Roles of caveolin-1 gene in metastasis of Human Head and Neck Cacinoma

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Roles of caveolin-1 gene in metastasis of Human Renal Cell Cacinoma

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요 약

약성 중양의 가장 중요한 생물학적 특성은 이들 중양 세포가 원발 부위에서 다른 부위로 이동, 즉 전이(metastasis)된 후 새로운 환경에서 적응 증식할 수 있는 능력을 가지고 있다는 것이다. 초기 암의 생물학 적인 특성의 변화를 유도하는 유전자와 발현은 전이 암의 특정을 분석 하는데 매우 중요하다. 차동 발현 유전자를 탐색하기 위해 시도한 subtractive hybridization에 의하여 탐색된 caveolin-1 유전자는, 신장 암 환자의 암 조직에서 정상 조직에 비해 70% 이상이 caveolin-1 mRNA의 높은 발현을 보였고, 전이 능력이 높은 SN12PM6에서 SN12C보다 증가된 수준으로 나타났다. 이러한 결과를 바탕으로 신장 암에서 caveolin-1 유전자와 암 전이와의 관계 및 역할을 조사하고자, 전이성이 낮은 SN12C에 caveolin-1 유전자를 transfection하여 전이 관련 유전자인 bFGF, VEGF, Type IV collagenase등의 발현을 분석하였다. 전이성이 낮은 SN12C에 caveolin-1 유전자를 cloning하여 transfection한 결과, 전이 관련 유전자 (bFGF, VEGF, type IV collagenase)들의 발현이 parental SN12C보다 높게 항 상됨이 관찰되었다. In vitro의 결과를 animal model을 통하여 확인하기 위해, transfected된 HRCC 세포를 실험 동물 (BALB/c-nude mice)에 주입하여 폐 전이를 관찰하였다. 유도된 전이 model (lateral tail vein injection)과 자발적 전이 model (renal subcapsule injection) 모두에서 caveolin-1 transfected된 SN12C가 parental에 비해 전이성이 증가함을 확인하였다. 이와 같이 caveolin-1 유전자의 다량 발현이 신장 암 전이와 관련이 있다는 연구 결과를 바탕으로, 암 전이 예측 표식으로서의 사용 가능성을 판단하기 위 해 신장 암 환자의 조직을 이용한 immunohistochemistry를
실시하였다. 환자의 약 70%가 caveolin-1 유전자의 발현을 관찰할 수 있었으며, 이들의 전이 진단은 추후 추적 관찰에 의해 판단될 것이다. 따라서, caveolin-1 유전자는 신장 암 전이에 관련되어 지며 caveolin-1 유전자의 연구가 신장 암 환자의 전이를 예측하는 진단 표식의 가능성까지 계속 진행되어야 한다고 사료된다.
ABSTRACT

The characteristics of malignant tumor cells are described by having metastatic potentials and resistance to chemotherapy. Generally these cells are easily able to adapt and proliferate in new environmental conditions. Becoming metastatic tumor is the changes of biological behaviors of tumor cells by environmental signals. Environmental signals may trigger to induce certain gene expression resulting in change of biologic behavior. To identify the differentially expressed genes between normal kidney tissues and neoplastic kidney tissue, the subtractive hybridization analyses were performed. As results, caveolin-1 gene was found. The expression level of this gene was examined between normal kidney tissues and tumor kidney tissue using northern analyses. Caveolin-1 gene expressed in 70% (7/10) of cancer tissues but not in all of normal kidney tissues. Additionally, the expression of caveolin-1 gene was increased in high metastatic clone (SN12PM6) when compared with low metastatic clone (SN12C). These results indicate that the expression of caveolin-1 gene in human renal cell carcinoma (HRCC) may relate to metastasis. Therefore, the stable HRCC cell lines, which strongly expressing caveolin-1 gene, were established and then used these cells for animal experiment in order to determine the metastatic function of caveolin-1 gene. In western analyses, SN12C transfected with caveolin-1 gene (SN12C-caveolin-1) induced the expression of metastatic genes such as bFGF, type IV collagenase, and VEGF. And in vivo, SN12C-caveolin-1 showed the high metastatic potentials when compared with their parental cells (SN12C) in both induced and spontaneous metastasis analyses. Present data suggested that the
expression of caveolin-1 gene is strongly related to metastasis and it also may induce the metastasis of tumor cells in HRCC. In addition, to investigate the possibility of caveolin-1 gene as a prognostic marker for determining metastasis, immunohistochemical analyses were performed. The results indicate that the expression of caveolin-1 gene may be not related to metastatic gene expression. Consequently, to investigate the use of caveolin-1 gene in HRCC as a metastatic prognostic marker, further studies are needed.
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INTRODUCTION

Human renal cell carcinoma (HRCC) is well known for its unpredictable behavior and tendency to recur and metastasize after treatment (1). The estimated incidence of HRCC was approximately 29,900 cases in the United States in 1998 and the proportion of HRCCs is increased continuously between CT scanning and ultrasonography have become widely available (2, 3). Despite significant improvements in diagnosis, surgical techniques, general patient care, and local and systemic adjuvant therapies, HRCC is one of the most difficult urological neoplasms to cure, because most deaths from HRCC are due to metastases that are resistant to conventional therapies. Not only patients with HRCC already have metastasis at the time of diagnosis in 25 - 37% of cases and but also distant metastasis occurs with in 10 years approximately 60% of all cases after curative nephrectomy (4, 5, 6). To increase survival rate of patients, several treatments such as radiation therapy (7), chemotherapy (8) and immunotherapy (9, 10) have been developed for controlling the growth of metastatic tumors and/or locally recurrent cancer cells.

