

Galectin-1 and Its Involvement in Hepatocellular Carcinoma Aggressiveness

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Hepatocellular carcinoma is one of the most common cancers worldwide. Despite several efforts to elucidate hepatocellular carcinoma molecular pathogenesis, it is still not fully understood. To acquire further insights into the molecular mechanisms of hepatocellular carcinoma, we performed a systematic functional genomic approach on human HuH-7 and JHH-6 cells. The subsequent analysis of the differentially expressed genes in human specimens revealed a molecular signature of 11 genes from which we selected the *LGALS1* gene, which was overexpressed in hepatocellular carcinoma. The expression analysis in humans of Galectin-1 (Gal-1), the protein encoded by *LGALS1*, showed a Gal-1 preferential accumulation in the stromal tissue around hepatocellular carcinoma tumors. Moreover, a significant association between increased expression of Gal-1 in hepatocellular carcinoma and the presence of metastasis was observed. Interestingly, Gal-1 overexpression resulted in an increase of cell migration and invasion. In conclusion, this study provides a portfolio of targets useful for future investigations into molecular marker-discovery studies on a large number of patients and functional assays. In addition, our data provide evidence that Gal-1 plays a role in hepatocellular carcinoma cell migration and invasion, and we suggest that further studies should be conducted to fully establish the role of Gal-1 in hepatocellular carcinoma pathogenesis and evaluate Gal-1 as a potential molecular therapeutic target.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and is the third most common cause of cancer-related death. Globally, the 5-year survival rate of HCC is <5%, and approximately 598,000 HCC patients die each year (1). Although surgery remains the only and the most effective therapeutic approach for HCC, most HCC patients are still ineligible for surgical intervention (2). For those who qualify for surgery, the subsequent improvement in long-term survival is only

modest, owing to a high rate of recurrence or intrahepatic metastases that develop through invasion of the portal vein or spread to other parts of the liver (3,4). To improve prognosis and treatment of HCC, information regarding the phenotypic and molecular changes associated with the development of this disease must be determined. Despite several previous efforts, our current understanding of HCC is still rather limited (5).

Gene expression profiling is a useful approach to elucidate the molecular events underlying HCC development

and to identify novel diagnostic markers as well as therapeutic and preventive targets. However, the selection of potentially important candidate genes for further studies (which are derived from lengthy gene lists generated from gene expression profiling studies) is challenging for several reasons, including the presence of many confounding factors in the gene expression profile data from human cancer tissues (differences in age, hospital care and treatment protocols; nonparallel progression of cancer; and environment factors). For this reason, to acquire further insight into the molecular mechanisms of HCC, we performed a systematic functional genomic approach to investigate human HuH-7 and JHH-6 HCC cells. The former are considered an acceptable model for differentiated HCC, whereas the latter are models for undifferentiated, more malignant liver

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cancer (6–10). With this approach and the subsequent gene expression analysis on human HCC specimens, we identified an HCC molecular signature consisting of 11 genes that may play important roles in hepatocarcinogenesis. Among these genes, we focused our attention on *LGALS1*, which was overexpressed in human HCC. *LGALS1* encodes the Galectin-1 (Gal-1) protein, which belongs to a family of soluble lactose-binding lectins (galectins) characterized by their affinity for β -galactoside moieties. Gal-1 is a multifunctional protein involved in various aspects of tumorigenesis (cell–extracellular matrix and cell–cell interactions, cell migration, angiogenesis, tumor–immune escape) and has been described as a promising cancer target (11–13). Gal-1 expression has been examined in several malignant tumors (14–24), and its correlation with tumor invasiveness and lymph node metastasis was demonstrated in breast cancer (25), neuroblastoma (26), oral squamous cell carcinoma and lung adenocarcinoma (27). Although Kondoh *et al.* (28) showed that the activation of *LGALS1* gene expression in HCC results from promoter hypomethylation, data on Gal-1 protein expression in HCC and its correlation to clinicopathological parameters were not available. The role of Gal-1 in molecular mechanisms leading to cancer development has been analyzed in several tumors (11) but surprisingly never in the molecular pathogenesis of HCC. In particular, it is still unknown whether Gal-1 affects the invasive ability of HCC as previously shown in other malignant tumors (25–27).

In this study, we performed a comprehensive Gal-1 protein expression analysis in resection specimens of patients with HCC, nontumorous liver tissues and normal liver tissues by using a clinically well-characterized tissue microarray (TMA) and correlated our findings with patient clinicopathological parameters. Moreover, we provide direct functional evidence demonstrating that Gal-1 affects the ability of tumor invasion in HCC cell lines.

MATERIAL AND METHODS

Cell Culturing

HuH-7 (differentiated human hepatoma) and JHH-6 cells (undifferentiated hepatocellular carcinoma) were obtained from Japan Health Science Research Resources Bank (Rinku Town, Osaka, Japan) and grown according to the manufacturer's protocol.

Microarray Analysis

RNA isolation from cell lines, gene profiling, microarray data processing and statistical analysis, as well as gene ontology analysis are described in detail in the online supplementary material. Briefly, we performed a microarray experiment in triplicate on JHH-6 and HuH-7 total RNA using U133Plus 2.0 GeneChip Human Genome (Affymetrix UK, High Wycombe, UK). The raw gene expression data (.CEL files) are provided as supplementary material. The quality control and the expression measures of each chip were performed using the *affy* package from the R Computing Environment (<http://www.bioconductor.org/>). The expression measures were performed using robust multiarray average (29). Gene expression analysis was performed by Cyber-T (<http://visitor.ics.uci.edu/genex/cybert/>). Significance levels were adjusted according to the Bonferroni method. Differentially expressed genes were classified according to the DAVID 2008 functional annotation system (<http://david.abcc.ncifcrf.gov>).

Real-Time Quantitative Polymerase Chain Reaction

Real-time quantitative polymerase chain reaction (QRT-PCR) experiments and statistical analysis are described in detail in the supplementary material. Briefly, we used TRIZOL reagent (Invitrogen, Milano, Italy) to isolate total RNA from cells, human HCC, cirrhosis surrounding HCC (SLC) and cirrhosis distal from HCC (DLC) specimens. cDNA was synthesized by random hexamers with the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen). QRT-PCR was performed

Table 1. Clinical findings of study patients (N = 20).

| | Mean \pm SD |
|------------------|---------------|
| Age, years | |
| All patients | 64 \pm 7 |
| Males | 63 \pm 8 |
| Females | 67 \pm 3 |
| | n (%) |
| Sex | |
| Male | 17 (85) |
| Female | 3 (15) |
| Male/female | 5.7/1 |
| Etiology | |
| HCV infection | 8 (40) |
| HBV infection | 3 (15) |
| Alcohol abuse | 8 (40) |
| Hemochromatosis | 1 (5) |
| Child-Pugh score | |
| A | 11 (55) |
| B | 6 (30) |
| C | 3 (15) |
| HCC grading | |
| G2 | 13 (65) |
| G3 | 7 (35) |

as previously described (30). *GAPDH*, *rRNA 18S* and *β -actin* mRNAs were used to normalize mRNA concentration. The statistical analysis was performed as previously described (30,31).

Patients

Twenty patients, followed at the Centro Studi Fegato (Trieste), who prospectively underwent surgical treatment of HCC, were included in the study. HCC, SLC and DLC specimens from each patient were collected at the time of surgery and snap frozen. HCC diagnosis was established based on international criteria for HCC diagnosis and treatment (32) and confirmed by histological analysis. HCC specimens were histologically classified according to the Edmondson and Steiner criteria (33). Patient clinical findings are summarized in Table 1. No patient had undergone chemotherapy before surgery.

