therapeutic strategies that inhibit hepatocarcinogenesis without blocking normal liver repair and regeneration.

Here, we present findings of gene expression profiling studies comparing HCC and normal liver tissue, as well as new integration of gene expression profiles of human and mouse HCC with global expression analyses across a time course of liver regeneration in mice. This use of integrative genomics identifies transcriptional networks that regulate liver regeneration and are implicated in hepatocarcinogenesis. Liver regeneration induced by partial hepatectomy (PH) is robust in rodent models, and captures the complex, physiological response to liver resection [Michalopoulos and DeFrances, 1997; Fausto et al., 2006]. Integration of published gene expression profiling studies of hepatocarcinogenesis and liver regeneration offers insights into molecular mechanisms, linking hepatocarcinogenesis and liver regeneration, and potential ways to circumvent the problems accompanying resection of the liver.

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GENE SIGNATURES IN HCC DEVELOPMENT

Global gene expression profiling using DNA microarrays has been widespread in cancer research for more than a decade. Our understanding of the molecular basis of HCC development relies in part on more than 300 published microarray studies of global gene expression in HCC compared to non-tumor liver samples [Iizuka et al., 2008]. A specific subset of genes correlates well with clinical features of HCC, offering candidate biomarkers for diagnosis, prognosis, and response to treatment, as well as novel therapeutic targets.

In one of the first microarray studies of liver cancer, gene expression profiles of 29 hepatitis B-virus positive HCC samples were compared to corresponding non-cancerous liver samples [Xu et al., 2001]. Genes with increased expression in HCC samples include several that act in DNA replication, for example, topoisomerase 2A (*TOP2A*), replication protein A3 (*RPA3*), or angiogenesis, for example, vascular endothelial growth factor (*VEGF*). In contrast, a number of enzymes that play roles in respiration, glycogen synthesis, lipid and amino acid metabolism, as well as proteins synthesized in liver, such as albumin and transferrin, are down-regulated in HCC samples. Detoxification enzymes, such as cytochrome P450 (CYP) superfamily members and glutathione *S*-transferases, are likewise decreased in HCC. The results of this study indicate that liver tumorigenesis occurs with decreased liver-specific functions, but whether this is a result of transformation or dedifferentiation is unknown.

A similar set of genes is deregulated in 20 paired HCC and non-cancerous liver tissues [Okabe et al., 2001]; however, this study further revealed that mitosis-associated genes, particularly those involved in the anaphase-promoting complex, were up-regulated in HCC. Some specific examples of activated genes, involved in metaphase–anaphase transition, are cell division cycle 23 homolog (*CDC23*), cyclin-dependent kinases subunit regulatory unit 1 (*CKS1*), and cyclin-dependent kinase 16 (*CDK16*). Among down-regulated genes in HCC, in addition to liver-specific enzymes and liver-synthesized functional proteins, lymphocyte antigen complex 6, locus E (*LY6E*) and retinol binding protein 1 (*RBP1*), which act in retinoic acid-mediated differentiation, are repressed. Interestingly, gene expression profiles of tumor tissue, compared to surrounding, non-cancerous liver tissue, identified a slightly different set of differentially expressed genes [Delpuech et al., 2002]. Here, a subset of genes involved in DNA-repair were up-regulated; whereas genes associated with immune response were down-regulated in HCC samples.

Studies with a larger set of samples are required to increase the generality of these findings with greater statistical significance. For example [Chen et al., 2002] used a hierarchical clustering algorithm to discriminate 102 primary HCC tumor samples and 74 non-tumor samples with respect to their gene expression pattern. In agreement with the findings of other studies [Okabe et al., 2001; Xu et al., 2001], genes encoding ribosomal subunits involved in DNA replication; proteins that promote cell cycle progression, notably in G2/M progression; and, regulators of mitosis, were up-regulated in tumor samples. Down-regulated genes featured liver-specific metabolic enzymes and liver-synthesized proteins, in agreement with and strengthening the results of previous studies with a larger sample population and the application of a hierarchical clustering algorithm as an analysis tool.