Although much progress has been made in the treatment of this cancer, metastasis is still the major cause of death for patients with solid malignancy (11). The process of metastasis is sequential and consists of a series of independent but
interrelated steps. The outcome of metastasis is dependent on both the intrinsic properties of tumor cells and the various homeostatic factors of host (12). To accomplish metastasis, tumor cells must complete all the steps, as follow; angiogenesis, motility, invasion, survival in the circulation, arrest in a distant capillary bed, and extravasation into and multiplication within the organ parenchyma (11). Recent studies in human malignancies have shown that the metastatic potential of cells correlated directly with the over expression of several genes that encode for epidermal growth factor receptor, basic fibroblast growth factor, interleukin-8, type IV collagenase, and multidrug resistance (13, 14, 15, 16). For example, type IV collagenase is important for tissue invasion. The activating of this gene is strongly correlated with the production of metastases (13, 17). Specifically, in situ hybridization and immunolocalization techniques provided evidences that the expression level of type IV collagenase did increase during progression from the benign to the malignant phenotype in the breast (18), colon (19), prostate (20), and gastric cancer (21).

Recent studies demonstrated that angiogenic genes are upregulated in various metastatic cancers (22, 23). Vascular endothelial growth factor (VEGF) is known to be an endothelial cell-specific powerful mitogen that involved in tumor neovascularization (24, 25). VEGF is released by tumor cells and marcrophages and binds to endothelial cell specific receptors. Binding of VEGF to receptor
promotes the proliferation of endothelial cell, subsequently to produce tubular formation of endothelial cells (26). Consequently, increasing VEGF resulting in hyperpermeability of microvessels leads to distant metastasis by facilitating tumor cell migration through the blood vessel wall (27). For example, breast cancer can entailed substantial development of new blood vessels within the tumor tissue having metastatic potential (28). Basic fibroblastic growth factor, transforming growth factor-β and tumor necrosis factor-α are also well known as angiogenic factors (27). One of factors, b-FGF, is a very potent mitogen and chemoattractant for capillary endothelial cells in vitro and it is also a mitogen for many other cells of mesodermal or neuroectodermal origin (29). b-FGF is distributed over a wide range tissues, including adrenal gland, kidney, placenta and various tumors (30). Neovascularization is also associated with a switch to the export of b-FGF in the multistep development of fibrosarcoma (31).

In the study of immunohistochemical analyses, the degree of neovascularization may closely correlate with an aggressive behavior of tumor cells as well as help to predict the risk of metastasis. Based on these results, it suggests that the expression levels of VEGF and bFGF could be powerful prognostic factors for determining the metastasis of tumor cells. However, it is not sufficient to understand the biologic behaviors of metastatic cells. Subsequently, identification of new valid prognostic indicators will improve the ability to
identify patients at risk of recurrence and to elucidate the biologic behaviors of metastatic cells.

To discover metastasis-related differently expressed genes, several genes were previously selected using a subtractive hybridization. One of them was caveolin-1. Caveolin is a family of highly conserved integral proteins of caveolae that are 50-100nm vesicular invaginations of the plasma membrane (32). Caveolin-1 among the caveolin family is a principal component of caveolae membranes and can serve as a marker for the caveolae (33). So far, the role of caveolin-1 is still remained unknown. Recent studies have been reported that caveolae have been implicated in vesicular trafficking events and signal transduction process. In caveolae, the interaction of caveolin-1 with signaling molecules is mediated via a membrane-proximal region of caveolin, termed the “caveolin-scaffolding domain” (34). Through this domain, caveolin-1 can fuctionally suppress the GTPase activity of hetero-trimeric G-proteins and also inhibit the kinase activity of Src-family tyrosine kinases. The caveolin-binding motifs that includes several crucial aromatic amino acid residues have been deduced in both tyrosine and serine/threonine kinases, as well as in eNOS (endothelial isoform of nitric oxide synthase) (35, 36). Also, caveolin-1 has been implicated in signalling pathway through the p42/44 MAP kinase pathway. And, in aspects of the tumor progression, caveolin-1 expression is reduced or absent in oncogenically
transformed cells (37) and is significantly reduced in human breast cancer cells compared with their normal mammary epithelial counterparts (38). These findings suggested that caveolin-1 is the missing tumor-suppressor gene (34, 35, 36, 38). However interestingly, Yang et al. (39) recently reported that elevated level of caveolin-1 may be associated with prostate-cancer lymph-node metastases, raising the possibility that caveolin-1 could also act as oncogene. In addition, they confirmed that caveolin-1 expression is linked to hormone-resistant prostate cancer in mouse model system (40). Up-regulation of caveolin-1 gene also represented in multidrug resistant cancer cells (41, 42). Based on these findings, the roles of caveolin-1 in cancer cells have to be verified.

Consequently, in this study, the role of caveolin-1 gene is investigated in HRCC as well as the relationships between expression of caveolin-1 gene and becoming metastatic tumors. For this purpose, two different clones of HRCC cells having different metastatic potentials were used. In addition, to understand the functions of caveolin-1 in HRCC, caveolin-1 gene was transfected into SN12C and SN12PM6, and stable cell lines expressing caveolin-1 gene were established. Using these cells, the study for metastatic potentials in in vivo was performed to determine whether an increased caveolin-1 expression could induce the HRCC potentials. Immunohistochemical analyses were also applied for caveolin-1 gene using paraffin-embedded tissues of HRCC to determine the possibility as a
prognostic factor for predicting the metastasis of HRCC. Taken together, the results in *in vitro* and *in vivo* indicated that the expression of caveolin-1 gene is strongly related to metastasis.
MATERIAL AND METHOD

Animals

Female athymic BALB/c nude mice were purchased from the Charles River Japan Inc.. The mice were housed in laminar flow cabinets under specific pathogen-free conditions and kept in conventional rooms with controlled photo period (12:12 L:D, lights on at 08:00h, approx. 300lux at 0.8m above the floor), temperature (23±1 °C), relative humidity (55±5%) and ventilation (15 air changes per hour) and used when 8 weeks of age. Mice were maintained in according to guide for the care and use of laboratory animals and Institute of Laboratory Animal Resources (National Research Council, USA).

Cell lines

SN12C, a human renal cell carcinoma cell line, was isolated from a primary renal cell carcinoma that was granular in cell type with extensive invasion of perinephric fat by Naito et al. (43). The highly metastatic SN12PM6 cell line was established from a spontaneous lung metastasis produced in a nude mouse by SN12C parental cells growing in the kidney (44) (Fig. 1).

