

Characterization of hepatocellular carcinoma cell lines based on cell adhesion molecules

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Abstract. Many studies which focus on the molecules and mechanisms related to the characteristics of the cancer have been performed. In particular, cell adhesion molecules (CAMs) are known to play a central role in the adhesion of cancer cells to vascular endothelial cells. In this study, the expression of CAMs in hepatocellular carcinoma (HCC) cell lines was analyzed and correlated with the characteristics of various HCC cell lines. Eight human HCC cell lines were used in this study. We analyzed the expression of ICAM-1, E-selectin and the integrin subunits of HCC cell lines by western blot analysis and ELISA kit. We estimated the expression of integrin- $\alpha 5$ using western blot analysis and RT-PCR to compare the expression at the gene level with the protein level. In addition, we determined the expression of TGF- $\beta 1$, as one of the markers for the cellular activity compared to the levels of expression with the expression of integrin- $\alpha 3$ and - $\alpha 5$. ICAM-1 was highly expressed in all of the cell lines except SNU398 and Hep3B, which exhibit a more aggressive nature among the studied HCC cell lines. E-selectin and integrin subunits varied in all HCC cell lines. In particular, integrin- $\beta 2$ was highly expressed on all HCC cell lines. In conclusion, the levels of expression of the CAMs may not affect cellular activity, morphology or tumorigenicity. However, most HCC cell lines show various expressions of CAMs, suggesting that HCC cell lines expressing the major CAMs remain candidates for molecular targeted therapy, which may need to be patient-tailored for therapy according to the molecular profile.

Introduction

Hepatocellular carcinoma (HCC) is an aggressive tumor that frequently occurs in the setting of chronic liver disease and

cirrhosis. Even though surgical resection is the treatment of choice, HCC is usually discovered under unresectable condition due to underlying liver disease and/or late diagnosis from the painless and slow growing nature. At the time of presentation less than 40% of patients in the world, fulfill criteria for curative treatment (resection, transplantation, local ablation) and only 20% are eligible for chemoembolization (1,2). Thereby, overall prognosis of HCC is significantly poor and in fact, it is a 3rd leading cause of cancer related death worldwide, resulting in more than 660,000 deaths/year, even though HCC is the 5th most common malignancy (3). A number of treatment modalities such as transcatheter arterial chemoembolization (TACE), selective internal radiation therapy (SIRT), radiofrequency ablation (RFA), percutaneous ethanol injection (PEI) and systemic chemotherapy, have been performed on patients with unresectable HCC (4-6). For patients with advanced unresectable HCC who are unsuitable for locoregional therapy, systemic chemotherapy is the only applicable treatment. However, HCC has been considered to be a relatively chemotherapy-refractory tumor (7,8). Recently, molecular target therapy which is focused on the typical nature of the cancer, discriminated from normal cell, has been raised as one of the options for the treatment of HCC. In practice, sorafenib which is a multi-targeted orally active small molecule tyrosine kinase inhibitor (TKI) is established as new standard systemic treatment for advanced HCC (9). In this regard, many studies on the relation of cancer cells and molecules in tumor invasion and metastasis have been performed. Especially, cell adhesion molecules such as cadherin, selectin, integrin and immunoglobulin superfamily (IGSF) are known to have the central role in cancer cells relating to the adhesion with vascular endothelial cells (10,11). Although the role and clinical usefulness of adhesion molecules are being investigated in many cancer cell lines, reports on its roles in HCC cell lines are limited. In this study, the expression status of the adhesion molecules in HCC cell lines is analyzed and any correlations between characteristics (tumorigenicity, morphology, etc.) of HCC cell lines and CAM expression, as well as CAMs themselves were investigated.

Materials and methods

Cell lines and culture methods. The human hepatoma cell lines (SNU-354, SNU-368, SNU-398, SNU-449, SNU-739,

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SNU-886, HepG2, Hep3B) were used. They (SNU-354, SNU-368, SNU-398, SNU-449, SNU-739, SNU-886, Hep3B) were grown *in vitro* in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 U/ml penicillin. HepG2 cell line was grown in DMEM medium supplemented with 10% heat-inactivated FBS, 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 U/ml penicillin. All of cell lines were incubated at 37°C in a humidified atmosphere containing 20% O₂ and 5% CO₂ in air referred to as the normoxic condition.

Immunoassay of AFP and transforming growth factor (TGF)- β 1. Human hepatoma cell lines (1x10⁶ cell) were cultured for 24 h in media. Cell culture supernatant was assayed for levels of AFP and TGF- β 1. Cell supernatants media were collected for measurement of total amounts of AFP with Quantikine ELISA kits (R&D Systems, MN, USA) and TGF- β 1 with 96-well Enzyme Immunoassay kit (Assay Designs, PA, USA). According to the manufacturer's instructions, to measure the amounts of total TGF- β 1, AFP was present in the conditioned media. The samples for measuring the amounts of total TGF- β 1 were activated by mild acid treatment to convert any latent TGF- β 1 to the active form, and then ELISA was performed.

