The Roles of Leukocyte Cell-Derived Chemotaxin 2 Gene in Hepatocellular Carcinoma Invasion and Angiogenesis

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Abstract

Hepatocellular carcinoma (HCC) is the fifth commonest cancer worldwide and the second leading fatal cancer in Southeast Asia. The five year survival rate for HCC patients is only about 40% to 50%, and the major poor prognostic factors include vascular invasion and large tumor size. Using high throughput technology suppression subtractive hybridization (SSH) and oligonucleotide microarray of human membrane/secreted proteins (TMSEC microarray), we found that LECT2, is a 16-kDa protein that consists of 133 amino acids and three intramolecular disulfide bonds, was associated with less vascular invasion in HCC patients. Univariate analysis of LECT2 mRNA level and various risk factors in 127 patients with HCC showed that LECT2 was highly correlated with overall survival and smaller tumor size. LECT2 overexpression did not influence hepatoma cells growth property, but strongly reduced tumorigenesis in subcutaneous xenograft model in vivo. Immunohistochemical staining revealed that tumors from LECT2-transfectant displayed less vasculaization as compared to vector control tumors. In addition, we purified LECT2-Fc recombinant proteins, and found that rhLECT2 could strongly and directly inhibit migration, invasion, and angiogenesis in vitro and in vivo. In conclusion, we first established that LECT2 is a promising prognostic molecular marker and could be a potential therapeutic protein drug for HCC invasion and angiogenesis.

Keywords: LECT2, hepatocellular carcinoma, invasion, angiogenesis
Background and Goals

HCC epidemiology and research motivation:

Hepatocellular carcinoma (HCC) is one of the most common types of cancer in the world, with annual incidence of approximately 1 million cases.\(^1\) In Taiwan, it continues the leading cause of cancer-related death among men with 46.5 deaths/100,000 and the second among woman with 18.1 deaths/100,000 in 2006.\(^2\) The major treatment for HCCs is surgical resection. Other treatment modalities includes alcohol injection, transarterial chemoembolization, microwave therapy, radiofrequency ablation therapy, and liver transplantation, etc. But the overall five year survival is only 40% to 50%.\(^3\) High cancer recurrence is still the major cause of death of HCC patients. According the literature, the five year recurrence-free survival was only 25-27%.\(^4\) The major poor prognostic factors included vascular invasion, high $\alpha$-FP, large tumor size, multiple tumor or tumor satellitosis, etc.\(^4,5\) Among the various literature reports with multivariate analysis, vascular invasion of the tumor is the major contribution of high recurrence and poor survival.\(^4,6\) Therefore, identifying differentially expressed genes between vascular-invasion and non-vascular invasion of HCCs is important.

The advantage of SSH and application of SSH with microarray:

Investigation of changes in gene expression in a biological system can be carried out with a variety of available tools that promise the identification of differentially expressed transcripts between two populations of mRNA. These include differential analysis of library expression, differential display, representational difference analysis, enzymatic degradation subtraction, linker capture subtraction, and serial analysis of gene expression, etc.\(^7\) Despite the fact that these methods have proven successful in isolation of differentially expressed genes, they all possess some intrinsic drawbacks. In addition to their specific limitations, a common feature is the inability to isolate rare transcripts. A novel technique called suppression subtractive hybridization (SSH) could generate an equalized representation of differentially expressed genes irrespective of their relative abundance.
SSH was first put forward by Diatchenko et al. in 1996. The basic principle was that common cDNA in the paired materials were subtracted by subtractive hybridization and then suppressive PCR was carried out to amplify cDNA fragments specially expressed in tester. The advantage of SSH was the design of two different adapters and the introduction of suppression PCR with the result that the differentially expressed cDNA fragments were amplified. It allows two subtractive hybridizations in the forward and reverse direction. Some mRNA expressed with low abundance could be detected, which provided clues for the further gene sequencing and identification. With the development and improvement of SSH technique, it has become one of the most effective techniques for identifying and cloning differentially expressed genes. Since it was established, more than two hundred papers have been published concerning about carcinogenesis, tumor metastasis, inflammation, apoptosis, and tumor therapy. Recent advances in technology, especially in the area of high-density of cDNA or oligonucleotide microarrays, have allowed us to simultaneously monitor the gene expression profiles of thousands of genes. Recently, there were several papers using SSH combined with microarray technology to identify and clone the novel genes in cancer biology and other disease.

The preliminary research results and correlation with research direction of this project:

In our preliminary research work, we have successfully used suppression subtractive hybridization and microarray technique to identify 20 differentially expressed genes between vascular-invasive and non-vascular invasive hepatocellular carcinoma. One of these twenty significant genes is leukocyte cell-derived chemotaxin 2 (LECT2, NM_002302) gene.

LECT2 gene information and the relation of LECT2 and HCC:

There’re several reasons for us to notice this gene’s function in HCC. LECT2 protein was originally identified from the culture fluid of the human T cell line SKW-3 in the process of screening for a novel neutrophil chemotactic protein in 1996 by Yamagoe et al. Subsequent isolation of the cDNA encoding LECT2 showed that its mRNA is specifically expressed in human adult and fetal liver and human
hepatoma cell lines. LECT2 is a 16-kDa protein that consists of 151 amino acids and three intramolecular disulfide bonds. Different amount of LECT2 secreted into the culture medium have been observed in various human hepatoma cell lines, including HuH-6, HepG2, and PLC/PRF/5, etc. The human LECT-2 gene consists of four exons and three introns. It has been mapped to chromosome 5q31.1-q32, where loci contain a cluster of several genes encoding immuno-regulatory cytokines, including IL-3, IL-4, GM-CSF, and IL-5.

