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Communication

Molecules and Cells

Systems-Level Analysis of Gene Expression Data Revealed NR0B2/SHP as Potential Tumor Suppressor in Human Liver Cancer

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Nuclear receptors (NRs) play pivotal roles in cell growth, proliferation, differentiation and homeostasis. Recent progress demonstrates that NR is tightly linked to human disease such as cancer, diabetes and obesity. Here we explore NR expression profiles in human tissue using systematic approaches. NR gene profiles reveal that individual NR has its own gene expression signature depending on tissue type. Of many organs, NRs expression is enriched in liver. Expression of many NRs was significantly changed in liver cancer. Notably, *NR0B2/SHP* expression level was significantly decreased in human liver cancer but not in normal liver. In addition, expression of SHP is well associated with good prognosis. SHP gene network analysis based on microarray data in liver cancer shows that SHP regulates cell proliferation and metabolism related gene sets. Our systematic approaches suggest that loss of SHP expression in liver might be key genetic events during hepatocarcinogenesis.

INTRODUCTION

Nuclear receptor (NR) is the largest number of transcription factor family regulating target gene expression (Mangelsdorf et al., 1995). It has pivotal roles in development, cancer, toxicology, reproduction, and metabolism (McKenna et al., 2009). So far, fourty night known receptors in human and 48 in mouse have been identified (Bookout et al., 2006). NRs are divided into those which bind to steroids (glucocorticoids, progestins, mineralocorticoid androgens, and estrogens), steroid derivates (vitamin D3), non-steroids (thyroid hormone, retinoids, prostaglandines), and receptors for which no ligand has been found yet (Giguere, 1999). Genetic or epigenetic alterations of NRs including gene fusion and over-expression were frequently observed in human cancers including breast cancer, prostate cancer, colon cancer, thyroid cancer and leukemia (Lupien and Brown, 2009). For instance, acute promyelocytic leukemia (APL) is genetically characterized by a translocation that involves the PML gene on chromosome 15 and the transcription factor retinoic acid receptor alpha (RARα) on chromosome 17 (Altucci et al., 2007; Doucas and Evans, 1996), resulting in the PML-RAR fusion protein and t(2;3)(q13;p25) a translocation identified in a subset of human thyroid follicular carcinomas, results in fusion of the DNA binding domains of the thyroid transcription factor PAX8 to domains A to F of the peroxisome proliferator-activated receptor (PPAR)γ1 (Kroll et al., 2000). In case of breast cancer or prostate cancer, estrogen receptor (ER) or androgen receptor (AR) is highly over-expressed (Heinlein and Chang, 2004; Herynk and Fuqua, 2004). Thus, antagonist of ER and AR has been widely used for cancer patient treatment (Jordan, 2008; Tran et al., 2009).

Developing genomic approaches, we are able to uncover the unknown function of genes and get new information of thousands genes (Tomlins et al., 2008). Comparative genomic hybridization (CGH), single nucleotide polymorphism (SNP), methylation and mRNA gene expression array tools have been widely used for screening the genome (Kulasingam et al., 2010; Lee and Thorgeirsson, 2006). Currently, series of array data are publicly available from GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo/). Thus, publicly available data sets are good resources to explore the gene function in human disease (Tomlins et al., 2008). For instance, using *in silico* meta analysis with genomic data, Willson and Giguere identified that ER and GATA3 reciprocally influenced downstream genes (Wilson and Giguere, 2008). To elucidate NRs function using *in silico* analysis, we used mRNA gene expression data sets. Since NRs functions as transcription factor, they are highly correlated with downstream genes via direct or indirect binding to target genes at the transcription level. In the current study, we re-analyzed the mRNA gene expression data to identify NR function in human cancer.

MATERIALS AND METHODS

Data process

Microarray data set from human normal tissue s were downloaded from author web site (http://home.ccr.cancer.gov/oncology/oncogenomics/). Liver cancer data set were downloaded from GEO (Gene Expression Omnibus accession codes: human microarray platform, GPL1528; human HCC microarray

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data, GSE1898 and GSE4024). All the data were normalized with quintile normalization method in R-program and used for analysis.