Multiple studies integrate and compare global transcription profiles of HCC samples of different histological grades with precancerous nodules. A group of 3,084 genes were associated according to tumor-grade among a set of 50 hepatitis B virus (HBV) seropositive hepatocellular nodular lesions, consisting of low-grade dysplastic nodules (LGDN), high-grade dysplastic nodules (HGDN), and primary HCCs [Nam et al., 2005]. Biological function annotation revealed that specific genes with increased expression in HCC samples, compared to dysplastic nodules, are involved in DNA replication, chromatin remodeling, cell proliferation, and protein synthesis. In contrast, a subset of genes with decreased expression levels in HCC, compared to dysplastic nodules, act in fatty acid and lipid metabolism, detoxification pathways and synthesis of complement and coagulation factors, underscoring a loss of primary liver function with increased malignancy. Additional analyses of early HCC compared to dysplastic nodules in a set of 65 tissue samples taken from 38 patients infected with hepatitic C virus (HCV) offer similar findings [Wurmbach et al., 2007]. In combination, these gene expression profiles reveal highly similar sets of genes enriched in conserved biological functional categories that are deregulated in liver carcinogenesis.

COMPARATIVE FUNCTIONAL GENOMICS OF HCC

Although groups of genes may be highly discriminatory between subgroups of HCC, testing them in human samples as potential therapeutic targets may not be feasible. At this point, comparative functional genomics offers a means of focusing on signature genes by their level of evolutionary conservation. Cross-species comparison of candidate gene lists between mouse models of HCC and human HCC samples prioritizes a list of genes to test as signature-based hypotheses in mouse models [Lee and Thorgeirsson, 2006]. For example, expression levels of genes among 68 HCC samples from seven different mouse models and 91 human HCC samples were clustered, based on the similarity of expression patterns for orthologous genes [Lee et al., 2004]. This analysis shows that gene expression profiles in human HCCs, from a subgroup associated with better survival, cluster with profiles determined for HCC mouse models of transgenic over-expression of Myc, E2f1, or both Myc and E2f1 (Myc/E2f1) in the liver [Conner et al., 2000, 2003]. In contrast, expression profiles of HCC that develops in a transgenic Myc/Tgfα mouse model, with over-expression of Myc and Tgfα in the liver [Murakami et al., 1993], and DEN-induced murine HCCs [Poirier, 1975] are comparable to human HCCs from the poorer survival subgroup.

Although HCC samples from different mouse models in the same subgroup may show similar expression profiles, distinct mechanisms may drive tumor development in each. Further studies suggest deregulation of distinct metabolic pathways occurred in Myc, E2f1, and Myc/E2f1 transgenic mice models in the early steps of hepatocarcinogenesis [Coulouarn et al., 2006]. A more extensive study integrated gene expression data from rat and mouse hepatic

cells with human HCC samples [Lee et al., 2006]. They clustered rat fetal hepatoblasts and adult hepatocytes, mouse hepatocytes, and 61 HCC samples from Chinese individuals, with respect to expression patterns of orthologous genes. Interestingly, the authors observed that 14 cases of HCC co-clustered with rat fetal hepatoblasts; however, the remaining cases co-clustered with rat adult hepatocytes or mouse hepatocytes in a different subgroup. This observation led them to identify new subtypes of human HCC samples: HB and HC subtypes. Next, they clustered an independent cohort of 139 patients into the poor survival and better survival subgroups, previously defined [Lee et al., 2004]. Although patients from both HB and HC subtypes were classified with the poor survival subgroup, patients from HB subtype showed worse prognosis.

Identification of mice models that recapitulate human conditions in HCC development enables testing of potential therapeutic targets in a defined genetic background. cIAP1 and YAP were suggested as new players in both mice and human hepatocarcinogenesis in a search for common recurrent gene amplifications [Zender et al., 2006]. Next, the authors tested their hypothesis and found that overexpression of cIAP1 or YAP enhanced tumor development; whereas, knockdown of cIAP1 or YAP prevented tumor growth in vivo in mice. Taken together, these expression studies and cross-species comparison offer important support for specific testing in these mouse models, mimicking each subgroup of human HCC and furthering hypothesis-driven studies of HCC.