Some chemotactic proteins have been shown to exert physiological functions in vivo other than the original activity demonstrated in vitro. Although LECT2 has been originally demonstrated to have a chemotactic function in vitro, its biological functions has not been thoroughly clarified. LECT2 is identical with chondromodulin II isolated from fetal bovine cartilage, and was identified as a growth stimulator for chondrocytes and osteoblasts. LECT2 was found a homologous to chicken mim-1 (myb-induced myeloid protein-1) gene product. The mim-1 gene is regulated not only by myb but also by C/EBP, a transcription factor that is abundantly expressed in the liver.

Except above mentioned multifunction, LECT2 also was found involved in several pathologic conditions. Segawa, et al found the reduction of LECT2 expression of mouse liver may be inversely regulated by TNF-α and/or IFN-γ mRNA expression in the Con A-induced hepatic injury. Saito, et al generated LECT2 -/- mice. They found NKT cells in the liver increased significantly in LECT2 knockout mice when compared with wild-type mice. The deficiency of LECT2 led to severe liver injury, after treated with Con A in LECT2 -/- mice, possibly because of excessive expression of IL-4 and Fas ligand by the increase in the number of hepatic NKT cells. Their results suggested that LECT2 might regulate the homeostasis of NKT cells in the liver.

In the literature, very few research results were reported about the role of LECT2 in HCC. In 1999, Uchida, et al, using immunohistochemistry, found the expression of LECT2 became weaker with the progression of multistep hepatocarcinogenesis.
In 2001, using cDNA microarray to identify genes involved in viral carcinogenesis and tumor progression, Okabe, et al. even did not notice this gene at all, although in their microarray results of figure3B revealed significant difference of LECT2 gene expression between vascular invasion and non-vascular invasion HCC. In 2004, French research people, used suppression subtractive hybridization (SSH) to identify target genes regulated by Wnt/β-catenin signaling. They injected adenovirus that encodes for an oncogenic form of β-catenin or with control adenovirus that encodes for LacZ or GFP into mice. The liver was removed 48 hours after injection. RNA was extracted. After SSH, they screened LECT2 was one of the target genes related to activation of β-catenin. They also used Huh 7 and HepG2, which differ in their β-catenin status, to proved LECT2 was a downstream target gene of β-catenin. Although they used real time RT-PCR trying to identify LECT2 expression difference between β-catenin mutation and without mutation HCC samples, their results cannot be definitely concluded.

Summary of literature reviews and unresolved problems:

Above all, LECT2 is a multifunctional protein. It involved chemotaxis, cell proliferation, inflammatory process, immuno-modulation, carcinogenesis, and tumor behavior. But the function and mechanism of LECT2 in these pathophysiologic conditions, especially in concerning with HCC is not clear.

Purposes

Taken together, the purposes of this proposal are (1) To identify and validate the differentially expressed genes between vascular-invasive and non-vascular invasive hepatocellular carcinoma using SSH and microarray technique. (2) To uncover and prove the functional role of LECT2, one of the 20 differentially expressed genes, on the tumor behavior of HCC through in vitro, and in vivo experiments. (3) To analyze statistically the relationship of the expression level of LECT2 gene in HCC with the clinicopathologic factors of these patients. (4) To dissect the signal-transduction molecular mechanism of LECT2 on inhibition of invasion or angiogenesis ability of HCC.
Materials and Methods

**Patients and tumor tissue collection**

All patients with primary, single hepatocellular carcinoma (HCC) with tumor size smaller than 5 cm were included. All tumor tissue samples would be collected at the National Taiwan University Hospital following approval by Institutional Review Boards and written informed consent. Tissues were snap frozen in liquid nitrogen immediately after surgical resection and stored at -80°C. The clinicopathologic factors of these patients were listed in Table 1. These 9 patients were divided into two group. Group A (Vascular-invasion group) had 4 patients with tumors of portal-vein invasion. Group B (Non-vascular invasion group) had 5 patients with tumors of no vascular invasion.

**Preparation of total RNA and polyA RNA from HCC**

Total RNA was extracted from frozen liver tumor tissues using Trizol reagent (Gibco BRL, USA). And mRNA was isolated from total RNA using the Oligotex TM mRNA kit (Qiagen, USA) according to its manufacture’s instructions. The integrity of RNA and mRNA was checked on a 1% agarose gel. RNA was pooled from individuals in each group to normalize for individual differences. The same amount of RNA from four patients’ tumors with vascular-invasion (Group A) were pooled together. Also RNA from five patients’ tumors with non-vascular invasion (Group B) were pooled together.

**Suppression subtractive hybridization (SSH)**

SSH was performed by using PCR-Select™ cDNA subtraction kit (Clontech Lab. Inc, USA) according to the recommendations of the manufacture. Two way SSHs were performed as Figure 1 showed. In the forward SSH, pooled RNA of vascular-invasive tumors (Group A) was used as the tester, and pooled RNA of non-vascular invasive tumors (Group B) was used as the driver. After forward SSH (A-B), the differentially-expressed, up-regulated cDNA of the group A relative to group B would be enriched. In the reverse SSH, group A was used as the driver and group B as tester. After reverse SSH (B-A), the differentially-expressed cDNAs of the
group B relative to group A would be enriched. This means that the down-regulated gene expression of group A vs. group B would be identified. In brief, the SSH procedure were as follows:

**Double strand cDNA synthesis**

Two μg of pooled RNA in each group was reverse transcribed to produce first-strand cDNA and then was amplified using SMART PCR cDNA Synthesis Kit (BD Biosciences) as manufacturer’s protocols. The SMART amplification method relies on the dC tailing of full-length cDNA by reverse transcriptase. The SMART oligonucleotide binds to the Cn tail of the cDNA, which is extended by the reverse transcriptase following template switching. PCR amplification of the full-length cDNA uses primers specific for the SMART oligonucleotide and the modified oligo(dT) sequences. The amount of PCR thermal cycle was optimized to ensure that the ds cDNA was amplified and stopped in the exponential phase.