Hierarchical clustering
Hierarchical clustering was performed on the normalized microarray by calculating Pearson's centered correlation coefficient followed by average linkage analysis using Cluster II software and visualized with TreeView software (Eisen et al., 1998).

Survival analysis
HCC patients were used in survival analyses with previously published data (n = 139) (Lee et al., 2004b; 2006). Association of *SHP* expression states (rank ordered and split into halves: high/low) relative to survival was tested using the log-rank test and results were visualized using Kaplan-Meier survival plots as performed previously (Lee et al., 2006).

Pathway analysis
To generate SHP gene network in liver cancer, we selected SHP correlated genes using Pearson's correlation co-efficient value. Among the 2603 genes correlated with SHP, 180 genes which has high correlation with SHP (correlation value more than 0.35), were applied for ingenuity pathway analysis (IPA). Among the pathways, four major pathways were presented in Fig. 3.

qRT-PCR
Liver cancer patients samples were previously described (Lee et al., 2004b; 2006). RT-PCR was assayed by using real-time qRT-PCR with Taqman primers specific to NR0B2/SHP gene (Applied Biosystems). Real-time PCR was performed using the 7700HT Real-Time PCR System with a 96-well block module (Applied Biosystems). Cycling conditions were 45°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative amounts of mRNA were calculated from the threshold cycle (CT) number using expression of cyclophilin A (PPIA) as an endogenous control. All experiments were triplicated and the values averaged.

RESULTS AND DISCUSSION

Nuclear receptors expression profiling in multiple human organs based on microarray data
In order to explore organ-specific regulation of NRs in normal physiological condition, we analyzed expression patterns of NRs in multiple human tissues. Although recent report showed that NRs expression profiles in normal mouse tissues (Bookout et al., 2006) human NRs expression pattern might be different from those of mouse. Thus, we explored NRs profiles in human normal tissues Previously, Son et al. (2005), reported mRNA gene expression profiles of multiple human organs. This data includes 158 tissues from 19 organs. Using expression data of NR genes in this data set, we carried out hierarchical clustering analysis to group NRs according to their expression patterns in multiple organs. As shown in Fig. 1A, NRs expression pattern was very diverse throughout whole organs. Based on expression pattern, NRs were composed of five major clusters. Cluster I (PXR, PPARα, COUP-TFa, COUP-TFβ, Reverb β, AR and ER) and Cluster IV (RORα, DAX-1 ERRβ, NOR1, LRH-1, GCNF and HNF4γ) well represent reproduction physiology as previously reported (Bookout et al., 2006). Interestingly, AR and ER were expressed not only in uterus, ovary and ureter (reproductive tract) but also in liver. This property is very interesting since this expression pattern is specific in human tissue but not in mouse. In case of Cluster II, RXRβ, GR, COUP-TFα and RARα were ubiquitously expressed throughout whole organs. TRβ, RXRγ, PR, TR2, TR4, Reverb β, TRα, TLX, RARα, Nurr1, RARγ, Reverb α and RORβ classified as Cluster V were highly expressed in central nerve systems (CNS). Most interestingly, Cluster III including SHP, HNF4α, FXR, VDR, LXRβ PXR, Nurr77 and PPARγ was highly enriched in liver as well characterized previously (Bookout et al., 2006), suggesting that these liver specific NRs play a pivotal role governing liver physiology. Since NR is a transcription factor regulating down-stream genes via direct interaction on the target promoter region, we investigated how many genes correlated with liver-specific NRs as potential downstream target genes. Pearson's correlation coefficient value was applied for selecting correlation genes with liver specific NRs. Figure 1B shows that three hundreds nine genes including *ABCA6, ABCC2, ABCG5, PCK, APOB, CYP8B1, CYP2B6* and *CYP27A1*i were also highly correlated with liver specific NRs (Supplementary Table 1). These genes were well characterized as liver specific NRs down-stream target genes (Moore et al., 2006), suggesting that outcomes of our *in silico* analysis well reflect physiological characteristics of NRs.