TMA Construction and Immunohistochemistry

The files of the Institute of Pathology, University Hospital Basel, and the De-

Table 2. Tumor-node-metastasis classification of HCCs.

| Staging parameter | n (%) |
|-------------------------------|------------|
| Tumor stage | |
| 1 | 61 (29.9) |
| 2 | 63 (30.9) |
| 3 | 48 (23.5) |
| 4 | 9 (4.4) |
| Not available | 23 (11.3) |
| Metastasis stage ^a | |
| 0 | 161 (78.9) |
| 1 | 32 (15.7) |
| Not available | 11 (5.4) |
| Node stage | |
| 0 | 178 (87.3) |
| 1 | 15 (7.4) |
| Not available | 11 (5.4) |
| Grading | |
| G1 | 79 (38.7) |
| G2 | 92 (45.1) |
| G3 | 29 (14.2) |
| Not available | 4 (2.0) |

^aThe presence of metastasis in human HCC specimens was assessed by autoptic observation.

partment of Pathology, University of Milan, School of Medicine and Humanitas Clinical Institute, were searched for samples of HCCs and dysplastic nodules (DN) obtained during the period from 1988 to 2007. In total, 204 HCCs, 5 HGDNs and 2 LGDNs were retrieved. Sufficient paraffin-embedded tissue for TMA construction was available in all cases. In 189 specimens, hepatic parenchyma surrounding neoplastic or dysplastic nodules was also sampled. Tumor characteristics, clinical findings and histopathology of surrounding liver tissue are summarized in Tables 2, 3 and 4. The male to female ratio was 3.4:1, mean age at diagnosis was 69.9 years (range 10–93 years).

For TMA construction, the number of punches per tissue type and patient ranged from 1 to 3. One punch was collected per patient on average (median 1.0) for each of the tissue types. We performed immunohistochemical analysis as previously described (34) using a mouse monoclonal anti-galectin-1 antibody (1:250; Novocastra, Newcastle upon Tyne, UK) detected by an antimouse

Table 3. Etiology of HCCs and DN^a.

| Etiology | HCC, n (%) | DN, n (%) |
|--|------------|-----------|
| Alcohol abuse | 39 (19.1) | 2 (28.6) |
| HBV infection | 28 (13.7) | 1 (14.3) |
| HCV infection | 63 (30.9) | 3 (42.9) |
| HBV + HCV infection | 2 (1.0) | 0 |
| HBV infection + alcohol abuse | 2 (1.0) | 0 |
| HCV infection + alcohol abuse | 2 (1.0) | 0 |
| Primary biliary cirrhosis + alcohol abuse | 1 (0.5) | 0 |
| Autoimmunhepatitis | 1 (0.5) | 0 |
| Hemochromatosis | 1 (0.5) | 0 |
| Hemochromatosis + alcohol abuse | 1 (0.5) | 0 |
| α_1 -Antitrypsin deficiency | 3 (1.5) | 0 |
| α_1 -Antitrypsin deficiency + HCV infection | 1 (0.5) | 0 |
| Unknown | 42 (20.6) | 1 (14.3) |
| Not available | 18 (8.8) | 0 |

^aHCC = 204; DN = 7.

peroxidase-conjugated EnVision antibody (Dako, Baar, Switzerland). Sections were counterstained with hematoxylin. Negative control consisted of primary antibody omission.

Gal-1 immunoreactivity was analyzed independently by two pathologists and was scored semiquantitatively by evaluating the number of positive hepatocytes and stroma cells over the total number of hepatocytes and stroma cells, respectively. Scores were assigned by use of 10% intervals and ranged from 0% to 100%. The staining intensity was semiquantitatively recorded as 0, 1, 2 or 3. A total of 197 HCC samples were divided into two groups based on the staining intensity: 0 and 1 (n = 153) versus 3 and 4 (n = 44). The absolute frequencies were compared by use of the Pearson chi-square test. Quantitative clinical data were compared by use of the Mann–Whitney test. $P \leq 0.05$ was considered significant.

Immunoblotting

Total protein extracts were prepared and used for immunoblotting as previously described (35) by use of goat anti-galectin-1 (0.2 μ g/mL; R&D Systems, Minneapolis, MN, USA), rabbit anti-Syk (1:1000; Cell Signaling Technology, Danvers, MA, USA) and rabbit anti-phospho-Zap-70 (Tyr319)/Syk (Tyr352) antibodies (1:1000; Cell Signal-

ing Technology) detected by horseradish peroxidase-conjugated antigoat and antirabbit antibodies (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A mouse anti- β -actin antibody (1:5,000; Sigma-Aldrich, Milan, Italy) was used as a control for equal loading.

Construct Preparation

We isolated total RNA from JHH-6 cells using TRIZOL reagent (Invitrogen). cDNA was synthesized by oligo(dT) with a SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen). The *LGALS1* cDNA was amplified by specific primers for *LGALS1* (5'-GAGTCTTCTGACAGCTGGTGC-3' and 5'-TGATTTCAGTCAAAGGCCACAC-3') with Pfu DNA Polymerase (Promega, Milan, Italy) and cloned to the plasmid pBluescript *EcoRV*. After sequencing, the 500-bp *BamHI-XhoI* fragment of *LGALS1* cDNA was cloned to the plasmid pcDNA3.1 *BamHI-XhoI* (pcDNA3.1-LGALS1 plasmid).

Table 4. Nontumorous liver tissue.

| Liver parenchyma | n (%) |
|------------------|------------|
| Liver cirrhosis | 155 (82.0) |
| Liver fibrosis | 9 (4.8) |
| Regular | 21 (11.1) |
| Not available | 4 (2.1) |

Table 5. Differentially expressed genes in HCC cell lines.