Protein extract and western blot analysis for the measurement of E-selectin, ICAM-1 and integrin- α 5. For protein isolation, 5x10⁵ cells were solubilized in 500 μl chilled RIPA buffer (ELPIS-Biotech, Taejeon, Korea). Samples were centrifuged at 13,000 rpm for 5 min and the supernatant were used. The protein concentrations were determined using a Pierce Protein Assay Regent (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA). Each protein sample was mixed with Laemmli's sample buffer (Bio-Rad Laboratories, Hercules, CA, USA)

For western blotting, total protein (15 μg) was run in each lane of an SDS-PAGE gel. After completion of gel electrophoresis, protein was transferred to a nitrocellulose membrane (PROTRAN; Whatman, Dassel, Germany) over 2 h using a blotting apparatus at 4°C. The membranes were blocked for 30 min in a blocking buffer containing Tris-buffered saline (TBS) and 5% skim milk, incubated with each specific primary antibody at 4°C overnight. The blots were probed with E-selectin (1:1,000, Biovision), ICAM-1 (1:1,000, Santa Cruz Biotechnology, Inc.), integrin- α 5 (1:1,000, Cell Signaling Technology). The membranes were rinsed for 20 min with washing buffer and re-incubation for 1 h at room temperature in blocking buffer with HRP-conjugated secondary antibody (1:2,500, Santa Cruz Biotechnology, Inc.), and then washed with TBS-T for 30 min. The wash buffer containing TBS, and 0.2% Tween-20 was changed every 5 min during primary and secondary washing procedures. Immunoreactive proteins were visualized using the ECL Plus Western Blotting Detection Reagent (Amersham Biosciences, Pittsburgh, PA, USA). Optical density analysis of proteins was performed with the Image-J software.

Measurement of integrin- α subunits. The relative levels of human integrin- α 2, - α 3, - α 5 on the surface of cells were measured by InnoCyte™ α 2, α 3, α 5 Integrin Detection kit (Calbiochem, EMD Chemicals, Inc., Darmstadt, Germany). Measurement of integrin- α 2, - α 3, - α 5 was used according to the manufacturer's instructions. We used cell density between

250,000 and 500,000 cells/ml. Measuring the fluorescence of the sample using a fluorescence plate reader at excitation wavelength of ~485 nm and an emission wavelength of ~520 nm.

Measurement of integrin- β subunits. Cell adhesion arrays were performed using Beta Integrin-Mediated Cell Adhesion Array kit (Chemicon, USA). Beta Integrin-Mediated Cell Adhesion Array kit used mouse monoclonal antibodies generated against human β integrins/subunits (β 1, β 2, β 3, β 4, β 6, α V β 5 and α 5 β 1), that are immobilized onto a goat anti-mouse antibody coated microtiter plate. Cell adhesion assays was carried out as previously described. Briefly, the plates were rehydrated with PBS per well for 10 min at room temperature before use. Common total cells were used 1x10⁶ cells/ml. Cells were collected and resuspended in Assay Buffer. Then 100 μl of cell suspension were added into each substrate coated well, and plates were incubated for 2 h at 37°C in 5% CO₂ incubator. Unattached cells were gently washed away with Assay Buffer. The attached cells were stained with cell stain solution for 5 min, destained with distilled water, solubilized in extraction buffer to each well and quantified the absorbance at 560 nm by the micro-plate reader.

RNA isolation and semi-quantitative RT-PCR for integrin- α 5. To quantify adhesion molecules mRNA generation, cDNA samples were analyzed by semi-quantitative reverse-transcription PCR. Total-RNAs were isolated from cell culture, using TRIzol (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. For cDNA synthesis, 1 μg of total-RNA in each hybridization sample was used to synthesize the first strand cDNA with RT-PCR system (Promega, Madison, WI, USA). Then 10 μl product was used as the template to amplify specific fragments in a 100 μl reaction mixture under the following conditions: denaturation at 95°C (5 min); 25 cycles of 94°C (30 sec), 52-60°C (30 sec) and 72°C (30 sec); then 72°C extension (7 min). Primers for integrin- α 5 cDNA were 5'-ACCAAGGCCCCAGCTCCATTAG and 3'-GCCTCA CACTGCAGGCTAAATG, yielding an amplification product of 375 bp. β -actin was amplified at the same tube as an internal control. The primers were: 5'-CCCCAGGCACCAGGGCG TGA and 3'-GGTCATCTTCTCGCGTTGGCCTTGGGGT, yielding amplification product of 870 bp. The 10 μl RT-PCR reaction product was analyzed by electrophoresis on a 1.5% agarose gel. Relative mRNA expression was normalized to the expression level of β -actin. Optical density analysis of mRNA was performed with the Image-J software.

Results

HCC cell lines. Previously, eight of the human hepatocellular carcinoma cell lines SNU-354, SNU-368, SNU-398, SNU-449, SNU-739, SNU-886, HepG2, Hep3B had been characterized on the origin, morphology, differentiation, grade, cellular products, protein expression, and tumorigenicity, by centers which had created these cell lines and by other authors. These are summarized in Table I (12-15). The SNU-398 is the only anaplastic cell line in this study and Hep3B made numerous metastases in the lungs in the tumorigenicity study *in vitro*. Otherwise, HepG2 is a noninvasive cell lines. The HBx gene which is known to be associated with the development of HCC in the HBV patients

Table I. General characteristics of HCC cell lines (13-16).