GENE EXPRESSION PROFILING IN REGENERATING LIVER

Several studies underscore the similarities, at a molecular level, between liver regeneration and hepatocarcinogenesis: one study showed that hepatoma cells implanted into rat remnant liver after PH, exhibited an intrinsic rate of growth and malignant transformation directly correlated with the degree of tissue resection [Shi et al., 2011]. Implicated in this transformation are growth factors, such as hepatocyte growth factor (HGF), augmenter of liver regeneration (ALR), transforming growth factor-α (TGF-α), and cytokines interleukin-6 (IL-6) and tumor necrosis factor (TNF), which are expressed and secreted during liver regeneration. Additionally, deletion of multidrug resistance p-glycoprotein 2 (Mdr2) in mice led to chronic, hepatic inflammation, and development of HCC, in a process accelerated by PH [Barash et al., 2010]. This mouse model simulates a human clinical condition where HCC occurs as a result of chronic liver regeneration, and directly links inflammation, regeneration, and tumor development in the liver.

Gene expression profiles of changes induced during liver regeneration are valuable resources for comparison and integration. For example, the early stages of rapid response to liver resection were assessed by a microarray study of RNAs expressed in hepatocytes during the first 4 h after two-thirds PH in comparison to a Sham surgical control [Su et al., 2002]. The time period of 0–4 h is sometimes referred to as a priming phase [Fausto et al., 2006], and brackets the remarkable transition that occurs when more than 90% of hepatocytes leave a quiescent state (G_0) and move into an early G_1 phase of cell cycle. This microarray study revealed that specific transcription factors, proteins involved in stress and inflammatory responses, cytoskeletal and extracellular matrix modifiers, as well as regulators of cell cycle entry, changed their expression levels more than twofold [Su et al., 2002].

Over a longer time-course of 0–48 h after two-thirds PH, differentially expressed genes (up- or down-regulated >2-fold at any time point) were classifiable into five distinct clusters [Arai et al., 2003]. Genes involved in protein production and post-translational processing are activated shortly after surgery; whereas secreted proteins are activated more gradually; and, genes involved in intermediate metabolism remained repressed. Microarray-based gene expression profiling at specific time points of G_0, early G1 and mid-G1, and S phases of the regenerative cell cycle, corresponding to 0, 2, 16, and 40 h after PH in mice [White et al., 2005], revealed that two main functional categories of genes significantly changed their expression. Genes associated with steroid and lipid metabolism were quickly decreased post-PH, and remained decreased throughout 40-h post-PH time course. Genes implicated in nucleotide and protein synthesis, as well as cytoskeletal organization, were elevated at 16-h post-PH and remained elevated throughout the time course. In all such studies, there are multiple sources of variation, such as individual differences between mice, species-specific variability, PH-specific variables, for example, anaesthetic and surgical procedure, the microarray platform used, and the design of each study. Nevertheless, these results are logical based on physiological and molecular studies of liver regeneration.

CLASSIFICATION OF HCC-ASSOCIATED GENES BY EXPRESSION DURING LIVER REGENERATION

We performed integrative analysis of expression profiling of HCC and liver regeneration to determine conserved molecular mechanisms. The first study we used for these comparisons is one that determined global gene expression patterns of 68 HCCs from seven different transgenic and knockout mouse models [Lee et al., 2004]. HCC-associated genes were identified as differentially expressed genes in HCC liver samples, compared to liver samples of wild-type (WT) mice. We used a hierarchical clustering algorithm to classify HCC-associated genes identified in this dataset. Then, in order to compare the processes of HCC and liver regeneration, we used a second study where liver tissue was collected at 0, 0.5, 1, 2, 4, 24, 38, and 48 h after PH or sham surgery [Singh et al., 2011].