| | | Downregulated genes | | | | | | |
|--------------|---|---------------------|---|-------------------|----------------------|----------------------|--|---|
| AFFY_ID | Gene | Gene symbol | mRNA NCBI accession number ^a | Fold ^b | P value ^c | Chromosomal location | Chromosomal aberration in HCC ^d | Epigenetic regulation in HCC |
| 203400_s_at | <i>Transferrin</i> | <i>TF</i> | NM_001063 | -8.988 | 0.042 | 3q22.1 | 3q22-24 (gain) | |
| 219465_at | <i>Apolipoprotein A-II</i> | <i>APOA II</i> | NM_001643 | -8.932 | 0.050 | 1q21-q23 | 1q21-23 (gain) | |
| 219466_s_at | <i>Apolipoprotein A-II</i> | <i>APOA II</i> | NM_001643 | -8.686 | 0.026 | 1q21-q23 | 1q21-23 (gain) | |
| 205892_s_at | <i>Fatty acid binding protein 1, liver</i> | <i>FABP1</i> | NM_001443 | -7.260 | 0.048 | 2p11 | | |
| 209560_s_at | <i>Delta-like 1 homolog (Drosophila)</i> | <i>DLK1</i> | NM_003836 and NM_001032997 | -6.914 | 0.017 | 14q32 | | Upregulation due to deregulation of methylation |
| 224588_at | <i>X (inactive)-specific transcript</i> | <i>XIST</i> | BX648323 | -6.746 | 0.002 | Xq13.2 | | |
| 214218_s_at | <i>X (inactive)-specific transcript</i> | <i>XIST</i> | BX648323 | -5.244 | 0.034 | Xq13.2 | | |
| 217073_x_at | <i>Apolipoprotein A-I</i> | <i>APOA I</i> | NM_000039 | -6.390 | 0.027 | 11q23-q24 | 11q23.1 (AI) | |
| 209505_at | <i>Nuclear receptor subfamily 2, group F, member 1</i> | <i>NR2F1</i> | NM_005654 | -5.292 | 0.054 | 5q14 | | |
| 209459_s_at | <i>4-Aminobutyrate aminotransferase</i> | <i>ABAT</i> | NM_020686 and NM_000663 | -5.195 | 0.020 | 16p13.2 | 16p13 (AI) | |
| 242375_x_at | <i>Phenylalanine hydroxylase</i> | <i>PAH</i> | NM_000277 | -5.103 | 0.015 | 12q22-q24.2 | 12q22-q23 (breakpoints of chromosome rearrangement) | |
| 204059_s_at | <i>Malic enzyme 1, NADP(+)-dependent, cytosolic</i> | <i>ME1</i> | NM_002395 | -5.029 | 0.038 | 6q12 | | |
| 200824_at | <i>Glutathione S-transferase pi</i> | <i>GSTP1</i> | NM_000852 | -4.999 | 0.045 | 11q13 | | Silenced by hypermethylation |
| 225424_at | <i>Glycerol-3-phosphate acyltransferase, mitochondrial</i> | <i>GPAM</i> | NM_020918 | -4.771 | 0.008 | 10q25.2 | | |
| 206130_s_at | <i>Asialoglycoprotein receptor 2</i> | <i>ASGR2</i> | NM_001181, NM_080912, NM_080913, and NM_080914 | -4.510 | 0.044 | 17p | 17p1-p2 (MDR); 17p13.1 (LOH of p53 locus); 17p11.2-p13.3 (AI); 17p12-pter (LOH) | |
| 205352_at | <i>Serine (or cysteine) proteinase inhibitor, clade I (neuroserpin), member 1</i> | <i>SERPINI1</i> | NM_005025 | -4.484 | 0.043 | 3q26.1 | 3q25-qter (gain) | |
| 211237_s_at | <i>Fibroblast growth factor receptor 4</i> | <i>FGFR4</i> | NM_002011, NM_022963, and NM_213647 | -4.228 | 0.050 | 5q35.1-qter | 5q35-qter (LOH) | |
| 201917_s_at | <i>Hypothetical protein FLJ10618 (solute carrier family 25, member 36)</i> | <i>SLC25A36</i> | NM_018155 | -3.026 | 0.031 | 3q23 | | |
| 201918_at | <i>Hypothetical protein FLJ10618 (solute carrier family 25, member 36)</i> | <i>SLC25A36</i> | NM_018155 | -2.862 | 0.043 | 3q23 | | |
| 207819_s_at | <i>ATP-binding cassette, sub-family B (MDR/TAP), member 4</i> | <i>ABCB4</i> | NM_000443, NM_018849, and NM_018850 | -2.035 | 0.001 | 7q21.1 | 7q21-32 (gain); 7q21.3-7q22 (amplification of CYP3A locus) | |
| 213198_at | <i>Activin A receptor, type IB</i> | <i>ACVR1B</i> | NM_004302, NM_020327, and NM_020328 | -1.963 | 0.023 | 12q13 | | |
| 1552474_a_at | <i>Guanidinoacetate N-methyltransferase</i> | <i>GAMT</i> | NM_000156, and NM_138924 | -1.749 | 0.023 | 19p13.3 | 19p13.3 (AI) | |
| 219569_s_at | <i>Transmembrane protein 22</i> | <i>TMEM22</i> | NM_025246 | -1.487 | 0.007 | 3q22.3 | 3q22-24 (gain) | |

Continued

Cell Transfection

One d before transfection, HuH-7 cells were plated in a 100-mm dish. At the

time of transfection, the cell confluence was 70%, and the cells were transiently transfected with 6 μ g pcDNA3.1-LGALS1

plasmid or the empty vector using TransIT-LT1 transfection reagent (Mirus, Madison, WI, USA). Forty-eight h after

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Table 5. Continued.

| | | Upregulated genes | | | | | | |
|--------------|---|-------------------|---|-------|-------|------------|--|--|
| 44120_at | <i>aarF</i> domain containing kinase 2 | <i>ADCK1</i> | NM_020421 | 1.349 | 0.037 | 14q24.3 | 14q24-q32 (LOH in less than 10% of HCC cases) | |
| 228737_at | Chromosome 20 open reading frame 100 (<i>TOX</i> high mobility group box family member 2) | <i>TOX2</i> | NM_032883 | 1.937 | 0.025 | 20q13.12 | | |
| 219587_at | Tetratricopeptide repeat domain 12 | <i>TTC12</i> | NM_017868 | 2.192 | 0.016 | 11q23.1 | 11q23.1 (AI) | |
| 212295_s_at | Solute carrier family 7 (cationic amino acid transporter, γ^+ system), member 1 | <i>SLC7A1</i> | NM_003045 | 2.323 | 0.034 | 13q12-q14 | 13q13-14 (LOH of Rb locus); 13q12.1-q12.3 (AI); 13q12-q13 (deletion of BRCA2 locus); 13q13.3-q22.1 (loss) | |
| 219582_at | Opioid growth factor receptor-like 1 | <i>OGFRL1</i> | NM_024576 | 2.582 | 0.026 | 6q13 | | |
| 233540_s_at | CDK5 regulatory subunit associated protein 2 | <i>CDK5RAP2</i> | NM_018249 and NM_001011649 | 2.812 | 0.054 | 9q33.2 | 9p13-9q33 (LOH) | |
| 212607_at | <i>v-akt</i> Murine thymoma viral oncogene homolog 3 (protein kinase B, gamma) | <i>AKT3</i> | NM_005465 and NM_181690 | 2.832 | 0.002 | 1q43-q44 | Amplification of AKT3 gene | |
| 208502_s_at | Paired-like homeodomain transcription factor 1 | <i>PITX1</i> | NM_002653 | 3.178 | 0.032 | 5q31 | 5q31 (amplification of SMAD5 gene) | |
| 201189_s_at | Inositol 1,4,5-triphosphate receptor, type 3 | <i>ITPR3</i> | NM_002224 | 3.315 | 0.011 | 6p21 | | |
| 217339_x_at | Cancer/testis antigen 1 | <i>CTAG1</i> | NM_139250 and NM_001327 | 3.650 | 0.020 | Xq28 | | |
| 211966_at | Collagen, type IV, α 2 | <i>COL1Va2</i> | NM_001846 | 3.743 | 0.038 | 13q34 | 13q34 (gain) | |
| 225147_at | Pleckstrin homology, Sec7 and coiled-coil domains 3 | <i>PSCD3</i> | NM_004227 | 3.830 | 0.049 | 7p22.1 | | |
| 1552390_a_at | Hypothetical protein FLJ39553 | <i>C8orf47</i> | NM_173549 | 3.922 | 0.033 | 8q22.2 | 8q22-24 (gain) | |
| 241418_at | cDNA DKFZp686B14224 (from clone DKFZp686B14224) | <i>LOC344887</i> | BX640843 | 4.222 | 0.029 | 3q27.2 | 3q25-qter (gain) | |
| 1553830_s_at | Melanoma antigen, family A, 3 | <i>MAGEA3</i> | NM_005362 | 4.296 | 0.007 | Xq28 | Demethylation of MAGEA3 gene | |
| 212223_at | Iduronate 2-sulfatase (Hunter syndrome) | <i>IDS</i> | NM_000202 and NM_006123 | 4.413 | 0.029 | Xq28 | | |
| 219279_at | Dedicator of cytokinesis 10 | <i>DOCK10</i> | NM_014689 | 4.716 | 0.039 | 2q36.2 | | |
| 223642_at | Zic family member 2 (odd-paired homolog, <i>Drosophila</i>) | <i>ZIC2</i> | NM_007129 | 4.745 | 0.025 | 13q32 | 13q32-qter (AI); 13q31-34 (gain) | |
| 204341_at | Tripartite motif-containing 16 | <i>TRIM16</i> | NM_006470 | 4.933 | 0.008 | 17p11.2 | 17p11.2-p13.3 (AI) | |
| 208966_x_at | Interferon, γ -inducible protein 16 | <i>IFI16</i> | NM_152501, NM_198928, NM_198929, and NM_198930 | 5.510 | 0.037 | 1q22 | 1q21-q22 (gain); 1q22-q23 (gain) | |
| 209288_s_at | CDC42 effector protein (Rho GTPase binding) 3 | <i>Cdc42ep3</i> | NM_006449 | 5.802 | 0.015 | 2p21 | | |
| 200600_at | Moesin | <i>MSN</i> | NM_002444 | 6.446 | 0.012 | Xq11.2-q12 | | |
| 204955_at | Sushi-repeat-containing protein, X-linked | <i>SRPX</i> | NM_006307 | 6.835 | 0.020 | Xp21.1 | Xp21-pter (gain) | |
| 201105_at | Lectin, galactoside-binding, soluble, 1 (galectin 1) | <i>LGALS1</i> | NM_002305 | 6.850 | 0.005 | 22q13.1 | Hypomethylation of LGALS1 gene | |
| 225275_at | EGF-like repeats and discoidin I-like domains 3 | <i>EDIL3</i> | NM_005711 | 7.133 | 0.009 | 5q14 | | |
| 204268_at | S100 calcium binding protein A2 | <i>S100A2</i> | NM_005978 | 7.183 | 0.039 | 1q21 | 1q21-23 (gain) | |
| 209160_at | Aldo-keto reductase family 1, member C3 (3- α hydroxysteroid dehydrogenase, type II) | <i>AKR1C3</i> | NM_003739 | 7.595 | 0.024 | 10p15-p14 | | |