Cells and culture conditions

The parental SN12C and metastatic variant SN12PM6 cell lines were purchased from Dr. Filder of Texas University M. D. Anderson Cancer Center,
USA. The cell lines were maintained as monolayers in Minimum Essential Medium (MEM) (GIBCO BRL Co.) supplemented with 10% fetal bovine serum (FBS), penicillin (50 units/ml), streptomycin (50μg/ml), fungizon (100μg/ml). Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. All of the cell lines were examined to confirm be free of Mycoplasma, reovirus type 3, pneumonia virus of mice, mouse adenovirus, murine hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (Miceobiological Associates, Bethesda, MD, USA).

Transfection analyses

The amplified caveolin-1 gene from human cDNA was inserted in an eukaryotic expression vector (pcDNA3.1/V5/His-Topo vector; Invitrogen Co.). And then it was used to determine the functional analysis in human cancer cells. 70% of HRCC cell lines grown in 100mm dishes was treated with a total of 5μg of DNA including lipofectamine reagent (5μg) (GIBCO BRL Co.). To select the stably expressed cells, drug selection in 1000μg/ml G418 (Neomycin, GIBCO BRL Co.) was performed for 21 days after transfection. Three weeks after treated with 1000μg/ml Neomycin, colonies were selected using cloning cylinders and then let it grow to harvest cells. These cell lines were stored at liquid nitrogen until used.

Preparation of tumor cell suspension for in vivo injection

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For in vivo injections, the SN12C or SN12PM6 cells were harvested from subconfluent cultures by a 1-minute treatment with 1×trypsin-EDTA (GIBCO BRL Co.). The flasks were tapped sharply to dislodge the cells, which were then washed in medium and then resuspended in Hank's balanced salt solution (HBSS) for injection. Only single-cell suspensions with viability of more than 90% were used for in vivo injections.

**Subcutaneous tumor cell injection**

Mice were injected subcutaneously (s.c.) with $1 \times 10^6$ viable cells in 0.4ml HBSS at the lateral aspect of the anterior thoracic wall. Tumor growth was measured weekly using caliper measurements and tumor–doubling time was calculated. The mice were sacrificed when the tumor reached 2cm in diameter or at 16 weeks after injection. Tumors were removed, weighed, and fixed in buffered formalin solution for histological examination. The presence of pulmonary and other visceral metastasis was determined.

**Assay for experimental pulmonary metastasis**

Cultured tumor cells were harvested as described above, and $1 \times 10^6$ viable cells suspended in 0.4ml of HBSS were injected into the lateral tail vein of nude mice. 6-8 weeks later, the mice were sacrificed, the lung fixed in Bouin's solution and the identification of peripheral tumor colonies determined under a dissecting microscope. All metastases were confirmed by histological examination of fixed
tissue sections stained with hematoxylin and eosin.

**Tumor-cell injection into the renal subcapsule**

Mice were anesthetized with methoxyflurane and placed in the left lateral decubitis position. A vertical incision was made in the right flank through the skin and peritoneum, exposing the lateral aspect of the kidney. The kidney was gently lifted out of the peritoneum and stabilized. A 27-gauge needle was inserted into the parenchyma from the lower pole of the kidney and advanced until its point reached just below the capsule, where tumor cells ($1 \times 10^6$ viable cells in 0.05ml HBSS) were deposited. A visible tumor formation between the renal parenchyma and capsule lacks significant bleeding or extrarenal leak of the injected tumor cell suspension were the criteria for a successful injection (43). (In some mice, leakage did occur subsequent to the injection, and these mice were eliminated from the study.) After injection, the kidney was returned to the abdominal cavity and the wound was closed in one layer with metal wound clips. The injected mice were sacrificed and autopsied when they became moribund. The kidney with tumors were removed and fixed in 10% buffered formaldehyde solution, formalin. Lung, liver and other organs suspected of metastasis were fixed in Bouin’s solution. The presence of HRCC growth was confirmed histologically.

**Tissue samples**

Both RCC patient’s samples and normal kidney tissues adjacent to the tumor
were obtained from Samsung Medical Center surgical room. Tissue specimens including renal cell carcinomas and normal kidney tissues were immediately frozen in liquid nitrogen and stored at \(-70^\circ\text{C}\) until used.

**RNA isolation and Northern blot analysis**

For cultured cells, total RNA was extracted using TRIzol Reagent (GIBCO BRL Co.). The procedures were as follow: Briefly, the cultured cell was dissolved in 1ml of TRIzol solution per 100mm dish and transferred lysates into DEPC treated eppendorf tube. 200ml of chloroform was added and separated clear supernatants using centrifugation at 13500 rpm for 10 min at 4°C. The clear supernatant was applied to be extracted using 500ml of chloroform and was then precipitated with 0.8 volume of isopropyl alcohol. The pellets were dissolved in 50ml of DEPC treated water and stored at \(-70^\circ\text{C}\) until used. For human cancerous and normal tissues, total RNA was extracted by the guanidine thiocyanate/cesium chloride method (45). Specially, the above procedure requires stringent conditions for preventing from the degradation of cellular RNA by RNase as follow: The use of gloves in every step, baked all of instruments at 200°C for 24 h, after DEPC treatment. All of solutions were treated with 1% DEPC for 24 h and then autoclaved to inactivate RNases. The intactness was checked by the intensity of the ribosomal RNA bands on 1% agarose/2.2 M formaldehyde gel. For analyzing intracellular levels of transcripts between
untreated cells and treated cells, 10mg of total RNA was electrophoresed on 1% agarose/2.2M formaldehyde gel and transferred to a Hybond™ N+ nylon membrane (Amersham Life Science Co.) using 20× SSC. Total RNAs was immobilized on the blot membrane using UV cross-linked with 150mJ/cm² using a GS Gene Linker UV Chamber (BIO-RAD Co.). The blots was stored at 4°C until used.