By integration of these datasets, we classified HCC-associated genes with respect to their expression profile over a 48-h period of liver regeneration. Two distinct groups of HCC-associated genes were apparent (Fig. 1A), and exhibited a high negative correlation. Approximately 63% of the first group of genes (41 out of 65) are up-regulated in HCC liver samples, compared to liver samples of quiescent WT mice. The top three significant biological function categories for these genes identified through the use of Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com) are cell death, cellular development, and cell cycle. Among these genes are *CDKN1A*, *MCM6*, *MAD2L1*, and *CCND1*, as

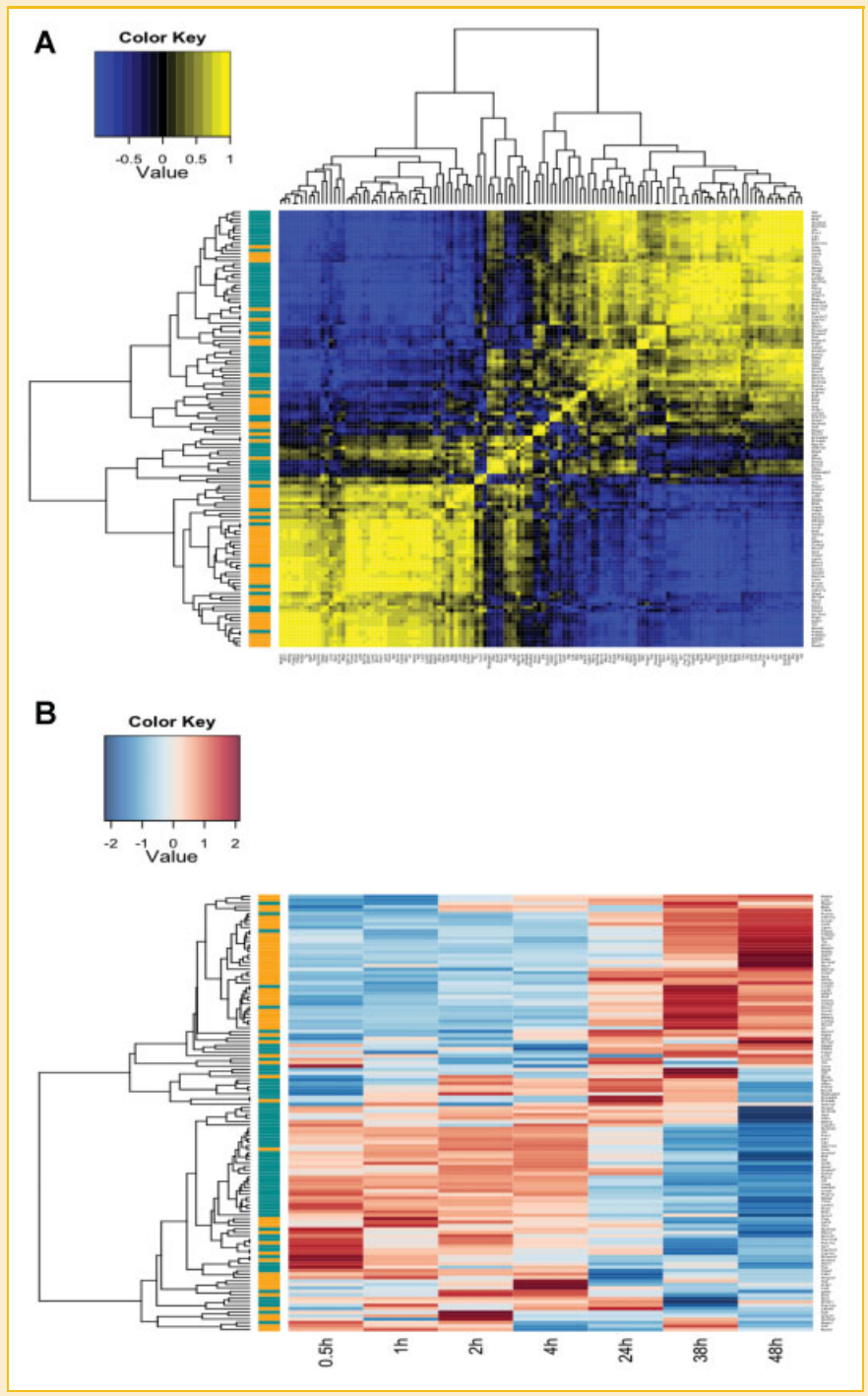

Fig. 1. A: Correlation plot of HCC-associated genes. Each point in the plot shows a pairwise Pearson's correlation coefficient of HCC-associated genes, based on the similarity of their gene expression levels at 0.5-, 1-, 2-, 4-, 24-, 38-, and 48-h post-PH. A high negative correlation is observed between two main distinct clusters, shown in the dendrogram at the left side. The upper cluster mostly includes down-regulated genes (cyan lines in the left bar) in HCC liver samples. In contrast, the lower cluster is enriched in up-regulated genes (orange lines in the left bar) expressed in HCC liver samples. B: The heatplot (genes vs. time points) shows normalized expression levels of HCC-associated genes at 0.5-, 1-, 2-, 4-, 24-, 38-, and 48-h post-PH. Columns represent time points post-PH and rows represent genes significantly down-regulated or up-regulated in HCC liver samples, corresponding to cyan and orange lines in the left bar, respectively.

well as *IGFBP1*, most of which are implicated in survival and proliferation. Similarly, during liver regeneration, the RNA levels of these genes increase within 24 h and remain elevated at 48-h post-PH, compared to 0–4 h (Fig. 1B).

In contrast, approximately 70% of the second group of genes (43 of 61) are down-regulated in HCC liver samples, compared to liver samples of quiescent WT mice. The top three categories of biological function tool in this group identified through the use of IPA software

(Ingenuity Systems) include lipid metabolism, vitamin and mineral metabolism, and molecular transport; including *EGFR*, *ACOX1*, *CSAD*, *TDO2*, and *GHR* (Fig. 1B). Likewise, the mRNA levels of these genes are lower at 24-h post-PH compared to 0–4 h, and remained decreased at 48-h post-PH. These results suggest that simply targeting and reducing cellular proliferation in HCC will not circumvent alterations that accompany both liver resection, as modeled by PH-induced changes in gene expression, and HCC. Therapeutic compensation for the specific types of metabolic processes, revealed here as decreased in both liver regeneration and HCC, may alleviate some of the collateral complications encountered during treatment of HCC.

INTEGRATIVE GENOMICS OF HCC