^aFor the genes that have alternatively spliced variants the NCBI accession numbers of each alternatively spliced variant are shown.

^bThe changes in gene expression of JHH6 versus HuH7 are shown as log2.

^cP value adjusted according to the Bonferroni method.

^dLOH, loss of heterozygosity; MDR, minimal deletion region; AI, allelic imbalance.

RESULTS

Gene Expression Profile in HCC Cells

The gene expression profile of JHH-6 cells compared with HuH-7 cells showed 47 (27 upregulated and 20 downregulated) significantly differentially expressed genes (Table 5). Two upregulated genes (*C8orf47* and *LOC344887*) were unknown. The genes belong to different functional groups (Figure 1A); 11 of 20 downregulated genes (55%) are known to be involved in metabolic pathways, and in particular *TF*, *APOA II*, *FABP1* and *APOA I* liver-specific genes were the most strongly downregulated. Conversely, the upregulated genes are known to be involved in several biological processes, and in particular in cell proliferation/differentiation/apoptosis (*AKT3*, *IFI16*, *LGALS1*, *TRIM16*), transcription regulation (*ZIC2*, *PITX1*, *TRIM16*), angiogenesis (*EDIL3*), cell-cell and cell-substrate interaction (*LGALS1*), and cell movement (*LGALS1*, *MSN*, *Cdc42ep3*). Several genes map to allelic imbalance chromosomal regions involved in HCC (Table 5). Interestingly, genes involved in cell proliferation (*IFI16*) and in transcription regulation (*ZIC2*, *PITX1*, *TRIM16*) mapped to allelic imbalance chromosomal regions involved in HCC. Moreover, previous data demonstrated that *DLK1*, *GSTP1*, *MAGEA3* and *LGALS1* gene expression in HCC is regulated by methylation status changes of the promoter (Table 5). The significant differential expression of these genes was validated by QRT-PCR using *GAPDH* and β -actin as housekeeping genes (Figure 1B, C).

Gene Expression Profile in Human HCC Specimens

We analyzed the expression profile of differentially expressed genes in 20 human HCC, SLC and DLC specimens by QRT-PCR using β -actin (Figure 2) and *rRNA 18S* (Supplementary Figure 1) as housekeeping genes. Eleven genes (*EDIL3*, *C8orf47*, *COLIVA2*, *AKR1C3*, *LGALS1*, *MSN*, *OGFRL1*, *PSCD3*, *SERPIN1*, *TRIM16* and *ZIC2*) showed a significant differential expression profile

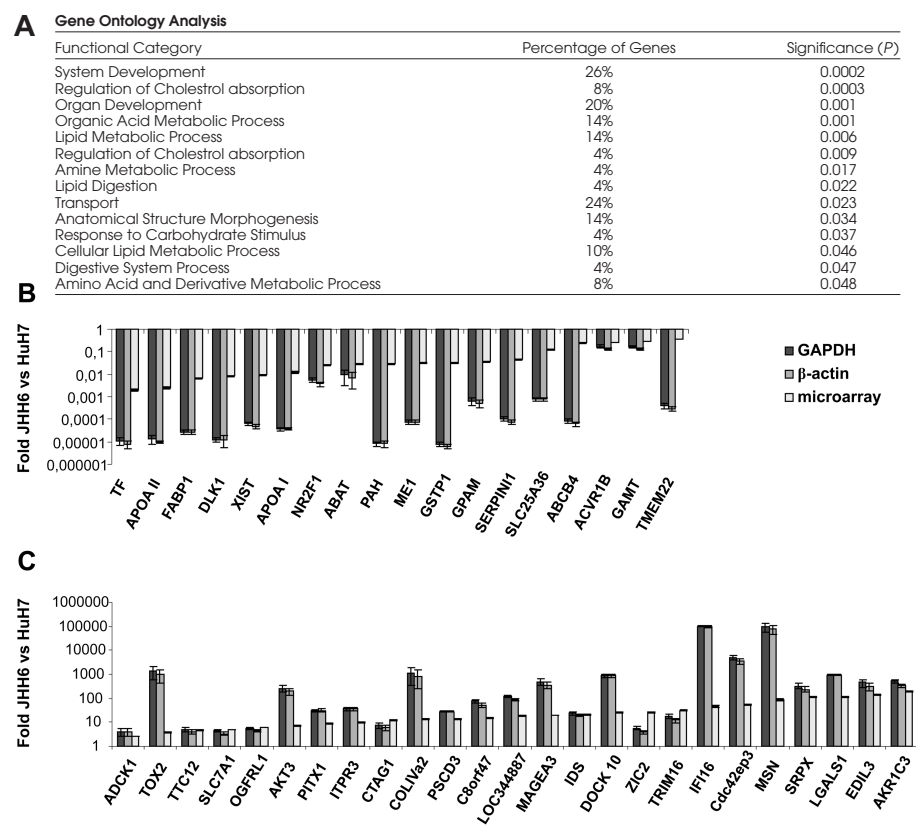


Figure 1. Gene expression profile in HCC cell lines. (A) Gene ontology analysis of genes identified by microarray analysis. Percentages indicate the percentage of genes within each functional category. Differential expression of downregulated (B) and upregulated (C) genes was validated by QRT-PCR. For each gene the change in expression of JHH-6 compared with HuH-7 (mean fold change = $2^{-(\text{average } \Delta\Delta\text{CT})}$) is shown relative to *GAPDH* (dark gray line) and β -actin (gray line). The fold from microarray analysis (light gray line) is also shown. Error bars are (mean fold change) $\times (2^{\text{SEM}} - 1)$. $P \leq 0.05$; Student *t* test.

transfection, the cells were briefly cultured for 72 h under the selection pressure of 3 mg/mL G418 (Gibco, Milan, Italy) to kill the untransfected cells. After 72 h, the cells were harvested; the *LGALS1* and Gal-1 expression levels were evaluated and cell invasion and migration assays were performed.