Hybridization

The blots were prehybridized in a solution containing 50% formamide, 5×SSC, 5×Denhardt’s solution (5g/L Ficoll type 400, 5g/L polyvinylpiruvinate, 5g/L BSA), 0.5% SDS, and 100μg/ml denatured salmon sperm DNA at 42°C for 2 h. Hybridization was performed at 42°C for 16-20 h with the α-dCTP ³²P-labelled denatured probes (20ng/ml, 107 cpm). After hybridization, the blots were washed three times at 37°C with 2×SSC/0.1%SDS (W/V) and were then finally washed at 50°C with 0.2×SSC/0.1%SDS (W/V). Autoradiography using X-ray film (Amersham Life Science, Co.) was carried out at -70°C with an intensifying screen.

DNA probes

Preparation of cDNAs

RNA (1-5μg) was incubated with 0.5μg of hexamer at 70°C for 10 min, chilled on ice and then incubation at 25°C for 10 min. The total RNA was
transcribed in a volume of 20μl reaction mixture containing 1×reverse transcriptase buffer (50mM Tris-Cl, pH 8.3, 75mM KCl, 3mM MgCl₂) provided by the manufacturer, 10mM DTT, 0.2mM dNTP, 5 units of cloned placental RNase inhibior, and 200 units of Superscript II reverse transcriptase (GIBCO BRL Co.). Reactions were performed at 42°C for 1h, followed by heating at 70°C for 15 min for inactivation of RNase H and were stored -20°C.

Reverse Transcriptase Polymerase Chain Reaction

1μl cDNA was used in a 20μl PCR reaction containing a final concentration of 1×Taq DNA polymerase buffer, 0.2mM dNTPs, 2mM MgCl₂, 0.5 M each primer (Table 1), and 2 units of Taq DNA polymerase (Boehringer Mannheim Co.). Reactions were preheated to 95°C for 5 min and then subjected to 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec. DNA amplification was performed in a Perkin-Elmer 9600 thermal cycler. For nested RT-PCR analysis, a 1μl of the primary PCR product was added to an identical reaction conditions except primers. Amplified products were separated onto 1% agarose gel and interesting DNA band were isolated using JETsorb Kit (GENOMED GmbH Co.). The isolated DNA bands were ligated into the cloning sites of the plasmid vector pcDNA3.1/V5/His-Topo and pCR 2.1-Topo (Invitrogen Co.) as described in manufacturer's procedure. The orientation of a cloned fragment was selected by PCR analyses using different primer
combinations. To confirm the DNA fragments in expression vector, all sequencing was performed on 5’ strand using a T7 Sequenase version 2.0 DNA sequencing kit according to manufacturer’s protocol (Amersham Life Science, Co.).

DNA probes

The following cDNA probes were used in this study: about 500 base-pairs EcoRI restriction endonuclease DNA fragment of pCR 2.1-TOPO plasmid containing the VEGF, bFGF, type IV collagenase genes; a 541 base-pairs BamHI-XhoI fragment of human caveolin-1 cDNA from pcDNA3.1/V5/His-Topo (Table 1). Each probe fragment was purified by agarose gel electrophoresis, recovered using JETsorb kit (GENOMED GmbH Co.), and radiolabeled with the Random Primed DNA Labelling Kit (Boehringer Mannheim Co.) using [α-32P]deoxyribonucleotide triphosphate (NEN, Ind.).

Protein analyses

All cell lines were grown up to 70% confluence to determine the expression of protein in the cells. As previously mentioned in molecular cloning all of protein samples was extracted using RIPA buffer (150mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, protease inhibitors). Proteins were solubilized with sample buffer containing 100mM Tris-Hcl (pH 6.8), 4% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 20%
glycerol, 0.2% bromophenol blue in deionized distilled water. After boiling for 5 min, proteins were separated by 10% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose filter with a constant current of 140 mA for 2 h. To normalize the protein concentration in each well, the protein concentration of the samples was measured with the bicinchoninic acid method using bovine serum albumin as a standard. This was done by electroblotting in glycine buffer (20% methanol, 0.1% SDS, 48 mM Tris base, 390 mM glycine). After transferring at 300 mA for 2 hours, the membrane was removed and blocked in TTBS buffer (50 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 0.05% Tween 20) containing 5% nonfat milk powder at 2 hours with gentle agitation. And then incubated for 1-3 h with a 1:500-diluted primary antibodies in TTBS containing 1% nonfat milk. After extensive washing with TTBS, the filters were then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulins (1:500 or 1:1000-diluted; Amersham Life Science Co.). The membrane was washed briefly with three times with TTBS solution and detected with an ECL system (Amersham Life Science Co.) according to the manufacturer’s instructions.

_in vitro cytostasis assay_

To determine the cytotoxicity of cancer cells against chemotherapeutic agents, MTT analyses performed. Briefly 70% confluent cancer cells were plated at a
density of $1 \times 10^3$ cells/well in 96-well plates in culture media after counting cancer cell with trypan blue staining using hematocytometer. Cell counts was performed three times at the same condition. To attach and let it grow, it incubated for overnight. Next day, the cultures were washed and then referred with medium (control) or medium containing different concentrations of doxorubicin (DXR). After 72 h, the cytotoxic activity was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT, Sigma Co.) assay. After incubation for 4 h in medium containing MTT at 0.42mg/ml, the medium was removed. Reaction products in cells were dissolved in DMSO. In this step, MTT converted to formazan by metabolically viable cells. It was monitored by an MR-5000 96-well microtiter plate reader (Dynatech) at 570nm. The percentage of cytostasis was calculated by the formula as follow: Cytostasis (%) = \[1 - \frac{B}{A}\] \times 100 \text{ where } A \text{ is the } A_{540} \text{ absorbance of the control cells and } B \text{ is the } B_{540} \text{ absorbance of the tested cells incubated in medium (46). In several control experiments, the number of viable cells counted by hematocytometer. The conversion of MTT to formazan directly correlated with the number of viable cells.}