Cell line	Origin	Differentiation (Edmondson-Steiner grade)	Tumorigenicity	HBxDNA detect	α -FP	TGF- β 1
SNU-354	Human HCC Asian male	II-III	No	Yes	Neg	Neg
SNU-368	Human HCC Asian male	II-IV	Yes	Yes	Neg	Pos
SNU-398	Human HCC Asian male	IV (anaplastic)	Yes	Yes	Neg	Neg
SNU-449	Human HCC Asian male	II-III	Yes	Yes	Neg	Pos
SNU-739	Human HCC Asian male	II-IV	No	Yes	Neg	Pos
SNU-886	Human HCC Asian male	II-IV	Yes	Yes	Neg	Pos
HepG2	Hepatoblastoma white male	-	No	No	Pos	Pos
Hep3B	Human HCC black male	-	Yes	Yes	Pos	Pos

HBx, X gene product of the human hepatitis B virus; HCC, hepatocellular carcinoma; α -FP, α -fetoprotein, TGF- β 1, transforming growth factor- β 1; Neg, negative; Pos, positive.

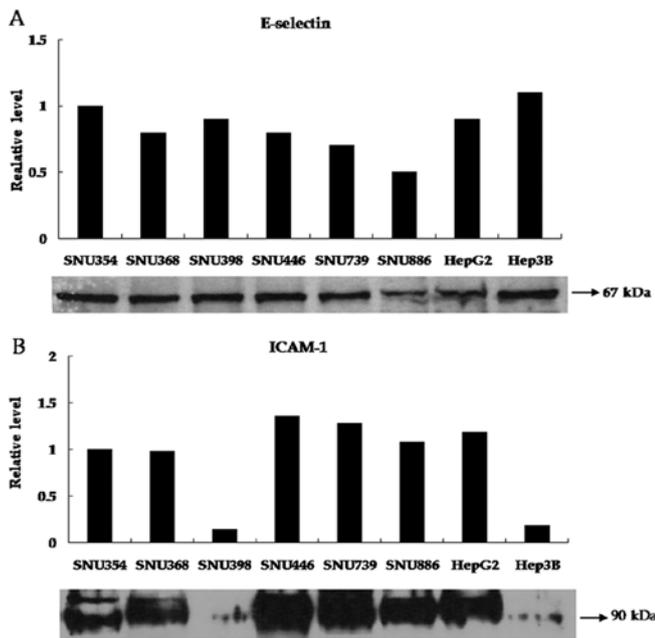


Figure 1. Expression of E-selectin and ICAM-1 in human hepatoma cell lines. (A) E-selectin was equivalently expressed in all HCC cell lines. (B) The ICAM-1 was expressed in all the cell lines as E-selectin, highly increased in SNU449, SNU739, SNU886 and HepG2 but it was very low in SNU398 and Hep3B.

is expressed in all cells line except HepG2, which is derived from the hepatoblastoma patient without HBV infection. SNU-354, SNU-739, HepG2 cell lines are not tumorigenic (15). The AFP and TGF- β 1, which represent the aggressiveness of the cell lines, were determined by immunoassay from the supernatant (16-18). AFP is expressed only in HepG2 and Hep3B. The TGF- β 1 was expressed in SNU-368, SNU-449, SNU-739, SNU-886, HepG2 and Hep3B.

E-selectin expression. E-selectin is expressed on the endothelium and accounts for leukocyte adhesion at inflammation or injury sites in response to inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1. Higher E-selectin levels was detected in patients with breast cancer,

ovarian cancer, GI cancer, lung cancer and colon cancer (19,20). Moreover, E-selectin also are closely correlated with the metastasis of HCC (21).

In this study, E-selectin was equivalently expressed in all HCC cell lines (Fig. 1A).

ICAM-1 expression. ICAM-1 is the transmembrane glycoprotein molecule of the immunoglobulin (Ig) superfamily which is characterized by five distinct Ig-like domains. As receptors of the integrin, it mediates cell-cell interaction and allows for signal transduction (22). ICAM-1 was reported as a significant prognostic factor for patients with HCC (23). The tissue and serum ICAM-1 could indicate the stage of HCC and estimate the potential of hepatoma cells for invasion and metastasis as well (24).

In this study, the ICAM-1 was expressed in all the cell lines as E-selectin, highly increased in SNU449, SNU739, SNU886 and HepG2 but it was very low in SNU398 and Hep3B (Fig. 1B).

Integrin subunits expression. Integrins are cell-surface glycoprotein receptors that are obligate heterodimers containing two distinct chains, the α (18 types) and β (8 types) subunits. They are the most dynamic and versatile of the cell adhesion molecules capable of forming more than 24 known combinations (25,26). Integrins play a significant role in the tumor transformation, tumor growth, invasion, migration, colonization of distant organ and angiogenesis (27,28). Integrin- α 1 β 1 and - α 2 β 1 were reported as the key regulator of HCC invasion (29) and integrin- α 3 β 1 is required for HCC migration and invasion (30).

In our study, we investigated ten types of the integrin subunits and heterodimers (Figs. 2 and 3). Integrin- α 2 expression was high on SNU-368, SNU-449. Integrin- α 3 was highly expressed on SNU-354, SNU-449, Hep3B (Fig. 2). For the integrin- α 5 expression, it increased similarly in SNU354, SNU398, SNU739, SNU886 and Hep3B in western blot analyses. It was somewhat different, but showed mostly similar levels in RT-PCR (Fig. 4).

For the integrin- β subunits, integrin- β 2 was highly expressed on all the HCC cell lines except HepG2. Whereas, expressions of integrin- β 3, - β 4, - β 6 were almost absent in most of cell lines (Fig. 3).