Cell Invasion and Migration Assays

We determined cell invasiveness by using chamber inserts (24-well format, polycarbonate membrane filter of 8- μ m pore size) (Corning, Milan, Italy), which were coated with 10 μ g matrigel (Matrigel Basement Membrane Matrix, BD Biosciences, Buccinasco, Italy). A total of 25,000 cells, transfected with pcDNA3.1-LGALS1 plasmid or the empty vector,

were seeded onto the upper chamber in DMEM medium without FBS. DMEM medium in the lower chamber contained 5% FBS served as a chemoattractant. After 2 h, cells migrating to the lower membrane surface were fixed and stained with hematoxylin/eosin. Cells were photographed and counted in six random fields per membrane at 10 \times magnification using a light microscope. The experiments were carried out in duplicate, and two independent sets of experiments were carried out. The migration assay was carried out as described in the invasion assay in the absence of matrigel.

All supplementary materials are available online at www.molmed.org.

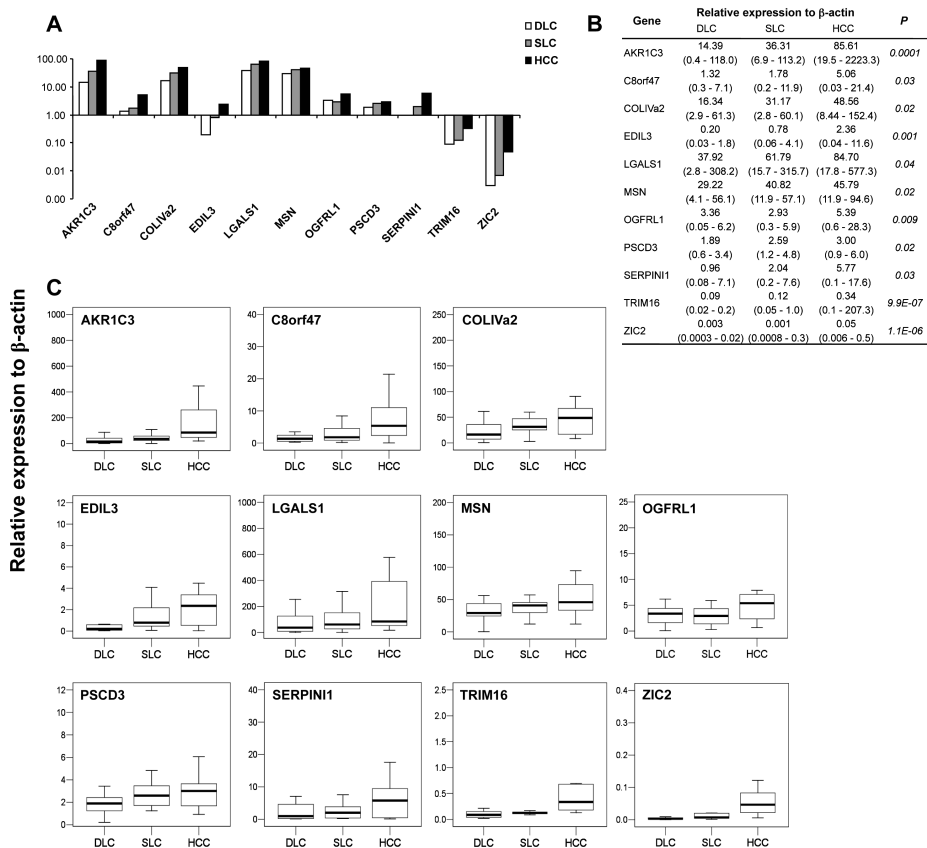


Figure 2. Gene expression profile in humans. The figure shows the statistically significant differentially expressed genes in human HCC, SLC and DLC specimens from 20 patients. (A) Trend of expression relative to β -actin for the 11 analyzed genes, (B) the median (minimum-maximum) values for each gene and (C) the box-plot of each gene are shown. *P* value was calculated by Kruskal-Wallis test.

with both β -actin (Figure 2B) and *rRNA 18S* (Supplementary Figure 1B). We confirmed the previously reported upregulation of *EDIL3* (36), *LGALS1* (28) and *SERPINI1* (37) genes and observed the upregulation of *AKR1C3*, *C8orf47*, *COL1A2*, *MSN*, *OGFRL1*, *PSCD3*, *TRIM16* and *ZIC2* genes. The upregulation of *SERPINI1*, *C8orf47*, *COL1A2* and *ZIC2* genes is in agreement with their location on chromosomal regions gained in HCC. Moreover, the *TRIM16* gene maps to an allelic imbalance chromosomal region involved in HCC (Table 5). We performed a gene ontology analysis on the 11 identified genes. As shown in Table 6, the most representative classes were GO:0007275 and GO:0048856. Both terms are present in *LGALS1* annotation. The re-

maining genes did not show a significant differential expression profile with both β -actin and *rRNA 18S* (data not shown).

Gal-1 Expression in Human Specimens

We analyzed the Gal-1 expression profile in human normal liver, cirrhotic tissue and HCC specimens by immunohistochemical analysis on TMA. Gal-1 was detected both in hepatocytes (hepatocyte expression) and stroma cells (stromal expression) in all three types of specimens (Figure 3A). Gal-1 hepatocyte expression was significantly higher in normal liver specimens than cirrhotic and HCC specimens ($P < 0.001$). The opposite trend was observed for Gal-1 stromal expression, with expression significantly lower in

normal liver specimens than cirrhotic and HCC specimens ($P = 0.006$) (Figure 3B). We compared Gal-1 hepatocyte or stromal expression of normal liver versus cirrhotic specimens, and of cirrhotic versus HCC specimens. A significant differential expression was found between normal liver and cirrhotic specimens relative to Gal-1 hepatocyte expression ($P < 0.001$; Mann-Whitney test).

A significant association was found between increased expression of Gal-1 in HCC and the presence of metastasis (odds ratio = 2.33; 95% confidence interval, 1.00–5.40; $P = 0.04$). No significant associations were found with other clinicopathological parameters (sex, age, size, grading [Edmondson] [33], tumor stage, node stage, etiology), although a trend of increased Gal-1 expression in HCC with vascular invasion was observed (odds ratio = 1.97; 95% confidence interval, 0.97–4.01; $P = 0.06$) (Table 7).