NRs expression profile reveals SHP role in liver cancer
Since expression of many NRs was changed in many cancers and their expression patterns were associates with clinical outcomes including overall survival, recurrence-free survival, and response to certain therapies (Kristensen et al., 2005; Yu et al., 2010), we next sought to catalog expression pattern of NRs and characterize them in human cancer, particularly in liver cancer. For analysis, we used gene expression data from 139 liver cancer tissues and 71 surrounding non-tumor tissues (Lee et al., 2004a). When hierarchical clustering analysis was applied to expression data of NR genes, liver tissues were largely subdivided into two groups: cancer tissues and normal liver tissues (Fig. 2A). Liver specific NRs (GR, TRβ, RORα, SHP, TR4, PXR, LXRα, HNF4α, PPARα and FXR) were highly expressed in normal liver while their expression was deceased in liver cancer tissues (Fig. 2A). Of liver specific NRs, SHP was dominantly expressed in normal tissue whereas its expression was diminished in liver cancer patients. While previous study with SHP knock out mouse model reported that SHP suppresses tumorigenesis and cellular proliferation by modulating *Cyclin D1 (CCND1)* expression (Zhang et al., 2008), its clinical relevance has never been assessed. Of interest, expression of SHP is not uniformly suppressed in liver cancer although overall expression of SHP is significantly lower in liver cancer than normal tissues using published data set (Lee et al., 2004b; 2006) (Fig. 2B). Additional data set also present similar result (Fig. 2C) (Chen et al., 2002). To validate these results, we carried our qRT-PCR with liver cancer tissues. As shown in Fig. 2D, *SHP* expression was high in normal tissues, while it was low in tumor tissues. Since our expression data clearly shows that SHP expression level is changed depending on clinical status, we explored the possibility that expression patterns of SHP might reflect different clinical outcome of liver cancer patients. We dichotomized liver cancer patients according to expression level of SHP in liver cancer tissues, and carried out survival analysis. As shown in Kaplan-Meier plot (Fig. 2E), SHP expression significantly associated with patient survival. The patients with lower expression of SHP showed significantly poorer survival than those with higher expression of SHP, suggesting that SHP may function as tumor suppressor in human liver.

Generation of SHP gene network in human liver cancer
Liver cancer is the fifth most common cancer in the world, ac-