**Ligation of cDNA fragments**

The SMART amplified cDNA pool was then digested with Rsa I restriction endonuclease to give approximate 600 base pair blunt end cDNA fragments. Rsa I-digested cDNA from tester was separated to two portions. Adapter 1 and adapter 2R were ligated separately to 2 μL of Rsa I-digested tester cDNA with 1:6 dilution in the presence of T4 DNA ligase at 16°C overnight followed by heating at 70°C for 5 min to inactivate the ligase. In order to determine the ligation efficiency, 1μL of adapter 1-ligated and adapter 2R-ligated cDNAs of the tester was diluted into 200μL H2O respectively and amplified in two separate 50 μL reactions. One reaction used GAPDH 3’, 5’primer, while the other used GAPDH 3’ primer and PCR primer1. PCR parameters were as follows: 30 cycles at 94 °C for 30 s, at 65 °C for 30 s and at 68 °C for 2.5 min. The products were examined by electrophoresis on a 1% agarose/EB gel.

**Subtractive hybridizations**

In the first hybridization, 1.5 μL of RsaI digested driver cDNA was mixed with 1.5 μL of diluted adaptor1- or adaptor2R-ligated tester cDNA. The samples were denatured at 98 °C for 1.5 min and immediately incubated in a thermal cycler at 68 °C
for 8 h. In the second hybridization, two kinds of sample resulting from the first hybridization were mixed in the presence of a freshly denatured driver cDNA. The samples were incubated at 68 °C for 18 h. After 200 μL of dilution buffer was added, the samples were incubated for an additional 7 min. Analysis of the subtraction efficiency was carried out using PCR amplification of GAPDH in diluted subtracted cDNA versus unsubtracted cDNA. PCR was performed for 35 cycles at 94 °C for 30 s, at 60 °C for 30 s and at 68 °C for 2 min. The products were monitored on a 1% agarose/EB gel for an aliquot which was removed from each reaction after 10, 15, 20, 25, 30, 35 cycles

**Suppression PCRs**

A 1 μL of diluted subtraction mixture was amplified with PCR primer1 in the primary PCR. The reaction mixture was incubated at 75 °C for 5 min to extend the adaptors and followed in turn at 94 °C for 25 s, 30 cycles at 94 °C for 10 s, at 64 °C for 30 s and at 71 °C for 1.5 min. The primary PCR mixture was diluted 10-fold and 1 μL from that was used in secondary PCR with nested PCR primer1 and primer2R. The conditions of the reaction were 15 cycles at 94 °C for 10 s, at 68 °C for 30 s and at 72 °C for 1.5 min. Eight μL products from each PCR reaction of the secondary PCR was analyzed on a 1% agarose/EB gel.

**Microarray**

After forward and reverse SSH, we will get two SSH libraries, A-B and B-A libraries.

1μg of the subtracted cDNA was labeled with Cy5 (A-B) and Cy3 (B-A) in a random priming reaction using Klenow fragment. Then the labeled Cy5 and Cy3 cDNA were hybridized to TMS (Tansmembrane and Secreted protein- relative genes) microarray (Taiwan Genome Sciences, Inc.). TMS microarray is the oligonucleotide microarray slide spotted with transmembrane or secreted protein-related genes. Hybridization signals were detected by GenePix 4000A Fluorescent Scanner. Image data collection and analysis was performed by GenePix Pro Software. The microarray experiment was performed twice for duplication.
Microarray data mining

TMS microarray had 10544 gene spots represented 4544 genes. Areas of the array with obvious blemishes were flagged and excluded from subsequent analysis. Raw data was normalized with LOWESS method using S-plus software. We set four criteria for selection of differentially expressed genes:

1. Signal to noise ratio > 3 times
2. The Cy5 signal > 1000 or Cy3 signal > 1000
3. \( \log_2 \text{Cy5/Cy3} > 3 \) or < -3
4. At least half of the repeated spots of the same gene in both microarray slides fulfilled the above 3 criteria

We have found 20 differentially-expressed genes after the preliminary experiments and data mining as shown in Table 2. One of the most interesting genes is LECT2. The next step in this project is to validate these 20 differentially-expressed genes through real-time RT-PCR. And further, we will correlate the combination of these genes and clinicopathologic outcome.

Real-time RT-PCR validation

To validate our microarray results and to further clarify difference in expression patterns for selected or interesting genes, we carried out real-time RT-PCR with SYBR green-based detection (ABI) using specific primer pairs that were run on an ABI 7700 fluorescent sequence detection system (Perkin-Elmer, Foster City, CA, USA) by using pooled RNA stock from vascular-invasive or non-vascular invasive group.