Gal-1 Contributes to Cell Migration and Invasion

The *LGALS1* gene expression in JHH-6 cells was significantly increased compared with HuH-7 cells ($P = 0.004$) (Figure 4A). In line with *LGALS1* gene expression, Gal-1 was expressed at a very high level in JHH-6 cells, whereas it was not detectable in HuH-7 cells (Figure 4B). To evaluate the contribution of Gal-1 to aggressiveness of HCC cells, the cDNA of *LGALS1* was amplified from JHH-6 total RNA and cloned in a pcDNA3.1 expression vector. HuH-7 cells transfected with the plasmid containing *LGALS1* cDNA showed a significant increase of *LGALS1* gene expression compared with cells transfected with empty vector (Figure 4C). In line with the increased *LGALS1* expression in the transfected cells, Gal-1 expression was significantly increased in HuH-7 cells transfected with the plasmid containing *LGALS1* cDNA compared with cells transfected with empty vector (Figure 4D). To determine the effect of Gal-1 on HuH-7 cell migration and invasion, *in vitro* cell migration and invasion assays were performed. The expression of Gal-1 strongly increased both the migration

Table 6. Functional annotation of 11 identified genes.^a

| Gene symbol | GOTERM ^b | KEGG pathways |
|-----------------|---|---|
| <i>MSN</i> | GO:0006928,cell motility; GO:0051674,localization of cell | hsa04670:Leukocyte transendothelial migration; hsa04810:Regulation of actin cytoskeleton |
| <i>OGFRL1</i> | — | — |
| <i>LGALS1</i> | GO:0007154,cell communication; GO:0007275,multicellular organismal development; GO:0016265,death; GO:0048468,cell development; GO:0048856,anatomical structure development; GO:0048869,cellular developmental process; GO:0050789,regulation of biological process; GO:0050794,regulation of cellular process | — |
| <i>C8orf47</i> | — | — |
| <i>PSCD3</i> | GO:0006810,transport; GO:0007154,cell communication; GO:0050789,regulation of biological process; GO:0050794,regulation of cellular process; GO:0051234,establishment of localization | — |
| <i>COL4A2</i> | GO:0006810,transport; GO:0007275,multicellular organismal development; GO:0009653,anatomical structure morphogenesis; GO:0016043,cellular component organization and biogenesis; GO:0048646,anatomical structure formation; GO:0048856,anatomical structure development; GO:0050789,regulation of biological process; GO:0051234,establishment of localization | hsa01430:Cell Communication; hsa04510:Focal adhesion; hsa04512:ECM-receptor interaction; hsa05222:Small cell lung cancer |
| <i>AKR1C3</i> | GO:0006091,generation of precursor metabolites and energy; GO:0044237,cellular metabolic process; GO:0044238,primary metabolic process | hsa00590:Arachidonic acid metabolism; hsa00980:Metabolism of xenobiotics by cytochrome P450 |
| <i>SERPINI1</i> | GO:0007275,multicellular organismal development; GO:0048856,anatomical structure development | — |
| <i>TRIM16</i> | — | — |
| <i>EDIL3</i> | GO:0007155,cell adhesion; GO:0007275,multicellular organismal development | — |
| <i>ZIC2</i> | GO:0006810,transport; GO:0007155,cell adhesion; GO:0007275,multicellular organismal development; GO:0009653,anatomical structure morphogenesis; GO:0009790,embryonic development; GO:0048066,pigmentation during development; GO:0048856,anatomical structure development; GO:0048869,cellular developmental process; GO:0051234,establishment of localization | hsa04340:Hedgehog signaling pathway |

^aGO terms in bold are in common with those of *LGALS1*.

^bGene ontology term (Biological Process, level analysis 2).

(Figure 4E) and invasion (Figure 4F) ability of HuH-7 cells transfected with the plasmid containing *LGALS1* cDNA compared with HuH-7 cells transfected with empty vector. In summary, this result showed that Gal-1 was positively associated with the invasiveness of HuH-7 cells.

Gal-1 Induces Syk Phosphorylation in HuH-7 Transfected Cells

To verify the activation of the Syk signaling pathway by Gal-1, we performed immunoblotting analysis on total protein extracts from HuH-7 cells transfected with empty vector and with the plasmid containing *LGALS1* cDNA. HuH-7 cells trans-

fectured with the plasmid containing *LGALS1* cDNA showed an increased phosphorylation of Syk protein compared with cells transfected with empty vector (Figure 5).

DISCUSSION

HCC is one of the most common and aggressive human malignancies (1). Al-

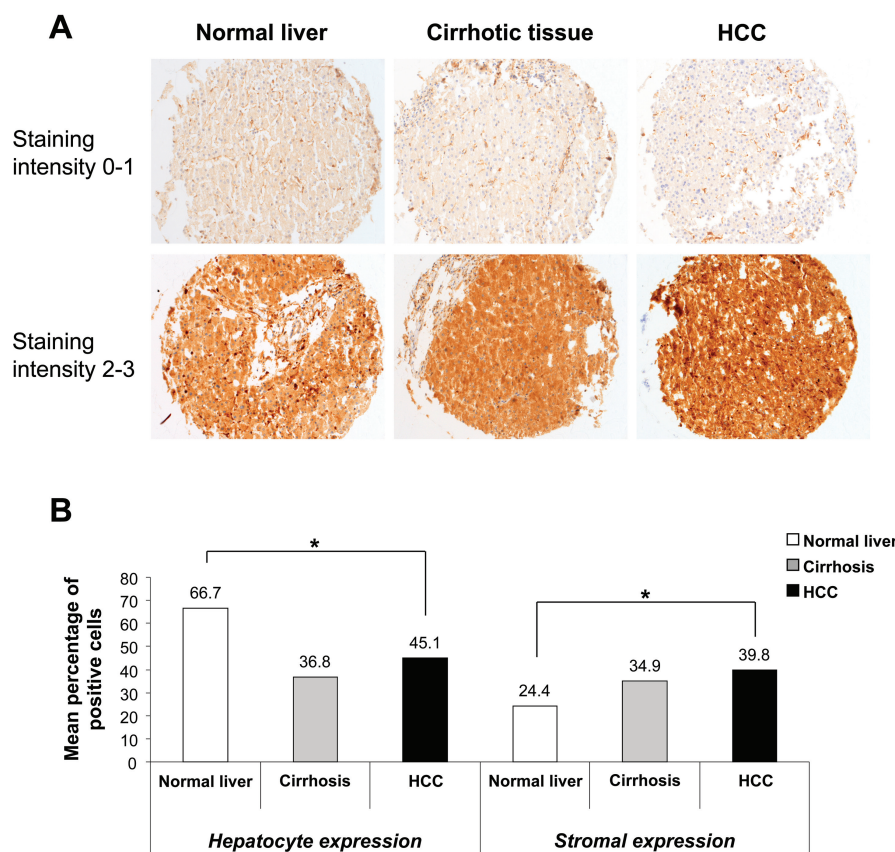


Figure 3. Gal-1 expression in humans. (A) Gal-1 expression in normal, cirrhotic and HCC liver specimens. The fold magnification was 200 \times . (B) The trends of Gal-1 hepatocyte and stromal expression in normal liver and cirrhotic and HCC liver specimens are shown. * $P < 0.01$, Kruskal-Wallis test.

though much is known about both the cellular changes that lead to HCC and the etiological agents responsible for the majority of HCC, the molecular pathogenesis of this disease is not well understood (5). Moreover, the severity of the disease and its clinical heterogeneity combined with the lack of good diagnostic markers and therapeutic strategies makes the treatment of HCC a major challenge.