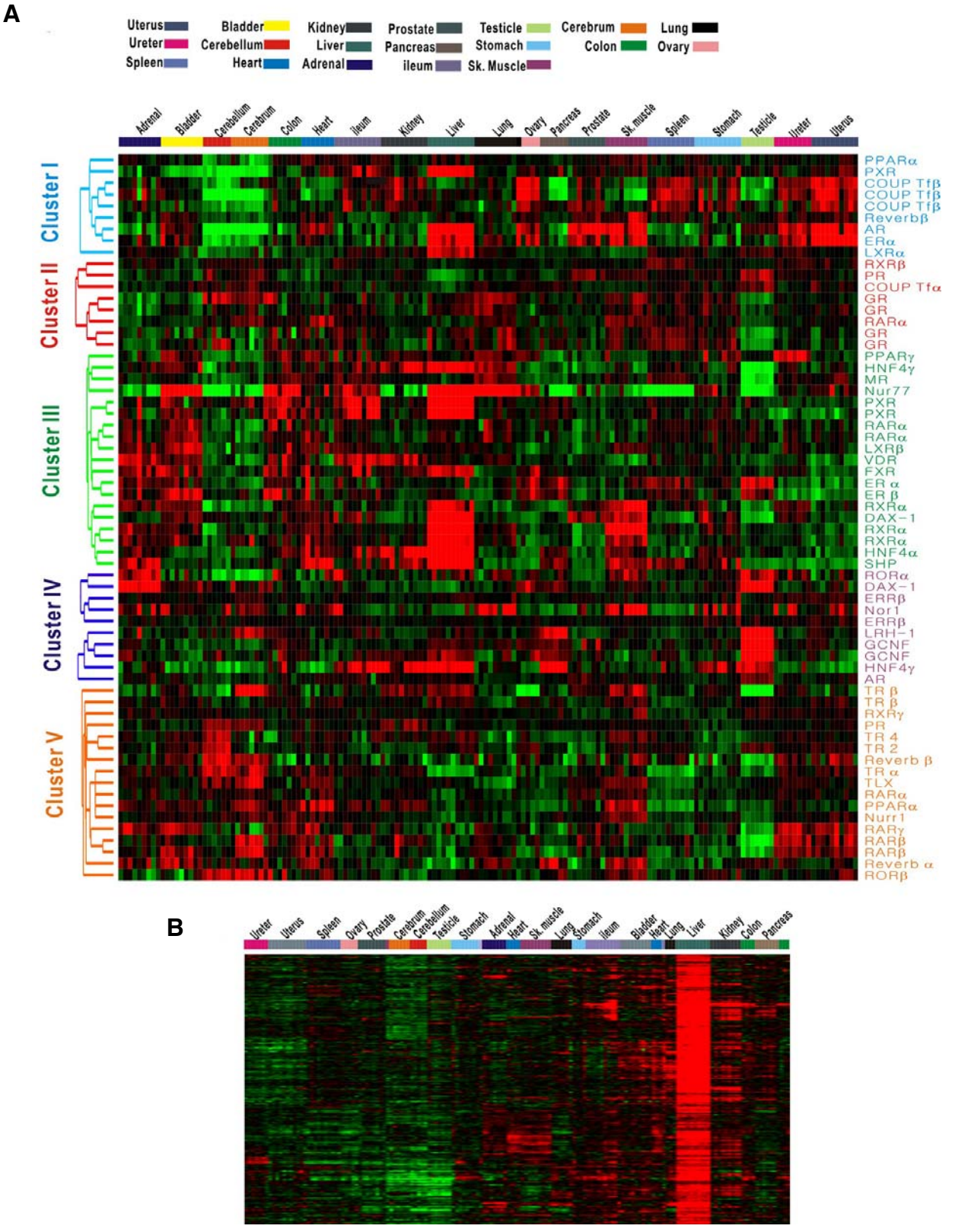

Fig. 1. Hierarchical clustering of nuclear receptors. (A) the tissue distribution profile of the NR superfamily in the multiple organs was evaluated by hierarchical clustering using Cluster II software as described in methods. Individual NRs segregate into five main clusters (labeled cluster I, II, III, IV and V) that define higher-order functional relationships. The dendrogram of NR clusters is color-coded for clarity. (B) average expression values of liver specific NRs (SHP, HNF4α, FXR, VDR, LXRβ PXR, Nurr77 and PPARγ) were applied for selecting correlated genes. Liver specific NRs (319 genes) based on correlation value were cluster and visualized.