**Immunohistochemical staining**

Formalin-fixed, paraffin-embedded tissues were cut at 5 \text{ \mu m}, mounted on charged slides, and dried. For immunohistochemical analysis, slides were deparaffinized and rehydrated in graded solutions of ethanol and distilled water.
Tissue sections were incubated with 3% H$_2$O$_2$ for 10min for endogenous peroxidase inactivity and blocked for 5min with blocking reagent (including The L.V. DAKO LSAB$^\circledR$ Kit, HRP). The primary antibodies, diluted to 1: 1000 using 0.05M Tris-Cl buffer, pH 7.2-7.6, containing 1% bovine serum albumin (DAKO$^\circledR$ Antibody Diluent), were applied and incubated for 1 h at room temperature. After several rinses in PBS, a secondary biotinylated anti-rabbit and anti-mouse immunoglobulins were applied sequentially for 10 min, followed by streptavidin conjugated to horseradish peroxidase in this Tris-Cl buffer for 10min. And then applied the substrate-chromogen solution (DAKO$^\circledR$ DAB Chromogen tablets) for 1-3 min. Slides were rinsed briefly in deionized water, counterstained with Harris’ hematoxylin, and mounted under coverslips. For negative controls, tissue sections were processed identically, except that normal rabbit serum was substituted for the primary antibodies. Pathological analyses were performed at Department of Pathology, Samsung Medical Center, Seoul, Korea. Immunoreactivity was assessed using routine light microscopy and intensity graded from - to ++.
RESULTS

Identification of differentially expressed genes in human renal cell carcinoma using subtractive hybridization

To elucidate the mechanisms of metastasis, subtractive hybridization was performed. Three different genes that may involve in the process of metastasis or carcinogenesis were obtained from these experiments. The expression patterns of these genes were confirmed using pairs of tumor kidney tissues (HRCC) and adjacent normal kidney tissues. One of isolated genes was differentiation-related gene (Drg-1). In northern analyses, Drg-1 gene was expressed differently between normal kidney tissues and HRCC tissues. The transcripts of drg-1 in 7 pairs of HRCC were three times higher than in normal kidney tissues (Fig. 2). But this expression was not different in 3 pairs of HRCC. Another genes encoded unknown sequence having zinc finger motifs and were also differentially expressed between normal kidney tissues and HRCC tissues (data not shown).

Expression patterns of caveolin-1 gene in human renal cell carcinoma

Recent studies have been reported that caveolin-1 gene is a powerful candidates for metastasis-related gene. In contrast, other reports have been suggested that caveolin-1 gene is a tumor suppressor. Presently the function of this gene is still controversial. Subsequently, the expression of caveolin-1 gene was examined using 10 pairs of normal kidney tissues and tumor kidney tissues. As shown in northern analysis, the caveolin-1 gene did express in Human renal cell carcinoma tissues. The results revealed that the expression of caveolin-1 was detected in 70% HRCC tissues but not in normal kidney tissues (Fig. 3). In
additional, this expression was tested among various cancer cells. In this test, the caveolin-1 expression was found in only SN12C but neither in breast cancer cell (MCF-7) nor in prostate cancer cell (LNCap) lines (Fig. 4). These data are suggested that caveolin-1 expression may be related to the developmental process of HRCC. Subsequently, the level of its expression was examined in two different clones of HRCC. As shown in figure 5, the level of caveolin-1 transcripts and proteins were increased in high metastatic clone (SN12PM6) when compared with low metastatic clone (SN12C). To verify the metastatic potentials in both cell lines, the expression level of metastasis related gene, such as VEGF, bFGF, and type IV collagenase, was examined using western analyses. As reported previously, the results showed that angiogenesis or invasiveness related genes were more expressed in SN12PM6 (Fig. 5 C, D, E). Therefore, the expression of caveolin-1 gene may be closely correlated with increasing metastatic potentials in HRCC cells.

Establishment of stable HRCC cell lines that were strongly expressing caveolin-1 gene

To investigate the function of caveolin-1 in HRCC, transfection experiments were performed. A 541 base-pairs human caveolin-1 cDNA (Fig. 6A) was cloned as followed; amplified caveolin-1 gene by reverse transcriptase polymerase chain reaction was inserted into a eukaryotic expression vector (pcDNA3.1/V5/His-TOPO). The expression of this gene was controlled under the activation of cytomegalovirus (CMV) promoter. After transfection with cloned DNA into both cells (SN12C and SN12CPM6) using lipofectamine, the transfected cells were selected using G418 for 3 weeks. Using genomic PCR, selected clones were
tested whether caveolin-1 was inserted into genomic DNA or not (Fig. 6B). Selected clones were also confirmed the expression of caveolin-1 gene using northern analyses and western analyses, respectively. In both experiments, increasing expression of caveolin-1 gene was found in SN12C but this gene expression was slightly increased in SN12PM6 (Fig. 7).

**In vitro, functional analyses of caveolin-1 gene in HRCC**

To examine whether caveolin-1 gene is promoting the metastasis of HRCC cells, the expression pattern of metastatic related genes were examined after transfection of caveolin-1 gene into both SN12C and SN12PM6. In figure 8A, as caveolin-1 gene was overexpressed in SN12C, the transcripts of bFGF were increased. In western analyses, elevated protein level of VEGF, bFGF, type IV collagenase was also detected in SN12C transfected with caveolin-1 gene (SN12C-caveolin-1) when compared with parental SN12C cells. However, in SN12PM6 transfected with caveolin-1 (SN12PM6-caveolin-1), increased protein level of bFGF was not detected.

**Up-regulation of caveolin-1 gene expression in drug resistant cells**

One of biologic behavior of metastatic cells may be the resistance to chemotherapeutic agents. It is very important to survive in the sequential metastatic pathway. Based on the above hypothesis, in this study, roles of caveolin-1 gene were investigated whether its expression is conferred to drug resistance or not. Using cisplatin resistant cell (H460/CIS) and parental cells (H460; lung cancer cell line), the expression level of caveolin-1 gene was examined. The results revealed that its expression in drug resistant cells was higher than in sensitive cells (Fig. 9A). Also, the level of caveolin-1 mRNA in
SN12C was gradually increased when increased the concentration of DXR treatment (Fig. 9B) In addition, MTT analyses was performed to estimate the cytotoxicity between SN12C and SN12C-caveolin-1. The results of MTT assay showed that SN12C-caveolin-1 was more resistant than SN12C (Fig. 10).