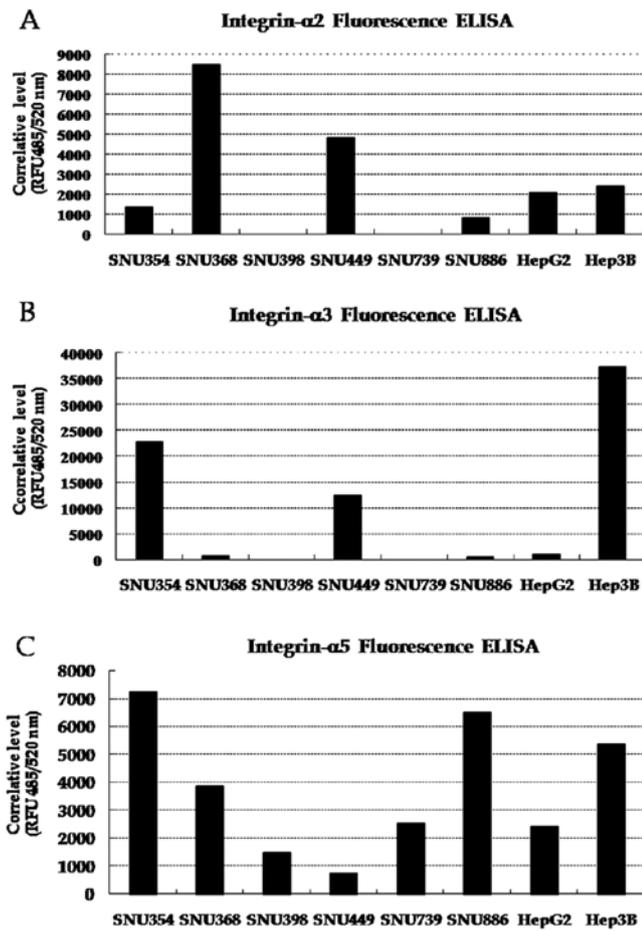


Figure 2. Expression of integrin α subunits. The relative levels of human integrin- α 2, - α 3, - α 5 on the surface of cells were measured by InnoCyte™ α 2, α 3, α 5 Integrin Detection kit. (A) Integrin- α 2 expression was high on SNU-368, SNU-449. (B) Integrin- α 3 was highly expressed on SNU-354, SNU-449, Hep3B. (C) For the integrin- α 5 expression, it increased similarly in SNU354, SNU398, SNU739, SNU886 and Hep3B.

The comparison between TGF- β 1 and integrin- α 3, - α 5 in HCC cell line. TGF- β 1 is a potent cytokine involved in a number of different functions such as epithelial mesenchymal transition, tissue morphogenesis, angiogenesis, and hence tumor progression, invasion and metastasis (31,32). Some studies show the expression of integrin- α 3, - α 5 is stimulated by TGF- β 1 and subsequently it enhances cell adhesion and migration in HCC cells (33-35).

In the present study, we investigated the expression of TGF- β 1 from the supernatant of HCC cell lines using an immunoassay. We found that the TGF- β 1 which is derived from HCC cell itself had no correlation with the expression of integrin- α 3, - α 5 on the HCC cell surface (Figs. 2 and 5).

Discussion

HCC is a most common primary liver tumor which often arises in the setting of underlying chronic liver disease and has a relatively poor prognosis (1-3). Most of the patients are detected as unresectable needing other therapeutic modalities, such as transcatheter arterial chemoembolization (TACE),

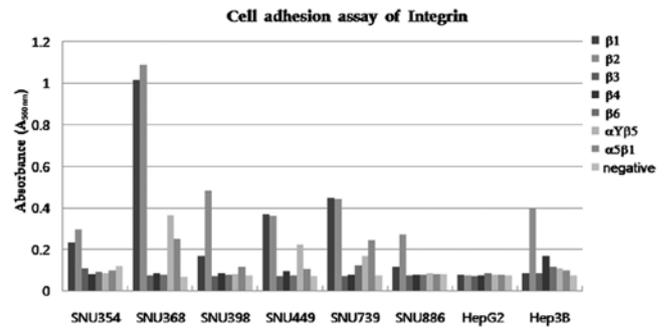


Figure 3. Expression of integrin- β subunits cell adhesion arrays were performed using β integrin-mediated cell adhesion array kit. Bar graph showing the subtype specific integrin expression. Integrin- β 2 was highly expressed on all HCC cell lines except HepG2. Whereas, expressions of integrin- β 3, - β 4, - β 6 were almost absent in most of the cell lines.

selective internal radiation therapy (SIRT), radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), systemic chemotherapy and liver transplantation. For patients with advanced unresectable disease who are unsuitable for locoregional therapy, systemic chemotherapy is the only applicable treatment, even though HCC has been considered to be a relatively chemotherapy-refractory tumor (4-6). In the era of the liver transplantation, HCC recurrence after liver transplantation has been emerged as the hot issue because the recurrence is the most common cause of posttransplant deaths in patients. Unfortunately, most cases of recurrence need systemic therapy, because hematogenous spread is the major route of tumor recurrence after liver transplantation (36,37).

Recently, molecular target therapy which is focused on the typical nature of the cancer discriminated from normal cell, has been raised as one of options for the treatment of neoplastic diseases (38). HCC is a highly vascularised tumor and express high levels of vascular endothelial growth factor (VEGF) on the surface of the tumor cell which makes vascular targeting approaches appealing for the treatment of HCC (39,40). Use of sorafenib, which is a multi-targeted orally active small molecule tyrosine kinase inhibitor (TKI), is a typical example of the molecular target therapy for HCC. The multicenter European randomized Sorafenib Hepatocarcinoma Assessment Randomized Protocol (SHARP) trial demonstrated a modest, though statistically significant, survival benefit for sorafenib over supportive care alone in patients with advanced HCC. These data established sorafenib monotherapy as the new reference standard systemic treatment for advanced HCC (9).