Cell Culture

Hepatoma cell lines (SK-Hep1, HCC36, Hep3B, HepG2, Huh 7, PLC/PRF5, 59T, Tong) were grown in DMEM medium (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) with 10% fetal bovine serum (FBS) (Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 5 % CO₂–95 % air. Cells were cultured according to the supplier’s recommendations. Adherent cells were detached from the culture dishes with trypsin-EDTA (Sigma, Deisenhofen, Germany).
**RT-PCR**

Reverse transcription of RNA isolated from cells was performed in a final reaction volume of 20μl containing total RNA (5μg) in First Strand Buffer with DTT (10 mM), dNTP (2.5 mM), Oligo (dT) 12-18 primer (1μg) and 200 units /μl Moloney murine leukemia virus reverse transcriptase (200 units). The reaction was carried out at 37 °C for 2 hrs, and was terminated by heating at 70 °C for 10 min. One microliter of the reaction mixture was then amplified by PCR using specific pairs of primers. 5-GATGATGATATCGCCGCGCT-3 (sense) and 5-TGGGTCATCTTCTCGCGGTT-3 (antisense) produced a 320 bp fragment product of the internal control gene: β-actin.

The PCR amplification was conducted in a reaction buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl$_2$, 167 μM dNTPs, 2.5 units of Taq DNA polymerase and 0.1 μM primers. The reactions were performed in a Biometra Thermaoblock (Biometra Inc. Florida, USA) using the following program: denaturing for 1 min at 95 °C, annealing for 1 min at 58 °C and elongating for 1 min at 72 °C for a total of 25-35 cycles according to the expression level; the final extension took place at 72 °C for 10 min. Equal volumes of each PCR sample were subjected to electrophoresis on a 1 % agarose gel, which was then stained with ethidium bromide and photographed under UV illumination.

**Western Blotting**

Proteins in the total cell lysate (40 μg of protein) were separated by SDS-PAGE in 10 % gels and transferred to a polyvinylidene difluoride membrane (Immobilon-P membrane; Millipore Corp., Bedford, MA) by electrotransfer. After the blot was blocked in a solution of 5 % skim milk, 0.1 % Tween 20, and PBS, membrane-bound proteins were probed with specific primary Abs and β-actin(Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was washed, and then incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min. Enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) were employed to depict the protein bands on membranes. This light is captured on Kodak X-OMAT Blue Autoradiography film.
**siRNA experiments**

Huh7 cells were used for siRNA transfection. Cells were plated at 2.3x10^5 cells per well in a 24-well tissue culture plate. Following 24 h in culture, cells were transfected with 40 nM LECT2 siRNA (Ambion siRNA ID 11330, and 11515), or SilencerTM Negative Control #1 siRNA using 8μl per well siPORT Amine Transfection Agent (Ambion) according to the manufacturer’s protocol. Forty eight hours after transfection, cells were used for migration, and invasion assay. And the cells were also harvested for protein and total RNA. LECT2 mRNA levels were measured by RT-PCR with 26S rRNA as the internal standard. All experiments were carried out in triplicate.

**LECT2 gene cloning**

HepG2 poly(A)^+ RNA was used as template for the synthesis of single-stranded cDNA. Degenerated 30-mer oligonucleotide were designed with sense 5'- TAA, AAC, CAA, AGC, TTT, TCC, ACC, AAA, GCC, CTC -3’, and anti-sense 5'- TTG, GCC, TTC, TCG, AGA, CAG, GTA, TGC, AGT, AGG -3’. These primers were subsequently applied in a RT-PCR reaction. The PCR product of the expected size was gel purified, and cloned into the pSecTag2/HygroA vector (Invitrogen) and sequenced.

**Transfection and established stable clone cells**

Human hepatoma cell lines were grown in DMEM medium (Invitrogen Corp., Carlsbad, CA), and culture media were supplemented with 10% fetal calf serum (FCS) (Bioserum, Victoria, Australia), 2mM l-glutamine, 100μg/mL streptomycin, and 100 units/mL pencillin (Invitrogen Corp.). Cell cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere. Adherent cells were detached from the culture dishes with trypsin-EDTA (Sigma Chemical Co.), and passage into new medium every 2-3 days before confluence. HUVEC were isolated from 2-5 umbilical cord veins, pooled, and established as primary culture in M199 containing 20% FCS. HUVEC were serially passaged (1:3 split ratio) and maintained in M199 contained 10% FCS, endothelial cell growth factor, porcine intestinal heparin, and antibiotics.
The expression vector LECT2 was constructed by placing the human LECT2 cDNA in the pSecTag2A eukaryotic expression vector containing the hygromycin B gene under the control of the same promoter. The LECT2-sense expression constructs were transfected into hepatoma cells using Lipofectin reagent (Invitrogen Life tech.). Stable cell populations were selected by 50μg/mL hygromycin B, and singles clones were confirmed to have prominent expression of LECT2 by RT-PCR and Western blotting analysis.

**Boyden Chamber Assay**

Invasion assays were performed using modified Boyden chambers with filter inserts for 24-well dishes containing 8-μm pores (Nucleopore Corp., Pleasanton, CA). Matrigel (40 μg; Collaborative Biomedical, Becton Dickinson Labware) coated filters were used for invasion assays. Cells (2.5×10^4) were plated into 100 μl of complete DMEM in the upper chamber, and the lower chamber was filled with 1 ml of DMEM. After 48 hr in culture, cells were fixed in methanol for 15 min and then stained with 0.05 % crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed in PBS. Cells on the underside of the filters were viewed and counted by microscopic inspection. Each clone was plated in triplicate per experiment, and each experiment was repeated at least three times.