This study was designed to acquire further insights into the molecular mechanisms of HCC. Several previous study groups have used microarray technology to explore the molecular mechanisms of HCC to possibly find markers or patterns for early detection and diagnosis and for stage classification and prognosis. A typical application of microarrays

in HCC study is to classify patients with different disease status. Such studies include the classification of cancer versus nontumor samples, high versus low histological grade, early versus late stage, favorable versus poor prognosis and HCC patients with HBV versus HCV infection. Gene expression signatures were identified to discriminate HBV-positive HCC from nontumor liver tissues (38) and DNAs from HCC in HBV (39) and HCV patients (40), and to monitor the pathological progression of HCV-induced hepatocarcinogenesis at each stage (cirrhosis, dysplasia, early HCC and advanced HCC) (41). Molecular profiling of HCCs unraveled molecular subtypes of HCC associated with specific signaling-pathway alterations (42,43). Moreover tumor-derived gene expression patterns

have been associated with patient survival (44), and gene expression signatures of nontumoral liver tissue adjacent to the tumor have been shown to be associated with survival and late recurrence of HCC (45) and with vascular invasion (46). Although these results showed promise, they appeared rather inconsistent, possibly owing to differences in experimental platforms and/or the underlying biological heterogeneity of the disease.

Our approach identified an HCC molecular signature of 11 genes. Although *EDIL3*, *LGALS1* and *SERPINI1* genes have been previously reported to be overexpressed in HCC (28,36,37), the remaining identified genes in this study have never previously been reported. Several of these genes are involved in important biological processes such as transcription regulation (*ZIC2*, *TRIM16*), angiogenesis (*EDIL3*), cell-cell and cell-substrate interactions (*LGALS1*) and cell movement (*LGALS1*, *MSN*), and accordingly these genes may play an important role in HCC development and progression. Functional studies are necessary to elucidate the role of these genes in HCC pathogenesis and determine their potential as molecular targets for the development of new HCC therapeutic approaches.

EDIL3 is a member of a family of extracellular matrix proteins with multiple EGF-like repeats. *EDIL3* is a minor splicing variant of *Del1*, for which there is extensive literature on its role in promotion of angiogenesis, *via* interaction with integrin $\alpha(v)\beta3$, and the metastatic process (47,48). *EDIL3* is induced by angiogenesis-related factors, including *VEGF1* (49), a factor known to be produced by HCCs (50). *PSCD3* belongs to the *PSCD* family, whose members are guanine nucleotide-exchange proteins, which serve as regulators of ADP ribosylation factors involved in vesicular trafficking and cytoskeletal regulation (51). Interestingly, the knockdown of *PSCD3* mRNA determined the mitotic arrest of the cell cycle (52). *ZIC2* belongs to the *ZIC* gene family of zinc-finger transcription factors, which

Table 7. Association analysis between Gal-1 expression in HCC specimens and clinicopathological parameters.

| | Staining intensity | | P |
|-----------------------------|--------------------|-------------|------|
| | 0 or 1 | 2 or 3 | |
| Sex, male/female | 118/35 | 32/12 | 0.55 |
| Age, years | (n = 151) | (n = 44) | 0.65 |
| Mean (SD) | 70.5 (11.7) | 69.1 (13.4) | |
| Median | 71.0 | 69.5 | |
| Specimen size, cm | (n = 141) | (n = 40) | 0.09 |
| Mean (SD) | 5.4 (4.5) | 6.7 (4.8) | |
| Median | 3.7 | 5.0 | |
| Grading (Edmondson) | (n = 149) | (n = 44) | 0.72 |
| I-II/III-IV | 62/87 | 17/27 | |
| Tumor stage | (n = 135) | (n = 39) | 0.13 |
| 1-2/3-4 | 97/38 | 23/16 | |
| Node stage | (n = 144) | (n = 42) | 0.22 |
| Lymph node invasion: no/yes | 135/9 | 37/5 | |
| Metastasis stage | (n = 144) | (n = 42) | 0.04 |
| No metastasis/metastasis | 125/19 | 31/11 | |
| Vascular invasion | (n = 128) | (n = 42) | 0.06 |
| No invasion/invasion | 85/43 | 21/21 | |
| Etiology | (n = 104) | (n = 31) | 0.07 |
| Viral/not viral | 75/29 | 17/14 | |

are essential for a wide array of developmental programs, including the central nervous system, muscle and skeletal development and the establishment of left-right asymmetry in the early embryo (53). Human mutations in *ZIC* family members determine many clinically significant congenital abnormalities (54). Moreover, aberrant expression of *ZIC2* has been observed in medulloblastomas (55), small cell lung cancers (56), endometrial cancer with node metastasis (57) and in bladder, colon and breast cancer (56). *OGFR1* encodes the opioid growth factor receptor-like 1, whose biological function is unknown. The opioid growth factor-opioid growth factor receptor interaction plays an inhibitory role in cancerous cell proliferation (58,59), cellular renewal in homeostasis, wound healing, development, angiogenesis and cancer (60). Moesin (membrane-organizing extension spike protein) is a member of the ERM (ezrin-radixin-moesin) protein family. These proteins act as molecular linkers between cell adhesion molecules and the cytoskeleton, regulating cell morphology. These proteins have also been implicated in regulating signaling mole-

cules (61). In particular, Moesin is localized in the intracellular core of microextensions known as filopodia, microvilli, microspikes, and retraction fibers. This subcellular distribution closely follows the dynamic changes in cell shape that take place when cells attach, spread and move spontaneously, or in response to extracellular signals. Therefore, Moesin could be involved in the dynamic restructuring of such microdomains by regulating binding interactions between the plasma membrane and the actin cytoskeleton (62). TRIM16 is a member of the RING-B box-coiled-coil protein family; its biological function has not been well characterized. However, evidence seems to suggest that TRIM16 may play a role in the response of tissues to differentiating agents (63), have a pivotal role in the retinoid anticancer signal (64,65) and play a role in innate immunity by enhancing the alternative secretion pathway of interleukin 1 β (66). AKR1C3 converts adrenal androgens such as androstenedione and dehydroepiandrosterone into intraprostatic testosterone. AKR1C3 regulates ligand access to steroid hormone and prostaglandin re-

ceptors and may stimulate proliferation of prostate and breast cancer cells (67,68). Increased expression of AKR1C3 has been shown to strongly correlate with prostatic carcinogenesis (69,70), malignant breast lesions (71) and endometrial cancer (72), indicating AKR1C3 plays a role in prostate, breast and endometrial carcinogenesis. Therefore, AKR1C3 is a potential target in treating prostate, breast and endometrial cancers (73).

We focused our attention on the *LGALS1* gene, which encodes Gal-1 protein. Although differential expression of *LGALS1* in human HCC specimens was not the most important one statistically, this gene is the most interesting among those identified because Gal-1 is involved in various aspects of tumorigenesis and has been described as a promising cancer target (11–13). The mechanisms related to how Gal-1 contributes to cancer progression and metastasis have been proposed: it regulates tumor cell growth (74), triggers the death of infiltrating T cells (75), suppresses T-cell-derived proinflammatory cytokine secretion (76), mediates cell-cell or cell-extracellular matrix adhesion (77,78), is involved in tumor angiogenesis (79) and promotes cancer cell migration (24,80).