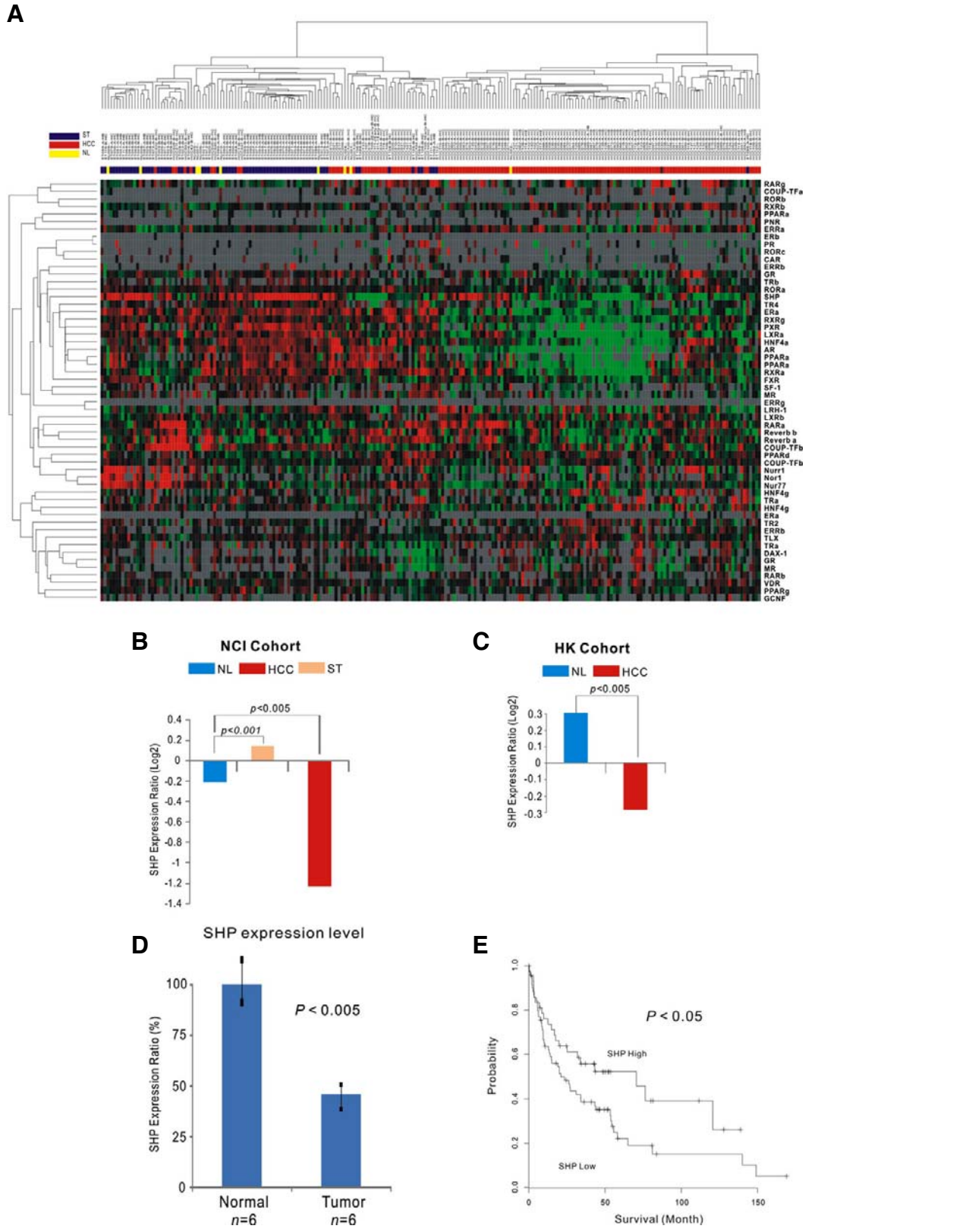

Fig. 2. SHP expression in liver cancer. (A) NRs expression profile in liver cancer was clustered and visualized as performed in Fig. 1. (B) and (C) liver cancer data sets (NCI and HK cohort) were classified into patients types and graph used to visualize SHP expression according to patient types. Statistical significance was calculated by ANOVA or *t*-test. (D) RNA from human liver tissues was used for qRT-PCR. Thirty nanogram of total RNA were analyzed by qRT-PCR using gene-specific primers as indi-cated. (E) Kaplan-Meier plots of overall survival of individuals with the *SHP* high and low from SHP expression level. Log-rank test was applied to estimate the significance of difference.