Identification of differentially expressed gene sets implicates specific signaling transduction pathways in liver tumorigenesis, and enhances our knowledge of the molecular pathogenesis of liver disease. A broader understanding of regulatory mechanisms requires integration of HCC gene expression profiling with diverse genomic data, including profiles of DNA methylation, histone modifications, specific transcription factors, RNA Polymerase (Pol) II, non-coding RNAs, and gene amplification. A recent study presents expression data from matched HCC tumor and normal samples integrated with genome-wide profiling of chromatin-enriched RNA Pol II, H3K4me3, and H3K27me3, as well as DNA methylation and gene copy number [Acevedo et al., 2008]. Integration of these different data led to an interesting conclusion that changes in gene copy number contribute more to hepatocarcinogenesis than the other analyzed factors. Further intersection of copy number and gene expression data for 103 HCC tumor samples uncovered gain in copy number at 6p21 as a new mechanism for increased expression of *VEGFA* in hepatocarcinogenesis [Chiang et al., 2008]. These insights may lead to better means of classifying disease states and insights into prognosis and treatment.

CONCLUSIONS

The large numbers of studies, where global gene expression profiling of HCC samples are compared to normal samples by DNA microarrays, have proven extremely useful. Although there are multiple sources of variation between studies, gene signatures in HCC development from different studies are enriched in conserved biological functions, offering insights into mechanisms of dysfunction. Further, gene expression profiles establish methods of classification for the highly heterogeneous HCC disease, which allow subdivision into more homogeneous subtypes that correlate well with clinical features, based on length of survival, stage of disease, and time to recurrence. Identification of these subgroups may lead the way to specific therapeutic interventions for each subgroup. However, testing potential therapeutic targets within these subtypes is problematic. In this way, the coherence shown by genomic profiling and integration, between rodent models mimicking human HCC and human HCC development, offer model systems for identifying and testing potential therapeutic targets, which will be useful in treatment of human hepatocarcinogenesis. By further imposing the process of liver regeneration, commonalities between rodent models and human HCC are increased. Undoubtedly, genomic data gathered under different physiological conditions, such as liver development, liver regeneration, and conditions that promote expansion of hepatic stem cells, and its intersection with multiple sources of gene expression profiling data will reveal additional commonalities and distinctions that offer insights into the complexities of HCC.

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