**Transendothelial migration assay**

HCC36/Neo and HCC36/LECT2 stable transfect cells were labeled with calcein AM (Molecular Probes, Eugene, OR) 4μg/mL at 37°C for 30 mins in calcein labeling buffer, cells were then washed twice with HBSS, and resuspended. Calcein AM-labeled cells (2×10^4 cells) were added to confluent HUVEC-coated wells in upper chamber of the transwell and incubated with medium, and allowed to migrate for 18 hours at 37°C incubator. Nonadherent cells were removed by washing HBSS three times, and cells on the upper side of the filters were removed with cotton-tipped swabs. The fluorescence was quantitated with Millipore fluorescence plate reader (Millipore Corp., Bedford, MA) using an excitation wavelength of 485 nm and an
emission wavelength of 530 nm. Each experiment was performed by three times, and the values were reported as the means ± SD.

**CAM assay**

The CAM (chorioallantoic membrane) assay was done according to Kim *et al.* (1998), except for a few modifications. 1x10^6 cells (HCC36) with or without LECT2 overexpression were inoculated onto a CAM of 10-day-old chick embryos. After 1, 2, 3, 7 days of incubation, the CAMs lining the cavity of the lower eggshell were used for the extraction of genomic DNA. The PCR produced an Alu-band of 224 bp. The bands from three separate experiments were quantitated by densitometric scanning. Control band intensity was assumed to be 100%; other intensities were compared with that.

**Animal and Subcutaneous Xenograft Models**

Eight-week-old female athymic nude mice (supplied by the animal center of the National Defence Medical Center) were housed under pathogen-free conditions and fed a diet of animal chow and water throughout the experiment. Mice randomly assigned to one of three groups (20 per group) were injected subcutaneously with LECT2-overexpression stable transfected SK-hep1, BNL cells. Tumors were measured every other day, starting on day 10 post injection when they had become palpable and visible. Tumor volumes were calculated using the equation: width^2 x length x 0.5. Thirty-two days after the injection, the subcutaneous tumors were excised, weighed and photographed, and a portion of each was placed in 10% formalin for paraffin embedding or snap-frozen in Optimum Cutting Temperature (OCT) solution (Miles, Elkhart, IN) in preparation for subsequent immunohistochemical analysis.

**Immunohistochemistry**

Tissue sections for immunostaining were obtained from formalin-fixed and paraffin-embedded primary tumors removed from hepatocellular carcinoma patients or frozen primary tumors produced in mice by subcutaneous injection of hepatocellular carcinoma cell lines. After three washes in PBS, the samples were
treated with goat anti-mouse IgG biotin-labeled secondary antibodies (Vector Laboratories, Inc., Burlingame, CA) at a dilution of 1:500 for 1 h at room temperature. Bound antibodies were detected using an ABC kit (Vector Laboratories Inc.). The slides were stained with diaminobenzidine, washed, counterstained with Delafield's hematoxylin, dehydrated, treated with xylene, and mounted. To quantify the angiogenesis, microvessel density was determined by staining tissue sections immunohistochemically for the pan endothelial cell antigen. Three highly vascularized areas per tumor were then evaluated at high magnification (200x). The total number of microvessels was determined for each area, and the average number recorded for each tumor.

**Immunoprecipitation and Western Blot Analysis**

The cellular lysates were prepared as described previously, and equal amounts of protein were incubated with specific antibody immobilized onto protein A-Sepharose for 2 h at 4 °C with gentle rotation. Beads were washed extensively with lysis buffer, boiled, and microcentrifuged. Proteins were resolved on SDS-PAGE and transferred to nitrocellulose membrane. After blocking, blots were incubated with specific primary antibodies. After washing and incubating with secondary antibodies, immunoreactive proteins were visualized using enhanced chemiluminescence detection (Amersham, Arlington Heights, IL). Where indicated, the membranes were stripped and reprobed with another antibody.

**Statistical Analysis**

Group comparisons were conducted using the chi-square test for categorical measures. Univariate logistical regression analysis was conducted initially to examine the level of LECT2 expression associated with various risk factors using the PROC LOGISTIC of the SAS. For those variables exhibiting a significant association in univariate logistical regression analysis, multiple logistical regression analysis was then employed. The survival probabilities after tumor removal between different strata were compared with log-rank test using PROC LIFETEST. Variables that had a significant effect in the univariate analysis were further examined by using Cox proportional hazards regression analysis as implemented in the PROC PHREG. P-values less than 0.05 were considered statistically significant.
**Results**

**Oligonucleotide microarray and RT-PCR results**

We used high throughput technology suppression subtractive hybridization (SSH) and oligonucleotide microarray of human membrane/secreted proteins (TMSEC microarray), there were twenty genes fulfilled the four selection criteria as described in methods. Eight genes were up-regulated in vascular-invasive HCCs and non-vascular invasive HCCs, and 12 genes were down-regulated in vascular-invasive HCCs. All these genes were listed in table 2. RT-PCR procedure was used to compare the amount of RNA in the pooled RNA sample of the vascular-invasive patients with that of the non-vascular invasive patients with primers of these selected genes.

**LECT2 suppressed cell invasiveness and transendothelial migration of HCC cells**

We found that the correlation between LECT2 expression and invasiveness in HCC cell lines. SK-Hep1, TONG, and HCC36 cells were highly invasiveness, and nearly no LECT2 level. To using 293T/Neo cells' conditioned media treated with HCC36, Hep3B, and HuH-7 cells, cell invasiveness were conspicuous decreased afetr 18 hours.