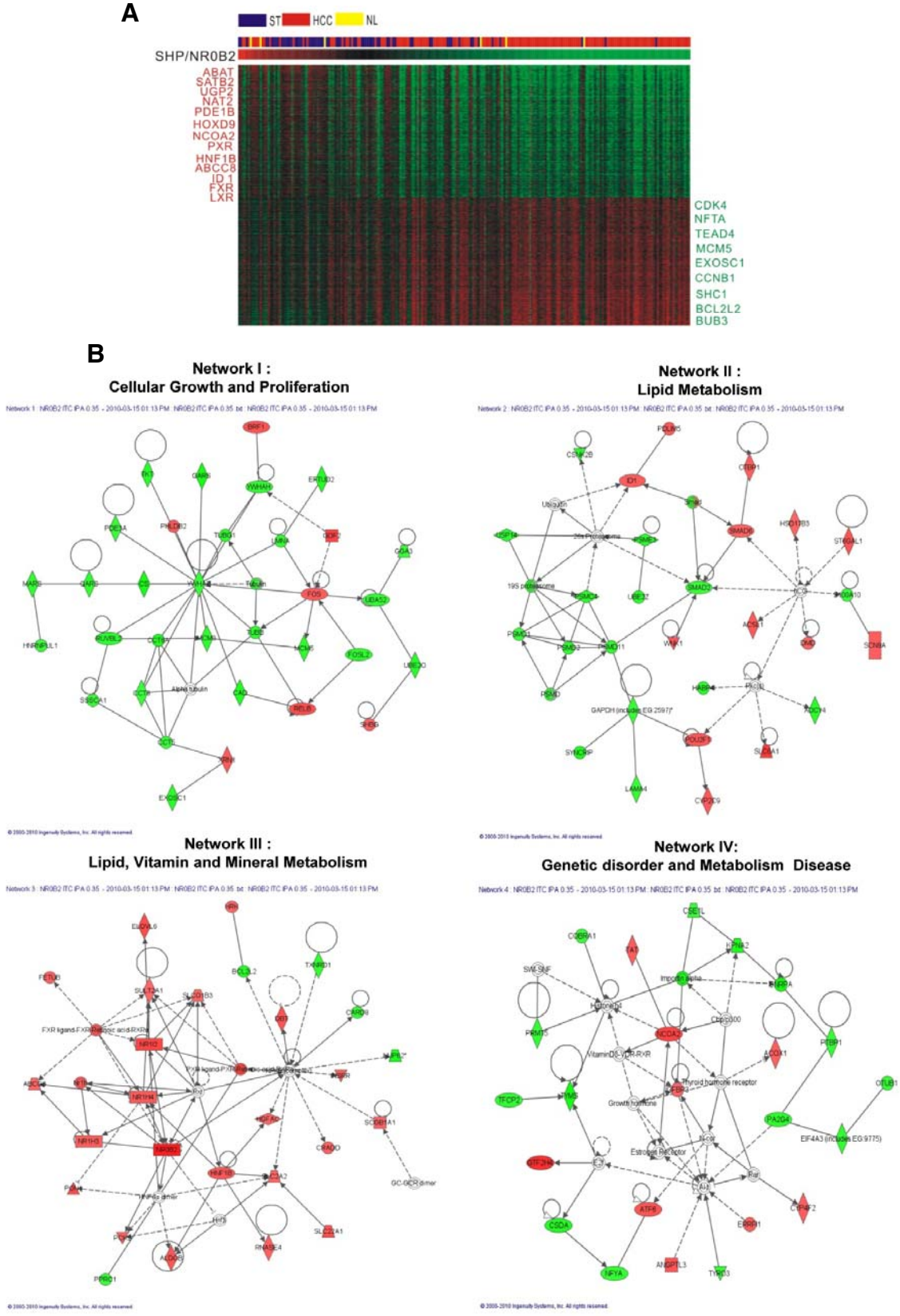

Fig. 3. Establishment of SHP gene network in liver cancer. (A) SHP correlated genes were selected using Pearson's correlation coefficient value. Correlated genes were clustered following correlation value. (B) one hundred eight genes were used for generating gene networks using IPA software. Red indi-cates high correlation with SHP. Green indicated low correlation with SHP.