In general, tumors are aggressive, with high metabolic rate and can invade surrounding healthy tissues and spread elsewhere in the body while the normal cell is tightly controlled by a variety of genetic, local and hormonal controls. Besides, invasive cells are less adhesive, more highly mobile than normal cells. During tumor progression, cell adhesion molecules (CAMs) have been reported to be related to the critical aspects in tumor invasion and metastasis involving adhesive interactions between tumor cells and between tumor and normal cells in the stroma (10,11). In this regard, many studies on the relation of cancer cells and cell adhesion molecules in tumor invasion and metastasis have been performed. However, the mechanisms

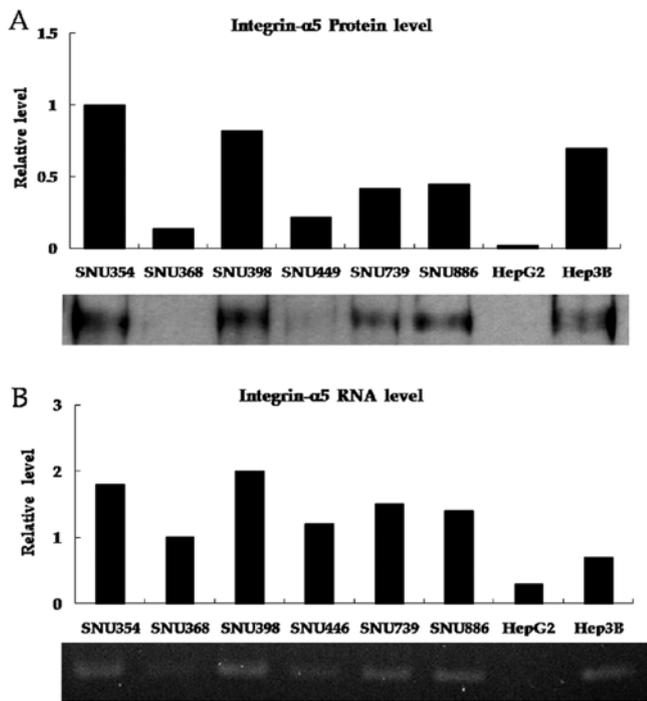


Figure 4. Comparison of integrin- α 5 expressions. (A) The expression of integrin- α 5 was estimated with western blot analysis and (B) with RT-PCR. The expression was somewhat different but showed mostly similar levels at the protein and the gene level.

of CAM responsible for tumor invasion and metastasis are not completely understood.

Cell adhesion molecules are cell surface proteins that account for cell-to-cell or cell-to-ECM interactions and are divided into four major groups: cadherins, integrins, selectins, and immunoglobulin superfamily (11). Here we investigated the expression status of the adhesion molecules in HCC cell lines and any correlations between the characteristics of HCC cell lines and CAMs expressions, as well as CAMs themselves. Consequently, this study might provide the basic frame for further studies.

E(endothelium)-selectin. The selectin family consists of single chain transmembrane glycoproteins that share similar properties to C-type lectins due to a related amino terminus and calcium-dependent binding. There are three subsets of selectins, E(endothelium), L(leukocyte), P(platelet)-selectin which are >60% identical in their structures (41). E-selectin, also named as CD62E, endothelial-leukocyte adhesion molecule (ELAM)-1 is expressed on the endothelium and accounts for leukocyte adhesion at inflammation or injury sites in response to inflammatory cytokines, such as TNF- α , IL-1, nuclear factor (NF)- κ B, and activator protein (AP)-1 (42-44).

In the tumors, E-selectin seems to be involved in the capturing of circulation tumor cells and organ-specific tumor spreading. Many studies show alterations in the expression of selectins and/or their ligands in several malignancies. Higher E-selectin levels were detected in patients with breast cancer, ovarian cancer, GI cancer, lung cancer and colon cancer (19,20). Moreover, the inhibition of E-selectin expression correlates with inhibition of metastasis (45,46). For the HCC,

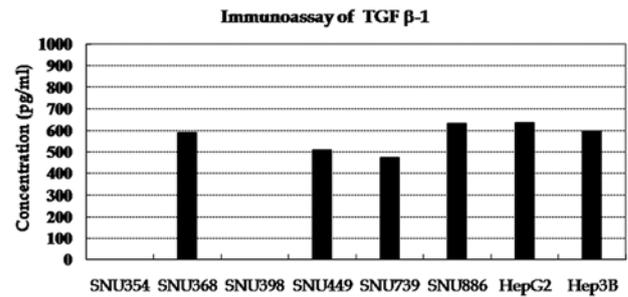


Figure 5. Expression of TGF- β 1. (A) Cell supernatants media were collected for measurement of total amounts of TGF- β 1 with 96-well Enzyme Immunoassay kit. We found that the TGF- β 1 which is derived from HCC cells had no correlation with the expression of integrin- α 3, - α 5 on the HCC cell surface (Fig. 2).