In vivo study for experimental animal model

To provide direct evidences that up-regulation of caveolin-1 gene is able to promote the metastasis in HRCC, each cell line was implanted into ectopic (subcutaneous, intravenous) and orthotopic (kidney) organs of nude mice (Table 2). Four of HRCC cells including control cells were injected to subcutis (s.c.) of BALB/c nude mice for determine the tumorigenicity. All of cells produced tumors in subcutis after 8 weeks. The s.c. tumors did not metastasize to lung tissue. To estimate the metastatic potentials, four cells including control cells were used. 1×10⁶ viable tumor cells was injected into intravenous (i.v.) and renal subcapsule (RSC), respectively. 4 cell lines produced tumors in lung by i.v. injection found in except for control and parental SN12C cell lines. The incidence of pulmonary metastasis and number of tumor colonies of SN12C-caveolin-1 were less than that pattern of parental SN12PM6 by the histopathological exmination of the tissues stained with hematoxylin and eosin. As injection of tumor cells into renal subcapsule, the tumor cells spontaneously metastasized in lung. Lung metastasis was less in all the cell lines when compared with i.v. injection. The control (cells transfected with vector only) and SN12C inoculated into kidney were less lung
tumors than the other cell lines. But SN12C-caveolin-1, SN12PM6 and SN12PM6-caveolin-1 produced the high level of spontaneous lung metastases. SN12C-caveolin-1 showed the high metastatic potentials than parental cell line. But there was no apparently different between SN12PM6-caveolin-1 and parental SN12PM6 in induced and spontaneous metastasis.

**Immunohistochemical analyses**

As shown in figure 3, the transcripts of caveolin-1 gene were solely detected in 70% of HRCC tissues. Based on this result, the present study investigated whether the expression of caveolin-1 gene can use as prognostic marker for determining micrometastasis or not. The results of immunohistochemical analyses revealed that the expression of caveolin-1 gene was positive in 70% of HRCC tissues. And both bFGF and type IV collagenase was also positive in four HRCC tissues but three HRCC tissues were negative. The rest of HRCC tissues, three tissues revealed negative signal of caveolin-1 gene, were not detected bFGF and type IV collagenase (Table 3). The representative results were shown in figure 11. The localization of caveolin-1 gene revealed that positive signals were found in both membrane and cytoplasm (Fig. 11). There was no correlation between caveolin-1 and metastasis-related gene (bFGF and type IV collagenase) immunoreactivity. The RCC patients that were detected caveolin-1 protein will be continuously observed until the RCC recurs.
DISCUSSION

Caveolin-1 is an integral protein of subcellular structures called caveolae, which are anatomical invaginations of the cell membrane. Caveolae plays important roles in signal transduction, molecular transport, and cellular motility and adhesion. In regard to signalling transduction, specific molecules which is able to be transformed have been associated with caveolae including members of the ras family, c-src, as well as the endothelin receptor (47). As a major component in caveolae, caveolin-1 may be functionally related to signal transduction pathways, as well as the transport of small molecules. Previous studies have been reported that caveolin-1 gene is closely related to the process of normal development (32, 34, 36). In normal tissues, caveolin-1 is particularly found in smooth muscle cells, adipocytes, and endothelium, but it is only minimally expressed or undetectable in normal prostatic epithelium. Caveolin-1 may also be involved in the attachment of cells to the extracellular matrix and possibly mediates cell motility, as it is complexed to glycosylphosphatidylinositol-linked proteins, such as the urokinase plasminogen activator receptor, under some condition (48). Although caveolin-1 is involved in numerous biological activities, direct evidence of roles for caveolin-1 in progression of human carcinoma has not been reported. One of recent studies demonstrated that overexpression of selected dominantly acting oncogenes resulted in suppression of caveolin-1 mRNA, protein levels, and fewer caveolae in fibroblastic NIH3T3 cells (37). Another study suggests that caveolin-1 may be involved with bridging integrin-mediated signaling with Shc and further downstream signal transduction
pathways that result in gene activities relevant to the metastatic cascade (49, 50). In present study, the expression of caveolin-1 gene was detected in HRCC tissues, but not in adjacent normal kidney tissues. This result suggests that increased caveolin-1 expression occur in progression of HRCC. To determine the metastatic potentials of localized HRCC cells, the expressions of metastastic genes (bFGF, type IV collagenase, VEGF) were examined. The results revealed that coexpression of caveolin-1 with metastatic genes were found in two different clones (SN12C, SN12PM6) (Fig. 5). In vitro, transfection analyses also revealed that elevated expression of caveolin-1 gene did increase the expression of metastatic genes. In conclusion, the elevated expression of metastatic genes by induction of caveolin-1 may help HRCC cells to be metastasized. The similar data discovered also in prostate and breast cancer (39). Yang et al. (39) identified the up-regulation of caveolin-1 gene in both mouse and human prostate cancer metastases and interestingly in primary and metastatic breast cancer. The above data suggested that increasing caveolin-1 expression occur earlier in progression of breast cancer relative to prostate cancer.