counting for an estimated half-million deaths annually (Parkin et al., 2001; Thorgeirsson and Grisham, 2002). Although the agents that cause liver cancer are known (i.e. HBV and HCV infections, aflatoxin exposure, alcoholism, non-alcoholic steatohepatitis, etc.), little is known about the cellular and molecular mechanism in liver cancer. SHP governs bile acid synthesis and glucose homeostasis in liver by regulating various nuclear receptors such as FXR, LRH-1, LXR and CAR (Goodwin et al., 2000; Moore, 2005; Lee et al., 2007). Since SHP functions as a transcription factor but lacks DNA binding domain, it regulates down-stream target gene via protein-protein interaction (Seol et al., 1996). To investigate genes whose expression might be regulated by SHP in liver cancer, we selected genes whose expression patterns are highly correlated with SHP as potential downstream targets by applying Pearson's correlation coefficient value. Two thousand six hundreds three genes were selected as SHP potential down-stream target genes (Fig. 3A). As we expected, SHP gene expression was high in normal liver and SHP negatively correlated genes were enriched in liver cancer. Since SHP has been well identified as transcriptional repressor inhibiting transcriptional activity, we hypothesized that negatively correlated genes would better reflect biological characteristics of SHP. Of the negatively correlated genes, genes (*CDK4, MCM5, EXOCS1, CCNB1, BUB3*, and *BCL2L2*) regulating cell growth and proliferation in cancer cells were included, suggesting that SHP might modulate cell proliferation in cancer cells by negatively regulating those genes directly or indirectly (Agarwal et al., 2009; Chen et al., 2010; Moller, 2003; Rizwani et al., 2009; Stegh et al., 2007; Turner et al., 2010). To investigate interaction of SHP correlated genes in liver cancer, we applied those genes for pathway analysis using Ingenuity Pathway Analysis (IPA) software. As shown in Fig. 3B, four major pathways were generated by IPA. Overall pathway can be classified into two major pathways such as lipid metabolism and cellular proliferation. While much is known that SHP is glucose or lipid sensor in liver, it was not fully understood that how SHP regulates cellular proliferation at the molecular level although earlier report showed that SHP modulates *CCND1* in liver cancer (Zhang et al., 2008). As shown in Fig. 3B, Network I and III present cell growth and proliferation pathway. These pathways showed that *MCM3, EXOCS1*, and *BCL2L2* were negatively correlated with SHP, suggesting that SHP is a negative regulator during cell proliferation via modulating subset of these genes that are known as bad prognostic factors in human cancer such as lymphoma and breast cancer (Agarwal et al., 2009; Moller, 2003). Since SHP negatively regulates cell proliferation related gene sets, poorer survival of liver cancer patients with low expression of SHP might be due to higher proliferation of cancer cells. Network II and IV was mainly related to lipid and glucose metabolism reflecting SHP property. Network III presents that SHP governs FXR (NR1H4), PXR (NR1I2), NR1H3 (LXRα) and HNF1B which are key regulator maintaining glucose level in liver and well identified as a glucose sensor, suggesting that *in silico* analysis well reflect *in vivo* physiology (Geyeregger et al., 2006; Kim et al., 2009; Ourlin et al., 2003). Network IV includes newly identified genes controlling transcriptional activity (*PA2G4, NFYA, TYMS, PRMT5, COBRA* etc.) (Supplementary Table 2). Generating SHP gene network with liver cancer data set enables to understand unidentified SHP's potential functions in liver cancer. We found that SHP participates on cancer cell proliferation and differentiation by governing many down downstream genes. In the current study, using genomics approaches, we demonstrate that SHP is a good prognostic factor by possibly inhibiting subset genes involved in cell proliferation. Our systematic approaches open the new possibility that *in silico* analysis will be useful approach to find out new pathway and unidentified function of genes. This finding would warrant further validation of potential new functions of important genes.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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