E-selectin and its ligands are closely correlated with the metastasis of HCC (21). Likewise, selectin is not only a key player in inflammation and thrombosis, but also an important molecule for the tumor growth and metastasis, suggesting it as potential target for cancer treatment. Our study also shows the comparable expression regardless of characteristics of the cell lines. It suggests that E-selectin could be a good therapeutic target for HCC.

Intercellular adhesion molecule (ICAM)-1. ICAM-1 is a 90 kDa transmembrane glycoprotein molecule of the immunoglobulin (Ig) superfamily which is characterized by five distinct Ig-like domains, a transmembrane domain and a cytoplasmic tail. ICAM-1 is a fundamental component in many immune-related processes and associates with receptors of the Integrin, thereby mediating cell-cell interaction and allowing for signal transduction (22,47,48). ICAM-1 was reported as a significant prognostic factor for patients with HCC (23) and the tissue and serum ICAM-1 could also indicate the stage of HCC and estimate the potential of hepatoma cells for invasion and metastasis (24).

In vitro, ICAM-1 is not expressed in the noncancerous hepatocyte area except the hepatocytes in the areas of periportal and intra-acinar inflammation whereas most of HCC cells (80-96%) express ICAM-1 on cell surface (49,50). In our study, six of eight HCC cell lines (SNU-354, SNU-368, SNU-449, SNU-739, SNU-886, HepG2) expressed ICAM-1. Expression level of ICAM-1 is significantly diminished in SNU 398, which is the only anaplastic cell line in this study and Hep3B which made numerous metastases in the lungs in the tumorigenicity study. This result suggests that the diminished ICAM-1 might facilitate the cell dissociation and tumor invasion. Consequently, HCC which expressed lower ICAM-1 in the cancer cells could be a more aggressive cancer.

Integrin subunits. Integrins are cell-surface glycoprotein receptors that are obligate heterodimers containing two distinct chains, the α (18 types) and β (8 types) subunits. They are the most dynamic and versatile of the cell adhesion molecules capable of forming more than 24 known combinations (25,26). For this reason, variable and controversial results have been reported.

In general, integrins play a significant role in the tumor transformation, tumor growth, invasion, migration, colonization of distant organ and angiogenesis (27,28). In terms of HCC, a new human HCC cell line with a highly metastatic potential was established from subcutaneous xenograft of a metastatic model of human HCC in nude mice, with high integrin level (51). But still another result showed integrin- β 1 downregulated in poorly differentiated HCC, whereas with relatively high activity in metastatic tumors and the presence of all integrins in cirrhotic liver (52). Integrin- α 1 β 1 and - α 2 β 1 were reported as the key regulator of HCC invasion (29) and integrin- α 3 β 1 is required for HCC migration and invasion (30). Another report shows that reduced expression of the integrin- α 5 subunit is correlated with more malignant phenotypes of human HC (53). There are many reports on different effects of integrin depending on subunits (54-57).

Here we investigated ten types of the integrin subunits and heterodimers (Figs. 2 and 3). In general, there is no correlation with the characteristics of HCC cell lines. However, particularly integrin- β 2 which is well known as the key molecule in the inflammatory process was highly expressed on all HCC cell lines except HepG2 (Fig. 3). It suggests that integrin- β 2 might have a significant role in tumor progression and it needs to be investigated further. All integrins are not expressed or very weakly expressed in HepG2 which is noninvasive cell line. It suggests that integrin might have some relation with tumor aggressiveness. We estimated the expression of integrin- α 5 using both western blot analysis and RT-PCR to compare the expression in the gene level with the protein level. It was somewhat different but showed mostly similar levels (Fig. 4). From the overall results of integrin, integrin also could be a good candidate for the therapeutic target for HCC in both gene and protein levels.

Transforming growth factor (TGF)- β 1 and integrin- α 3, - α 5. TGF- β 1, a polypeptide with multi-function, modulates a variety of cellular processes, such as proliferation, differentiation and apoptosis. The role of TGF- β 1 in HCC cells is not well understood (31,32).

Giannelli *et al* (34) and Katabami *et al* (35) elucidated the correlation between the TGF- β 1 and integrin- α 3 β 1. They showed the TGF- β 1 serum concentrations and integrin- α 3 expression are strongly correlated and concluded that TGF- β 1 may play an important role in HCC invasiveness by stimulating integrin- α 3. It was shown that TGF- β 1 also enhances the expression of integrin- α 5 β 1 and subsequently stimulated cell adhesion onto fibronectin (35).

In our study, we investigated the expression of TGF- β 1 from the supernatant of HCC cell lines using an immunoassay. We found that the TGF- β 1 which is derived from HCC cells had no correlation with the expression of integrin- α 3, - α 5 on the HCC cell surface (Figs. 2 and 5). It suggests that the secretion of TGF- β 1 which effects on the integrin- α 3, - α 5 might be controlled more by endothelial cells and/or inflammatory cells than the HCC cells.

In conclusion, this study shows various expressions of cell adhesion molecules in eight HCC cell lines. We found slight correlations between cell adhesion molecules and the characteristics of HCC, such as more aggressive cell line showing less expression of ICAM-1.

The level of the expression of the adhesion molecules may not affect the cellular activity, morphology or tumorigenicity. However, most of HCC cell lines show various expressions of cell adhesion molecules. It suggests that HCC cell lines that express the major adhesion molecules remain candidates for cellular target therapy, which may need to be patient-tailored according to their molecular profile in order to optimize clinical benefits for specific molecular subgroups.

Because of the limitations of the *in vitro* study, our results should be corroborated *in vivo* and many studies are needed to investigate the clinical correlations.