To further characterize the invasiveness suppressor function of LECT2 in hepatocellular carcinoma, in this study, we established a cellular model in which LECT2 cDNA stable expressed in HCC36 cells that lack endogenous LECT2 expression. The ectopic expression of LECT2 in stably transfected cells was confirmed by semiquantitative RT-PCR and Western blot analysis. To determine the effect of LECT2 on cell proliferation, we monitored the proliferation rate of LECT2 stably expressing HCC36 cells for 4 days in complete medium. We observed that HCC36/LECT2 were no different cell proliferation rate with HCC36/Neo cells.

The capability of invasion and migration is an important indicator of malignant transformation. We compared the invasion ability of HCC36/LECT2 and HCC36/Neo cells in complete medium culture. As expected, the LECT2 stably transfected cells exhibited a dramatically reduced ability to invasion on matrigel coated transwell. These data indicated that the reduction of cell invasiveness was not due to the different proliferation rate. Next, we determine the effect of LECT2 expression on the
transendothelial migration assay. The addition of HUVECs to the transwell model decreased the level of HCC36/LECT2 migration to around than HCC36/Neo cells.

**LECT2 suppressed cell intravasation ability of HCC cells in CAM assay**

The chorioallantoic membrane (CAM) model has long been used for the investigation of angiogenesis and oncogenesis [9]. Otherwise, many innovative studies [10] have established the chicken embryo as a useful model for the investigation of tumor cell metastasis. The ability of tumor cells to intravasation from primary tumor into the lower CAM. To assess the tumor suppressor function of LECT2, we compared the intravasation ability of HCC36/Neo and HCC36/LECT2 cells in CAM assay. We observed that human specific Alu repeat sequence detection by HCC36/Neo cells in the low CAM was abundant than HCC36/LECT2 cells. Our findings suggest that ectopic expression of LECT2 is sufficient to suppress invasion, transendothelial migration, and intravasation ability in HCC36 cells.

**LECT2 protein expression in tumorous liver tissues from HCC patients**

Representative results of the protein by Western blot analysis in Figure 7A. All non-vascular invasion tumors showed highly expression of LECT2 than vascular invasion tumor in liver tissues. Results of correlation of LECT2 expression with vascular invasion of HCC patients in Figure 7B.

**LECT2 expression affects neovascularization in HCC tumors**

To begin to explore the potential role of LECT2 in tumor angiogenesis, we stably transfected LECT2-overexpressing vector into the hepatocellular carcinoma cell lines, SK-hep1, and transfected small interference LECT2 (shLECT2) RNA-expressing vector into the hepatocellular carcinoma cell lines, Huh-7. Based on the results of this earlier work, it appears that SK-hep1 cells has almost zero level of endogenous LECT2 protein, while Huh-7 cells exhibit strong expression of LECT2. After hygromycin B or G418 selection, we obtained the LECT2-overexpressing cells, and the shLECT2-expressing Huh-7 cells significantly decreased the level of endogenous LECT2 protein as compared to the vector control cells. The *in vitro* growth properties of these transfectants and their vector control cells were then determined by MTT assay, suggesting that overexpression or knock-down of LECT2 level did not affect
tumor cell proliferation. We further investigated the effects of LECT2 on tumor growth in a xenograft tumor model in which mice were injected subcutaneously with these stable clones. Palpable tumors were first detected in all mice by 10 days after the cell injections. At 32 days, mean tumor volumes in mice injected with control cells, such as SK-hep1/neo and BNL/Neo, were significantly larger than in analogs injected with LECT2-transfected clones. Tumor sections were stained for CD31 (PECAM-1), revealing that microvessel density was significantly reduced in the xenograft tumors from the SK-hep1/LECT2 and BNL/LECT2 group as compared to the vector control group. The above animal data suggest that LECT2 overexpression may inhibit tumor growth in primary sites in hepatocellular carcinoma, possibly due to inhibition of tumor angiogenesis. Supportive data from clinical inspection of 14 patients revealed lower microvessel density in human hepatocellular carcinoma with high LECT2-expression level. By contrast, low LECT2 analogs had higher density.

**Effects of conditioned media of LECT2-transfected cells on angiogenesis**

We used *in vitro* assays to examine the role of LECT2 in microvascular endothelial cell migration and tube formation, assembly and alignment, which are involved complex process of angiogenesis. We subjected monolayers of HUVEC cells to a wound healing assay, and quantized the numbers of cells that had migrated into wounded zone after incubation for 48 hrs in 50% conditioned media (CM) of these LECT2 transfectants CM. LECT2 CM inhibited HUVEC cells migration significantly, and CM of silence endogenous LECT2 expression in Huh-7 cells increased HUVEC cells reparative response.

In summary, these findings indicated that LECT2 suppress the angiogenesis of HCC cells and suggest the feasibility of LECT2 expression as a promising prognostic molecular marker for HCC.
Discussions

LECT2 was originally noted for its possible neutrophil chemotactic activity [11]. In addition, it was independently reported to be a growth-stimulated factor for chondrocytes and osteoblasts, and was named chondromodulin II [12]. Using SSH and microarray, we found that expression of twenty genes significantly difference in vascular-invasive HCC patients compared with the non-vascular invasive. LECT2 is one of down-regulated genes in HCC patients with vascular invasion. To determine the function of LECT2 in HCC, we generated stably expressed LECT2 cDNA in HCC36 cells. Expression of LECT2 significantly suppressed cell invasion, transendothelial migration, intravasation ability and angiogenesis in HCC cells.