Metastatic cells have generally drug resistant phenotype for their growth and development under the cytotoxic environment (41). To determine the biologic behaviors of cancer cells, MTT analyses were performed between cells that is stably expressing caveolin-1 gene and the parental cells as a control (Fig. 10). The results indicate that the caveolin-1 gene increased the resistance in cytotoxic drug. Therefore, metastatic HRCC cells may require changes of biologic behaviors such as a characteristic property of drug resistance. Interestingly, one of data for roles of caveolin-1 in cancer cells has reported that the expression of caveolin-1 has a
growth-inhibitory effect on transformed cells (38). In fact, caveolin-1 was identified as one of over 20 candidate "tumor suppressor" genes that are down-regulated in the growth of breast cancer (51). In agreement with this, Lavie et al. (41) reported that the growth rate of HT-29-MDR cells (mean doubling time of 96 h) is significantly slower than that of their parental drug-sensitive cells (mean doubling time of 24 h). Hence, one functional consequence of the up-regulation of caveolin-1 in drug resistance is a slower rate of proliferation, which may provide cells with some protection against the action of cytotoxic drugs and thus contribute to their selective growth advantage under chemotherapy. These data suggest that cancer cells having phenotype of drug resistance could obtain the benefits for metastasizing cancer cells to other organs.

And, important parameters for cellular invasiveness, the expression of type IV collagenase in vitro, were activated by increased level of caveolin-1 expression. Although it is not clear whether type IV collagenase is the sole factor that determines the invasive phenotype of HRCC cells, it is proposed, based on our observations, that the HRCC invasive phenotype can be rescued by caveolin-1 transfection, possibly through the up-regulation of type IV collagenase activity. These results suggest that caveolin-1 should be thoroughly examined for its putative role in activating HRCC invasion and ultimately metastasis in vivo.

In both induced and spontaneous animal experiments, SN12C-caveolin-1 produced more lung metastasis when compared with control group. However, SN12PM6 having high metastatic potentials did not effect on lung metastasis by caveolin-1 gene. Based on these results, since SN12PM6 already have high metastatic potentials, more expression of caveolin-1 gene by transfection may not
effect on metastatic potentials in either induced or spontaneous metastasis of animal experiments.

Neovascularization and invasiveness have been implicated in the earliest step of metastasis, and there is emerging evidence that changes into these metastatic properties are permissive for biologic behaviors of cancer cells (11,12). Although several evidences support the hypothesis that up-regulation of caveolin-1 resulting from either genetic alterations or epigenetic effects is attribute to the progression of many cancers, the mechanism that caveolin-1 exerts metastatic function is still remained unknown. The best interpretation seems to be that metastasis of cancer cells mediated by caveolin-1 acts to activate cell migration. It is then conceivable that the increase of caveolin-1 protein would favor the migration of tumor cells from the original local site. However, other than neovascularization and invasiveness, caveolin-1 may regulate additional aspects of the tumor phenotype, including drug-resistant activity, as revealed in these studies. It is not clear whether these additional effects are a sequential result of the gain of angiogenesis and invasiveness or, alternatively, an independent process involving other cellular signaling events. In conclusion, it is possible that the expression of caveolin-1 gene may modulate activation of angiogenesis factors. Indeed, the present data provided a direct connection between the re-expression of caveolin-1 and the up-regulation of VEGF, bFGF, and type IV collagenase activity, important biological targets for therapeutic intervention.

This study is the first report that caveolin-1 gene plays a central role in activating the angiogenic and the invasive phenotype of HRCC cells. These results indicated that increased expression of caveolin-1 has been detected in
more than 70% of RCC patients and the expression of caveolin-1 gene was strongly related to the expression of metastatic genes (bFGF, VEGF, and type IV collagenase). Although the true percentage may be underestimated because of insufficient patient samples, the expression pattern of caveolin-1 gene may be used as an important therapeutic consideration.
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permeability factor (vascular endothelial growth factor) and its receptors in breast


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Figure 1. The morphology of human renal cell carcinoma (HRCC) cell lines.
Figure 2. The expression patterns of Drg-1 (differentiation related gene) in HRCC patient’s tissue and adjacent normal kidney tissues. 10 pairs of HRCC patient’s tissues are investigated. 
N : normal kidney tissue, T : tumor kidney tissue.
Figure 3. The expression patterns of caveolin-1 gene in RCC patient’s tissue and adjacent normal kidney tissues (A). 10 pairs of HRCC patient’s tissues are investigated. Equals loading of mRNA samples were monitored by hybridizing the same membrane filter with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA probe (B).

N: normal kidney tissue, T: tumor kidney tissue.
Figure 4. Caveolin-1 mRNA expression among several cell lines (B). Equal loading of total RNA was determined by the intensity of the ribosomal RNA bands on 1% agarose/2.2M formaldehyde gel (A). 1: SN12C, 2: MCF-7, 3: LNcap.
Figure 5. The expression of caveolin-1 gene in two HRCC cell lines having different metastatic potentials. A is northern analysis for caveolin-1 transcripts. The results of western analyses is shown as follows; B for caveolin-1, C for Type IV collagenase, D for bFGF and E for VEGF protein, respectively.
1 : SN12PM6, 2 : SN12C.
Figure 6. Establishment of stable cell lines expressing caveolin-1 gene. Caveolin-1 gene amplified from several cells and cancer tissues and then inserted into pcDNA3.1/V5/His-TOPO vector (A). After transfection, transfected cells were selected with G418 (1000 μg/ml) and then confirmed with genomic PCR (B).

A2774: ovarian cancer cell line, negative control: vector-only, positive control: vector inserted caveolin-1.
Figure 7. The expression of caveolin-1 mRNA and protein in HRCC cells and their transfectants. A: Northern blot, B: Western blot.
1: SN12C, 2: SN12C-caveolin-1, 3: SN12PM6, 4: SN12PM6-caveolin-1.
Figure 8. The expression of metastasis-related gene in HRCC cells and their transfectants. A: Northern blot for bFGF, B: Western blot.
1: SN12C, 2: SN12C-caveolin-1, 3: SN12PM6, 4: SN12PM6-caveolin-1.
Figure 9. Up-regulation of caveolin-1 gene in drug resistant cells. A is northern blot for lung cancer cell lines. 1: Drug sensitive cells (H460), 2: Cisplatin resistance cells (H460/CIS). B is northern blot for SN12C treated DXR. 1: SN12C, 2: SN12C treated with 0.5ng/ml of DXR, 3: SN12C treated with 1ng/ml of DXR.
Figure 10. MTT analyses for DXR resistance in SN12C.
Figure 11. The photographs of immunohistochemical analyses. The positive signal was detected in cytoplasm and cytoplasmic membranes. The results were confirmed by a pathologist. Each vertical sample is identical tissue of RCC patient. A, B, C are the results of immunohistochemistry for caveolin-1, type IV collagenase and bFGF antibody, respectively.