References

1. Bruix J and Sherman M: Management of hepatocellular carcinoma. *Hepatology* 42: 1208-1236, 2005.
2. Llovet JM and Bruix J: Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. *Hepatology* 37: 429-442, 2003.
3. El-Serag HB and Rudolph KL: Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 132: 2557-2576, 2007.
4. Bruix J and Llovet JM: Prognostic prediction and treatment strategy in hepatocellular carcinoma. *Hepatology* 35: 519-524, 2002.
5. Miller AB, Hoogstraten B, Staquet M and Winkler A: Reporting results of cancer treatment. *Cancer* 47: 207-214, 1981.
6. Therasse P, Arbuck S, Eisenhauer E, *et al*: New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 92: 205-216, 2000.
7. Leung TW, Patt YZ, Lau WY, *et al*: Complete pathological remission is possible with systemic combination chemotherapy for inoperable hepatocellular carcinoma. *Clin Cancer Res* 5: 1676-1681, 1999.
8. Lau WY, Leung TW, Lai BS, *et al*: Preoperative systemic chemoimmunotherapy and sequential resection for unresectable hepatocellular carcinoma. *Ann Surg* 233: 236-241, 2001.
9. Llovet JM, Ricci S and Mazzaferro V: Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 24: 378-390, 2008.
10. Makrilia N, Kollias A, Manolopoulos L, *et al*: Cell adhesion molecule: role and clinical significance in cancer. *Cancer Invest* 27: 1023-1037, 2009.
11. Jiang WG, Puntis MC and Hallett MB: Molecular and cellular basis of cancer invasion and metastasis: implication for treatment. *Br J Surg* 81: 1576-1590, 1994.
12. Llovet JM and Bruix J: Molecular targeted therapies in hepatocellular carcinoma. *Hepatology* 48: 1312-1327, 2008.
13. Park JG, Lee JH, Kang MS, *et al*: Characterization of cell lines established from human hepatocellular carcinoma. *Int J Cancer* 62: 276-282, 1995.
14. Lee JH, KU JL, Park YJ, *et al*: Establishment and characterization of four human hepatocellular carcinoma cell lines containing hepatitis B virus DNA. *World J Gastroenterol* 5: 289-295, 1999.
15. Knowles BB, Howe CC and Aden DP: Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* 209: 497-499, 1980.
16. Song TJ, Eisenberg DP, Fong Y, *et al*: Oncolytic herpes viral therapy is effective in the treatment of hepatocellular carcinoma cell lines. *J Gastrointest Surg* 10: 532-542, 2006.
17. Tangkijvanich P, Anukulkarnkusol N, Suwangool P, *et al*: Clinical characteristics and prognosis of hepatocellular carcinoma: analysis based on serum alpha-fetoprotein levels. *J Clin Gastroenterol* 31: 302-308, 2000.
18. Okumoto K, Hattori E, Tamura K, *et al*: Possible contribution of circulating transforming growth factor-beta1 to immunity and prognosis in unresectable hepatocellular carcinoma. *Liver Int* 24: 21-28, 2004.
19. Zhou L, Liu J and Luo F: Serum tumor markers for detection of hepatocellular carcinoma. *World J Gastroenterol* 12: 1175-1181, 2006.
20. Banks RE, Gearing AJ, Hemingway IK, *et al*: Circulating intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in human malignancies. *Br J Cancer* 68: 122-124, 1993.
21. Ye C, Kiriyama K, Mistuoka C, *et al*: Expression of E-selectin on endothelial cells of small veins in human colorectal cancer. *Int J Cancer* 61: 455-460, 1995.