We observed the increased expression of *LGALS1* in HCC specimens compared with cirrhotic tissues. Furthermore, we observed preferential accumulation of Gal-1 in the delicate stromal tissue surrounding neoplastic hepatocytes of HCC tumors. Because Gal-1 protein is secreted and can be found on the extracellular side of all cell membranes as well as in the extracellular matrices of various normal and neoplastic tissues (77,80–85), we hypothesize that the significant decrease of Gal-1 expression in the hepatocytes of HCC and its significant corresponding increase in the stromal tissue surrounding neoplastic hepatocytes of HCC could be due to the increased secretion of Gal-1 by neoplastic hepatocytes and its accumulation in the stroma surrounding HCC. In support of our hypothesis, an increased secretion of Gal-1 in conditioned medium

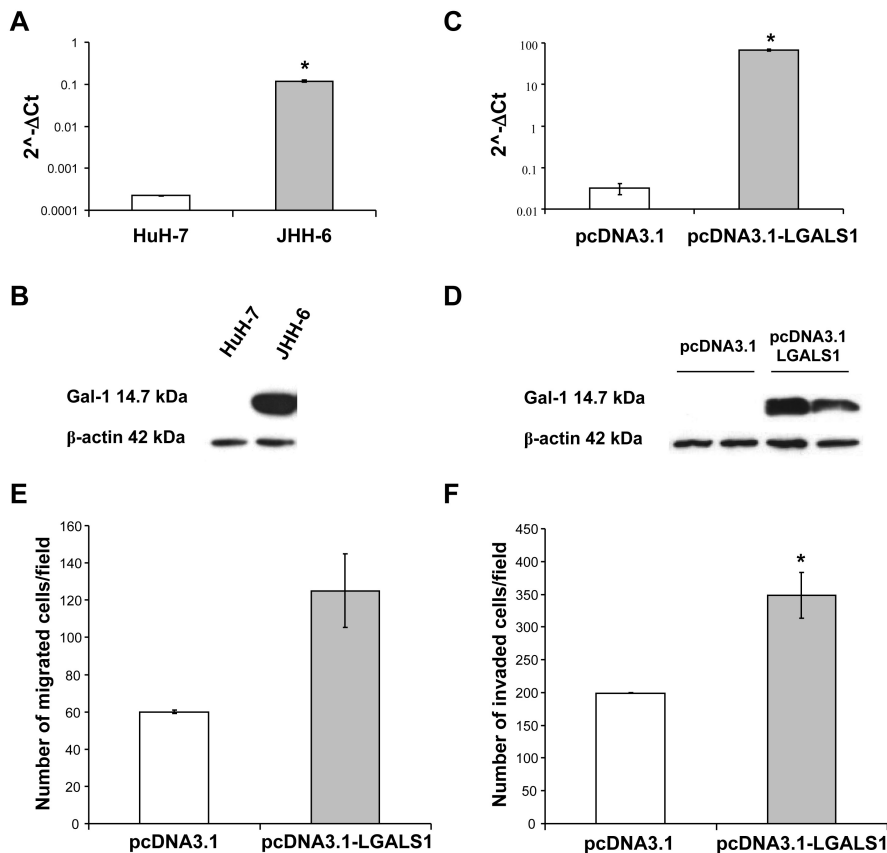


Figure 4. Effect of Gal-1 on migration and invasion. (A) *LGALS1* expression relative to β -actin in HuH-7 and JHH-6 cells. The histograms show the mean values of $2^{-(\Delta Ct)}$ in HuH-7 and JHH-6 cells. The error bars in the histogram were determined by SEM of ΔCt values. * $P = 0.004$, Student *t* test. (B) Immunoblotting analysis was performed on total protein extracts (30 μ g/lane) from HuH-7 and JHH-6 cells. A very high level of Gal-1 expression was observed in more aggressive JHH-6 cells. (C) *LGALS1* expression relative to β -actin in HuH-7 cells transfected with plasmid containing *LGALS1* cDNA or empty vector. The histograms show the mean values of $2^{-(\Delta Ct)}$ in HuH-7 transfected cells. The error bars in the histogram were determined by SEM of ΔCt values. * $P = 0.004$, Student *t* test. The graph shows one of three independent experiments with similar results. (D) Immunoblotting analysis was performed on total protein extracts (15 μ g/lane) from HuH-7 cells transfected with plasmid containing *LGALS1* cDNA or empty vector. The panel shows one of three independent experiments, performed in duplicate, with similar results. (E) Migration assay for the *LGALS1*-overexpressing HuH-7 cells. The *LGALS1* overexpression results in an increase in the average number of HuH-7 migrating cells transfected with plasmid containing *LGALS1* cDNA (number of migrated cells/field = 125; SEM = 19.83) compared with the migrating cells transfected with empty vector (control) (number of migrated cells/field = 60; SEM = 0.83). (F) Invasion assay for the *LGALS1*-overexpressing HuH-7 cells. The *LGALS1* overexpression results in a significant increase in the average number of HuH-7 invading cells transfected with plasmid containing *LGALS1* cDNA (number of invaded cells/field = 348.42; SEM = 35.08) compared with the invading cells transfected with empty vector (control) (number of invaded cells/field = 199.17; SEM = 0.5). * $P = 0.05$, Student *t* test.

of JHH-6 cells compared with HuH-7 cells was observed (data not shown). Our findings are in accordance with

previously reported studies showing Gal-1 increased expression in human thyroid (14–16), endometrium (17), head

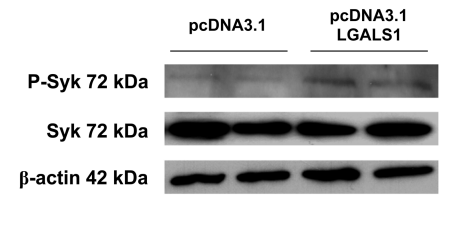


Figure 5. Gal-1 induces Syk phosphorylation. Immunoblotting analysis was performed on total protein extracts (15 μ g/lane) from HuH-7 cells transfected with plasmid containing *LGALS1* cDNA or empty vector. β -Actin was used as the loading control.

and neck (18), thymus (19), bladder (20), pancreas (21) and colon cancers (22) and cholangiocarcinoma (23) and glioma (24). Moreover, preferential accumulation of Gal-1 in the peritumoral stroma has been reported for thyroid (16), head and neck (18), colon (22), ovary (77) and prostate carcinoma (86). Gal-1 expression was significantly increased in HCCs with metastasis compared with those without metastatic disease. The correlation between Gal-1 expression and metastasis was validated by *in vitro* functional studies. Our findings show Gal-1 expression affects the invasiveness of HCC cells. In line with the findings of Fulcher *et al.* (87), the Gal-1-induced migration and invasion in HuH-7 cells were mediated by the stimulation of Syk phosphorylation. Therefore, our data correlate Gal-1 expression with tumor aggressiveness, which is in accordance with findings in breast cancer (25), neuroblastoma (26), oral squamous cell carcinoma and lung adenocarcinoma (27). Metastasis is a multistep process during which cancer cells escape from their original tissue, invade the surrounding tissues of the primary tumor, penetrate a lymphatic or blood vessel, reach distal sites, extravasate and colonize in the second organ (88,89). Increased invasiveness of cancer cells is a critical step in tumor metastasis and requires many changes; for example, cancer cells loosen their adhesion to neighboring cells and the extracellular matrix, degrade adjacent tissues and elevate their motility (90). Aberrant gene ex-

pression changes or gene mutations in tumor cells or carcinoma-associated stromal cells increase their malignancy and accelerate cancer progression. Several chromosomal aberrations and genes were positively related to HCC invasion and metastasis (91,92). Our data provide evidence that Gal-1 also affects the invasive ability of HCC cells. Therefore, further studies should be conducted both to fully unravel the role of Gal-1 in HCC pathogenesis and to evaluate Gal-1 as a molecular therapeutic target for HCC.

In conclusion, this study provides a portfolio of targets useful for future investigations into molecular-marker discovery studies on many patients and functional assays. These further studies could lead to the identification of markers that can be used to predict diagnosis and prognosis of HCC, may provide further insight into the mechanisms of pathogenesis and could also highlight new potential therapeutic targets for patients with this disease.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the re-

sults and discussion reported in this paper.

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