In screening of several HCC cell lines, it seems that the higher invasiveness, the lower of LECT2 gene expression in the HCC cell line shown in the following preliminary data. (Fig.2.) Next, we will clone LECT2 gene into pSecTag2/HygroA plasmid from HepG2 cell line (high LECT2 gene expression cell) or normal liver tissue. Then we will transfect vector or LECT2 plasmid into hepatoma cell lines, and select the stable clones. (HCC36, and SK-Hep1; these two cell lines were LECT2 low-expression cell lines) Then we will compare the functional influence of LECT2 with or without over-expression in these cell lines with evaluating the proliferation, migration, and invasion ability. We also will transiently transfec the plasmid into 293T cell, and collect the conditioned medium with high secreted LECT2 protein amount. And we will compare the functional influence of HCC36 or SK-Hep1 cells treated with high LECT2 conditioned medium or controlled conditioned medium.

This study performed transendothelial migration assay with HUVEC put on the upper side of Boyden chamber to mimic the situation of vascular invasion of cancer cell in vivo. And we compared the effect of LECT2 by using LECT2 with/without overexpression HCC cell lines (SK-Hep1/neo, SK-Hep1/LECT2).

We foremost found that LECT2 negatively regulation the cell invasion by inhibited phosphorylation of ERK1/2 protein. Tumor invasion and metastasis are multistepped and complex processes that include cell division and proliferation, proteolytic digestion of the extracellular matrix, cell migration through basement membranes to
reach the circulation system, and remigration and growth of tumors at the metastatic sites [38, 39, 40].

To analyze statistically the relationship of the expression level of LECT2 gene in HCC with the clinicopathologic factors of these patients. Our preliminary data revealed that a significant difference of LECT2 gene and protein expression levels between vascular-invasive and non-vascular invasive HCC. For this aim, we collected more surgical specimen of HCC or using the tissue samples from tissue bank. About 100 tumors were collected if it was primary, resected, unifocal HCC. None of these patients had received transhepatic arterial embolization or chemotherapy before surgery. Detailed pathologic assessment, and regular follow-up will be performed. All the clinicopathologic factors and prognostic outcome will be collected. The gene expression levels of LECT2 in hepatic tumors will be statistically analyzed with clinicopathologic factors and outcomes such as age, sex, HBsAg status, HCV, AFP level, tumor size, grade, stage, recurrence, overall survival, p53 mutation, beta-catenin mutation status.

In conclusion, our findings thus far have indicated that LECT2 significantly inhibited Met-downstream pathway mediated invasion ability. Expression of LECT2 suppressed cell invasion, transendothelial migration, intravasation and angiogenesis of hepatocellular carcinoma cells.
<table>
<thead>
<tr>
<th>Group</th>
<th>A (vascular invasion)</th>
<th>B (non-vascular invasion)</th>
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<tbody>
<tr>
<td>Sex (men:woman)</td>
<td>3:1</td>
<td>4:1</td>
</tr>
<tr>
<td>Age (mean)</td>
<td>50 y/o</td>
<td>54 y/o</td>
</tr>
<tr>
<td>Number</td>
<td>Single</td>
<td>Single</td>
</tr>
<tr>
<td>Size (mean)</td>
<td>1.5-4.7(2.9) cm</td>
<td>2.8-3.8(3.2)cm</td>
</tr>
<tr>
<td>Capsule</td>
<td>no capsule</td>
<td>no capsule</td>
</tr>
<tr>
<td>Grade</td>
<td>Grade2 4 patients</td>
<td>Grade2 5 patients</td>
</tr>
<tr>
<td>TNM</td>
<td>T3N0M0</td>
<td>T1N0M0</td>
</tr>
<tr>
<td>Stage</td>
<td>IIIA</td>
<td>I</td>
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Table 2. The selected differentially expressed genes between vascular-invasive and non-vascular invasive HCC patients.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession No.</th>
<th>Abbreviation</th>
<th>Mean ratio</th>
<th>log2</th>
<th>RT-PCR</th>
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<tr>
<td><strong>Down-regulated genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>transmembrane 4 superfamily number 4</td>
<td>NM_004617</td>
<td>TM4SF4</td>
<td>0.0286</td>
<td>44</td>
<td>Y</td>
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<tr>
<td>transmembrane 4 superfamily number 3</td>
<td>M35252</td>
<td>TM4SF3</td>
<td>0.0297</td>
<td>44</td>
<td>Y</td>
</tr>
<tr>
<td>occular development-associated gene, RHOEEM052</td>
<td>NM_021167</td>
<td>ODAG</td>
<td>0.0369</td>
<td>44</td>
<td>Y</td>
</tr>
<tr>
<td>LOC222189, mRNA</td>
<td></td>
<td></td>
<td>0.0402</td>
<td>44</td>
<td>failed</td>
</tr>
<tr>
<td>leukocyte cell-derived chemotaxin 2</td>
<td>AB017056</td>
<td>LECT2</td>
<td>0.0406</td>
<td>44</td>
<td>Y</td>
</tr>
<tr>
<td>regenerating islet-derived 1 alpha (pancreatic stone protein)</td>
<td>NM_002909</td>
<td>RISP1A</td>
<td>0.0451</td>
<td>44</td>
<td>Y</td>
</tr>
<tr>
<td>putative membrane protein LCCS4499</td>
<td>NM_019026</td>
<td>LOC54499</td>
<td>0.0641</td>
<td>34</td>
<td>Y</td>
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<tr>
<td>ADP-ribosyltransferase 4</td>
<td>X93826</td>
<td>DO</td>
<td>0.0731</td>
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<td>Y</td>
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<tr>
<td>apolipoprotein M</td>
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<td>APOM</td>
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<tr>
<td>SUMO/Ossanin specific proteases family member 3</td>
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<td>SENP3</td>
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<td>no difference</td>
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<tr>
<td>serum amyloid A4, constitutive</td>
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<td>SAA4</td>
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<tr>
<td>cadherin 17, LI cadherin (liver-intestine)</td>
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<td>CDH17</td>
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<td>failed</td>
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<td><strong>Up-regulated genes</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CD34 antigen</td>
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<td>CD34</td>
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<td>no difference</td>
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<tr>
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<td>HBG</td>
<td>19.0991</td>
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<td>no difference</td>
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<tr>
<td>bladder cancer associated protein</td>
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<td>BLCAP</td>
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<tr>
<td>Osteopontin</td>
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<td>SPP1</td>
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<tr>
<td>insulin-like growth factor 1 (somatomedin C)</td>
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<td>IGFB1</td>
<td>20.3854</td>
<td>702</td>
<td>Y</td>
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<tr>
<td>interferon, alpha-inducible protein 27</td>
<td>X67325</td>
<td>IFI27</td>
<td>67.4533</td>
<td>88</td>
<td>Y</td>
</tr>
<tr>
<td>BC007038, Homo sapiens amyloid P component</td>
<td>BC007038</td>
<td>APCS</td>
<td>95.0584</td>
<td>34</td>
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Figure 1. An overview of experimental design. Combining two-way (Forward and Reverse) SSH and microarray to identify differentially expressed genes between vascular-invasive and non-vascular invasive HCCs. SSH: suppression subtractive hybridization. TMSEC microarray: Oligonucleotide microarray with probes of transmembrane and secreted protein-related genes.
Figure 2. Analysis of LECT2 mRNA expression and invasiveness in HCC cell lines.