22. Zhang BH, Chen H, Yao XP, *et al*: E-selectin and its ligand-sLeX in the metastasis of hepatocellular carcinoma. *Hepatobiliary Pancreat Dis Int* 1: 80-82, 2002.
23. van de Stolpe A and van der Saag PT: Intercellular adhesion molecule-1 (Review). *J Mol Med* 74: 13-33, 1996.
24. Shimizu Y, Minemura M, Tsukishiro T, *et al*: Serum concentration of intercellular adhesion molecule-1 in patients with hepatocellular carcinoma is a marker of the disease progression and prognosis. *Hepatology* 22: 525-531, 1995.
25. Sun JJ, Zhou XD, Liu YK, *et al*: Invasion and metastasis of liver cancer: expression of intercellular adhesion molecule 1. *J Cancer Res Clin Oncol* 125: 28-34, 1999.
26. Stupack DG: The biology of integrins. *Oncology (Williston Park)* 21 (Suppl 3): S6-S12, 2007.
27. Luo BH, Carman CV and Springer TA: Structural basis of integrin regulation and signaling. *Annu Rev Immunol* 25: 619-647, 2007.
28. Giancotti FG and Ruoslahti E: Integrin signaling. *Science* 285: 1028-1032, 1999.
29. Stupack DG and Cheresh DA: Get a ligand, get a life, integrins, signaling, and cell survival. *J Cell Sci* 115: 3729-3738, 2002.
30. Yang C, Zeisberg M, Lively JC, *et al*: Integrin alpha1beta1 and alpha2beta1 are the key regulators of hepatocarcinoma cell invasion across the fibrotic matrix microenvironment. *Cancer Res* 63: 8312-8317, 2003.
31. Giannelli G, Bergamini C, Fransvea E, *et al*: Human hepatocellular carcinoma (HCC) cells require both alpha3beta1 integrin and matrix metalloproteinases activity for migration and invasion. *Lab Invest* 81: 613-627, 2001.
32. Hu PP, Datto MB and Wang XF: Molecular mechanisms of transforming growth factor-beta signal. *Endocr Rev* 24: 1128-1136, 1998.
33. Oft M, Heider KH and Berg H: TGF-beta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* 8: 1243-1252, 1998.
34. Giannelli G, Fransvea E, Marinosci F, *et al*: Transforming growth factor-beta1 triggers hepatocellular carcinoma invasiveness via alpha3beta1 integrin. *Am J Pathol* 161: 183-193, 2002.
35. Katabami K, Mizuno H, Sano R, *et al*: TGF-beta1 upregulates transcription of alpha3 integrin gene in hepatocellular carcinoma cells via Ets-transcription factor-binding motif in the promoter region. *Clin Exp Metastasis* 22: 539-548, 2005.
36. Xu Z, Shen MX, Ma DZ, *et al*: TGF-beta1 promoted epithelial-to-mesenchymal transformation and cell adhesion contribute to TGF-beta1 enhanced cell migration in SMMC-7721 cells. *Cell Res* 13: 343-350, 2003.
37. Schlitt HJ, Neipp M, Weimann A, *et al*: Recurrence patterns of hepatocellular and fibrolamellar carcinoma after liver transplantation. *J Clin Oncol* 17: 324-331, 1999.
38. Song GW, Hwang S and Lee SG: Liver transplantation in patients with hepatocellular carcinoma. *Korean J Gastroenterol* 55: 350-360, 2010 (In Korean).
39. Syrigos KN and Karayiannakis AJ: Adhesion molecules as targets for the treatment of neoplastic diseases. *Curr Pharm Des* 12: 2849-2861, 2006.
40. Villanueva A, Newell P, Chiang D, *et al*: Genes and signaling pathways involved in the pathogenesis of HCC. *Senin Liver Dis* 27: 55-76, 2007.
41. Huynh H, Nguyen TT, Chow KH, *et al*: Over-expression of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular carcinoma: its role in tumor progression and apoptosis. *BMC Gastroenterol* 3: 19, 2003.
42. Gollob JA, Wilhelm S, Carter C, *et al*: Role of Raf kinase in cancer: therapeutic potential of targeting the Raf/MEK/ERK signal transduction pathway. *Semin Oncol* 33: 392-406, 2006.
43. Siegel AB, Cohen EI, Ocean A, *et al*: Phase II trial evaluating the clinical and biologic effects of bevacizumab in unresectable hepatocellular carcinoma. *J Clin Oncol* 26: 2992-2998, 2008.
44. Hsu CH, Yang TS, Hsu C, Toh HC, *et al*: Efficacy and tolerability of bevacizumab plus capecitabine as first-line therapy in patients with advanced hepatocellular carcinoma. *Br J Cancer* 102: 981-986, 2010.
45. Vestweber D and Blanks JE: Mechanisms that regulate the function of the selectins and their ligands. *Physiol Rev* 79: 181-213, 1999.
46. Bird MI, Foster MR, Priest R, *et al*: Selectins: physiological and pathophysiological roles. *Biochem Soc Trans* 25: 1199-1206, 1997.
47. Koch AE, Halloran MM, Haskell CJ, *et al*: Angiogenesis is mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. *Nature* 376: 517-519, 1995.
48. Collins T, Read MA, Neish AS, *et al*: Transcriptional regulation of endothelial cell adhesion molecules: NF-kappaB and cytokine-inducible enhancers. *FASEB J* 9: 899-909, 1995.
49. Khatib AM, Fallavollita L, Wancewicz EV, *et al*: Inhibition of hepatic endothelial E-selectin expression by C-raf antisense oligonucleotides blocks colorectal carcinoma liver metastasis. *Cancer Res* 62: 5393-5398, 2002.
50. Gout S, Tremblay PL and Huot J: Selectins and selectin ligands in extravasation of cancer cells and organ selectivity of metastasis. *Clin Exp Metastasis* 25: 335-344, 2008.
51. Marlin SD and Springer TA: Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell* 51: 813-819, 1987.
52. Yang L, Froio RM, Sciuto TE, *et al*: ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha activated vascular endothelium under flow. *Blood* 106: 584-592, 2005.
53. Torii A, Harada A, Nakao A, *et al*: Expression of intercellular adhesion molecule-1 in hepatocellular carcinoma. *J Surg Oncol* 53: 239-242, 1993.
54. Momosaki S, Yano H, Ogasawara S, *et al*: Expression of intercellular adhesion molecule 1 in human hepatocellular carcinoma. *Hepatology* 22: 1708-1713, 1995.
55. Tian J, Tang ZY, Ye SL, *et al*: New human hepatocellular carcinoma cell line with highly metastatic potential (MHCC97) and its expressions of the factors associated with metastasis. *Br J Cancer* 81: 814-821, 1999.
56. Jaskiewicz K and Chasen MR: Differential expression of transforming growth factor alpha, adhesions molecules and integrins in primary, metastatic liver tumors and in liver cirrhosis. *Anticancer Res* 15: 559-562, 1995.
57. Yao M, Zhou XD, Zha XL, *et al*: Expression of the integrin alpha5 subunit and its mediated cell adhesion in hepatocellular carcinoma. *J Cancer Res Clin* 123: 435-440, 1997.