++ : strong positive, + : weak positive, - : negative.
## Table 1. Oligonucleotides for cloning

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<th>Gene</th>
<th>Strand</th>
<th>Name</th>
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Table 2. Tumorigenicity and lung metastasis of several RCC cell lines in BALB/c nude mice

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* 1x10⁶ viable tumor cells were injected into the subcutis or lateral tail vein of nude mice.

*Number of mice with tumor/number of injected mice.
Table 3. Immunohistochemistry analyses for the expression of caveolin-1, bFGF and Type IV collagenase in tumor kidney tissue. The localization of caveolin-1 protein revealed that positive signals were found in both cytoplasm and membrane.

+++ strong positive, ++ weak positive, +/- negative.

<table>
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<th>Type IV collagenase</th>
<th>Pathology</th>
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감사의 글

학부를 포함한 6년의 배움의 길에서 오늘의 논문을 완성하였습니다. 아직은 안타깝고, 부족한 면이 많지만 끝이 아닌 시작의 의미로써 더욱 더 많은 지식의 습득에 노력할 것을 다짐합니다.

한결같이 따뜻한 마음으로 바른 지식을 지도하여 주신 이혜영 교수님께 깊은 존경과 감사를 드립니다. 많은 배려와 격려를 아끼지 않으셨던 양서영 교수님께 진심으로 감사드립니다. 또한, 본 논문의 지도에 도움을 주신 류재혁 교수님께 깊은 감사드립니다. 그리고, 한홍의 교수님, 최병희 교수님, 조성호 교수님, 이창중 교수님, 조강현 교수님, 김정호 교수님의 커다란 가르침에 작은 실게임이 되지 않도록 노력하겠습니다. 진심으로 감사드립니다. 실험실 생활에 있어 실제적인 정보와 도움을 무조건적으로 배울 수 주신 유천진화학 연구실의 박사님들께 다시 한번 감사드립니다.

젊은 두 해의 기간동안 서로 의지가 되었고, 되어준 나의 친구 중은과 영진에게 고마움을 전합니다. 떠어진 한 옥타리 속에서 부대끼가며, 서로를 생각하고 위할 줄 알았던 혁이 오빠, 현환이 오빠, 세영이 오빠, 재광이 오빠, 봉준이 오빠, 헤미 언니, 수연이, 진경이를 비롯한 많은 선배님들과 후배님들께 모두 모두 감사드립니다. 대학 세내기 시절부터 지금까지 배놓을 수 없는, 많은 추억과 기억이 가득한 나의 서를 수영부와 친구 옥이에게도 고마운 마음을 전하고 싶습니다.
감사의 글

학부를 포함한 6년의 배움의 길에서 오늘의 논문을 완성하였습니다. 아직은 안타깝고, 부족한 면이 많지만 끝이 아닌 시작의 의미로써 더욱 더 많은 지식의 습득에 노력할 것을 다짐합니다.

한결같이 따뜻한 마음으로 바른 지식을 지도하여 주신 이해영 교수님께 깊은 존경과 감사를 드립니다. 많은 배려와 격려를 아끼지 않으셨던 양서영 교수님께 진심으로 감사드립니다. 또한, 본 논문의 지도에 도움을 주신 류재혁 교수님께 깊은 감사드립니다. 그리고, 한홍의 교수님, 최병희 교수님, 조성호 교수님, 이창중 교수님, 조강현 교수님, 김정호 교수님의 커다란 가르침에 작은 일개들이 되지 않도록 노력하겠습니다. 진심으로 감사드립니다. 실험실 생활에 있어 실제적인 정보와 도움을 무조건적으로 베풀어 주신 유전전화학 연구실의 박사님들께 다시 한번 감사드립니다.

젊은 두 해의 기간동안 서로 의지가 되었고, 되어준 나의 친구 정은과 영진에게 고마움을 전합니다. 뒤어진 한 울타리 속에서 부대끼가며, 서로를 생각하고 위할 줄 알았던 혁이 오빠, 현환이 오빠, 세영이 오빠, 제광이 오빠, 봉준이 오빠, 혜미 언니, 수연이, 진경이를 비롯한 많은 선배님들과 후배님들께 모두 모두 감사드립니다. 대학 새내기 시절부터 지금까지 배농을 수 없는, 많은 추억과 기억이 가득한 나의 서른 수영부와 친구 옥이에게도 고마운 마음을 전하고 싶습니다.
지난 1년간 갖춘 경험과 새로운 배움의 길을 열어준 진정화 선배님께 감사드리며, 편안한 마음으로 보살펴준 삼성의료원의 영원 언니, 지형 언니, 국주에게 고마움을 전합니다. 힘들고 지친 생활에 즐거움과 활력을 안겨주고 언제나 작은 보금자리의 행복을 느끼게 해준 유리 언니와 경민 언니, 아주 많이 감사드려요. 그리고, 항상 따뜻하게 안아주고 자상하게 일러주며 진지하게 알려주는 착한 운정 언니에게도 깊은 감사드립니다. 항상 부족한 저에게 세심한 배려와 가르침을 주신 안광성 박사님과 윤성수 선생님, 김진국 선생님께 진심으로 감사드립니다.

13년의 긴 시간속에서 서로를 잃지 않고 오랜 우정을 보여준 성은이에게 고마움을 전합니다. 가까운 곳에서, 필요한 힘과 용기를 심어주며 좀 더 성숙하고 당당한 사회인으로 나아갈 수 있도록 도와준 정원석 선배님께도 감사드립니다.

마지막으로, 끝임없는 사랑과 운정을 배풀어 주신 부모님께 감사드리고, 사랑스런 나의 동생들에게도 고마움을 전합니다.

1999년 12월
20세기를 마감하며

이현익