A: RNA were isolated from 8 HCC cell lines and normal liver tissue, cDNA synthesized and subjected to RT-PCR for analysis of LECT2 and S26 expression. B: Ability of invasiveness on 8 HCC cell lines. Cells (1×10^5) were plated on the matrigel-coated chamber and incubated with conditioned media. After 18 hours, cells were fixed with methanol and stained with 0.05% crystal violet. The invaded cells were counted from 40X microscopic fields. The assay were performed in duplicate, and the results represent mean values ± SD from three independent experiments.
Figure 3. Effect of invasiveness on HCC cell lines treated with 293T/neo or LECT2 conditioned media. LECT2 transient transfect 293T cells, after 48 hours, the conditioned media (serum-free) were harvested for HCC cell lines culture. HCC cells (1×10^5) were plated on the matrigel-coated chamber and incubated with conditioned media. After 18 hours, cells were fixed with methanol and stained with 0.05% crystal violet. The invaded cells were counted from 40X microscopic fields. The assay were performed in duplicate, and the results represent mean values ± SD from three independent experiments.
Figure 4. Analysis of cell proliferation in HCC36/Neo and HCC36/LECT2 cells.
A: Stable expression of LECT2 in HCC36 cells. Left, Western blotting; right, RT-PCR.
B: Cells (3×10^3) were palted in 96-well, and cultured for 24, 48, 72, 96 hours. Using 50 μg MTT, the color reaction was quantified with an ELISA plate reader.
Figure 5. Stable expression of LECT2 inhibited invasiveness and transendothelial migration ability in HCC36 cells.

A: Invasiveness of the HCC36/neo and HCC36/LECT2 cells were assayed with matrigel-coated invasion chamber. Invasive cells were stained, photographed, and counted under microscope.

B: Ability of HCC36/neo and HCC36/LECT2 cells transendothelial migration. Endothelial cells were full confluence pre-seed in upper chamber of transwell for 18 hours, HCC cells were incubated above the endothelial cells and allowed to transendothelial migrate toward the lower chamber. Migrate cells were photographed, and counted under fluorescent microscope.
Figure 6. Stable expression of LECT2 inhibited intravasation in the chick embryo model. Detection and quantization of HCC36/Neo and HCC36/LECT2 cells spontaneous metastasis. The indicated cells ($2 \times 10^4$) were suspended in serum-free DMEM in upper CAM and allowed to intravasate for 1-5 days. PCR amplification of Alu sequence in mixture of human and chicken CAM DNA from lower CAM.

Figure 7. LECT2 expression correlation with vascular invasion tumor in HCC patients. A: Western blot analysis for LECT2 expression in tumorous liver tissues of HCC patients. 293T/LECT2 cell lysate was used as the positive control. B: Correlation between LECT2 expression and vascular invasion tumor in HCC patients.
Figure 8. Effect of LECT2 in microvessel density expression of HCC sections.

Figure 9. Endogenous LECT2 inhibited tube formation in HCC cells conditioned medium.

Figure 10. LECT2 inhibited tube formation in HCC cells conditioned medium.
Figure 11. LECT2 inhibited SK-hep1 cells of tumor growth on Nude mice.

Figure 12. LECT2 inhibited mouse BNL cell of tumor growth on BALB/c mice.

Figure 13. LECT2 inhibited HUVEC migration in HCC cells conditioned medium.
Figure 14. LECT2-conditioned medium inhibited angiogenesis in CAM assay.

Figure 15. Purification of recombinant hLECT2 proteins.
References


