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Paired Cell Line Establishment and Characterization of Genes Expressed Differentially Between Paired Primary and Peritoneal Seeding Colorectal Cancer Cell Lines

동일 환자에서 기원한 원발 대장암 유래 세포주와 복막전이 대장암 세포주 수립과 차별 발현된 유전자의 특성분석

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Paired Cell Line Establishment and Characterization of Genes Expressed Differentially Between Paired Primary and Peritoneal Seeding Colorectal Cancer Cell Lines

By

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A thesis submitted to the department of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Science (Surgery) at Seoul National University College of Medicine

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Doctoral Committee

Professor _____ **Chairman**

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Abstract

In colorectal cancer, variable genetic mutations and polymorphisms of primary tumor has been studied. But genetic differences between paired primary and peritoneal seeding colorectal cancer cell lines were not well studied. Thus, the aims of this study are to establish cell lines derived from primary CRC tissue and peritoneal metastasis tissue of same patient and to identify differentially expressed genes between paired primary and peritoneal seeding colorectal cancer cell lines and to clarify the biologic characteristics of differentially expressed genes by functional study.

Three pairs of CRC cell lines were established from 3 primary tumors and 3 peritoneal metastatic tumors obtained from 3 Korean patients. And we initially identified the actin-binding protein Calponin 3 (h3-calponin, acidic calponin, CNN3) which was overexpressed in peritoneal seeding cell lines(SNU-2335D, SNU-2404B, and SNU-

2414B) compared to those paired primary cell lines(SNU-2335A, SNU-2404A, SNU-2414A) by using Affymetrix GeneChip® hybridization method. And Calponin 3 was highly expressed in other highly metastatic CRCs in western blot analysis. Interestingly, the Calponin 3 plasmid transfected cell lines tended to grow faster than the control plasmid transfected cell lines and *Calponin 3* knockout cell lines showed reduced invasion than that of control cell lines.

Our present results imply that the higher expression of Calponin 3 plays a role for increasing cell proliferation and invasion of CRC cells, and suggest that Calponin 3 can be linked to positive function in peritoneal metastasis of CRC.

Moreover the established three paired CRC cell lines in this study should be useful in investigations of the biological characteristics of CRC, particularly for investigations related to gene alterations associated with primary CRC and peritoneal metastatic CRC.

Key words: colorectal cancer, Calponin 3 (h3-calponin, acidic calponin, CNN3), cell line, peritoneal seeding

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Introduction

Colorectal cancer (CRC) is the second most common cause of cancer related deaths in developed countries. In Korea, the incidence of CRC is increasing annually and CRC is the third most common cancer (Korea Central Cancer Registry in 2009) (1). Recent progress in diagnostic and treatment technologies has improved the long-term survival of patients with early CRC, but the prognoses of patients with metastatic CRC remains unfavorable. Peritoneal metastasis is one of the major patterns of unresectability of CRC and as a result, is a cause of death in advanced CRC. Despite improvements in chemotherapy and surgical techniques, prognosis for peritoneal metastasis remains depressed; thus, detection of a new therapeutic target and/or protection for peritoneal metastasis has been the subject of intensive research.

Despite the fact that metastases are the major cause of CRC deaths, most of genetic studies of colorectal carcinogenesis have focused on

the differences between normal colonic mucosa and primary carcinomas. And there were a few studies on the genetic alteration of primary carcinomas to peritoneal metastases (2-7). The identification and characterization of genes that are differentially expressed in primary CRC cells and peritoneal metastatic CRC cells will provide important information that extend our understanding of the mechanisms responsible for peritoneal metastases (8, 9). Cancer cell lines play an important role in understanding the mechanism of cancer development and progression. Thus in these days, CRC cell lines established from human tumor tissues are widely used in various biological studies, including cancer biology and the development of new therapeutic modality. However, most established CRC cell lines have been obtained from primary tumor only or primary tumor and its lymph node metastasis. There were only a few cell lines derived from primary CRC tissue and peritoneal metastasis

tissue of same patient. Fortunately, we had some paired tissues derived from the different sites of the same patients and the cell lines were derived from the different sites of the same patients. In addition, we have had clinical informations of those patients and can review the matched tissue blocks.

Thus, the aims of this study are to establish cell lines derived from primary CRC tissue and peritoneal metastasis tissue of same patient and to identify differentially expressed genes between paired primary and peritoneal seeding colorectal cancer cell lines and to clarify the biologic characteristics of paired colorectal cancer cell lines from functional study of differentially expressed genes.

The presently established three paired CRC cell lines should be useful in investigations of the biological characteristics of CRC, particularly for investigations related to gene alterations associated with primary CRC and peritoneal metastatic CRC.

Materials and Methods

Establishment and maintenance of human CRC cell lines

Cell lines from pathologically proven colorectal adenocarcinoma and its peritoneal metastasis were established. Solid tumors were finely minced with scissors and dispersed into small aggregates by pipetting. Appropriate amounts of fine neoplastic tissue fragments were seeded into 25 cm^2 flasks. Most of the tumor cells were initially cultured in ACL-4 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS) (AR5). ACL-4 is a fully defined specifically formulated for the selective growth of human lung adenocarcinoma cells and has proven useful in the establishment of CRC and hepatocellular carcinoma cell lines. AR5 medium was prepared from RPMI 1640. ADF5 medium prepared by a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 supplemented with 5% heat-inactivated FBS was also used for the initial culture of tumor cells.

Cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS (R10). When heavy tumor cell growth was observed, initial passages were performed and following passages were performed every 1 or 2 weeks. Adherent cells were recovered while growth was subconfluent by treatment with trypsin, dispersed by pipetting and used for the passages. If stromal cell growth was noted in the initial cultures, differential trypsinization was used to obtain a pure tumor cell population. Cultures were maintained in humidified incubators at 37˚C in an atmosphere of 5% CO2 and 95% air. SNU-1, SNU-61, SNU-C2A and SNU-C4 cell lines obtained from the Korean Cell Line Bank (Seoul, Korea) were used as controls.

Nucleic acid isolation and synthesis of complementary DNA

Genomic DNA was extracted from the cell lines using G-DEX genomic DNA Extraction Kit (Intron Biotechnology), and RNA was extracted using the easy-BLUE total RNA Extraction Kit (Intron

Biotechnology). For complementary DNA synthesis, 2 μg of total RNA was reverse transcribed using random oligo (dT) primer, deoxynucleoside triphosphates and 1 μl (200 U) of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a final volume of 20 μl for 75 min at 42˚C, after a 10 min denaturation at 70˚C. Eighty microliters of distilled water was then added to the reverse transcription reaction, which was stored -20˚C until used.

DNA fingerprinting analysis

DNA was amplified using an AmpFlSTR identifiler PCR Amplification Kit (Applied Biosystems, Foster City, CA). A single round of PCR amplified 15 short tandem repeat markers (CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA) and an Amelogenin gender-determining marker at loci containing

highly polymorphic microsatellite markers.

Amplified products were analyzed using an ABI 3730 Genetic analyzer (Applied Biosystems). Additionally, DNA was PCR amplified at loci containing the highly polymorphic microsatellite markers D1S1586 and D3S1765. PCR products were denatured by 95% formamide and electrophoresed on a 7 M urea polyacrylamide gel for 2 h at 60 W. Gels were dried and visualized by autoradiography.

MSI and mutation analyses of MMR gene

For MSI analysis, BAT-25 and BAT-26 were evaluated by a capillary-based sequencing analysis. PCR was performed as described above, except that the forward primers were labeled with a fluorescent dye (FAM), and the labeled samples were run on an ABI 3730 genetic analyzer (Applied Biosystems). GeneMapper software (Applied Biosystems) was used to calculate the size of each fluorescent PCR

product. For gel-based MSI analysis, the desired fragments were amplified in the presence of [α-P^{32}] deoxycytidine triphosphate. The PCR products were denatured and separated on 6 M urea/7% polyacrylamide gels run at 60 W. To identify mutation in hMLH1 and hMSH2, we screened all coding regions and splicing sites by direct sequencing.

Cell cultures

Three paired cell lines (SNU-2335A, SNU-2335D, SNU-2404A, SNU-2404B, SNU-2414A, SNU-2414B) derived from the same patients were recently established in our laboratory. Thirty-six human CRC cell lines (SNU-61, SNU-81, SNU-175, SNU-283, SNU-407, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1040, SNU-1047, SNU-1197A, SNU-C1, SNU-C2A, SNU-C4, SNU-C5, Caco-2, COLO201, COLO205, COLO320, DLD1, HCT-8, HCT-15, HCT-116,

HT-29, LoVo, LS174T, NCI-H716, SW-403, SW-480, SW-620, SW-1116, WiDr, KM12C, KM12SM and KM12L4) were obtained from Korean Cell Line Bank. All cell lines were maintained in RPMI1640 media with 10% FBS at 37^{o}C and 5% CO_2.

RNA isolation and cDNA synthesis

Total RNA was isolated using easy-BLUE kits (Intron Biotechnology, Korea) from cultured and washed cancer cell pellets. Briefly, the protocol involves adding 1 mL of easy-BLUE to collected cells, vortexing vigorously and then adding chloroform. After centrifuging, the aqueous layer was transfer to a new tube. Isopropanol was added to precipitate RNA and RNA pellets obtained were washed with 70% ethanol. Pellets were then dissolved in DEPC-treated distilled water at 65 ^{o}C for 20 min and then stored at -70^{o}C. For cDNA synthesis, 2 μg

of total RNA and 1 μl of random primer were mixed and incubated at 70 ^{o}C for 10 minutes. Then, they were cooled down in ice for 5 minutes. The mixture that contained 4 μl of 0.1M DTT, 1 μl of 2.5mM dNTP, 1 μl of Superscript^{TM} II reverse transcriptase (Invitrogen, Camarillo, CA, USA) and 1 μl of DEPC water was added to the tube that included the mixture of total RNA and random primer and the reverse transcription reaction was performed. The conditions of the reaction were 1 hour 30 minutes at 42 ^{o}C and 15 minutes at 80 ^{o}C. Finally, 80 μl of DEPC water were added for cDNA dilution.

Microarray analysis

Total RNA was extracted using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). High quality RNA (RNA integrity number >9.0) was used for

expression microarray analysis in which 300ng of the total RNA was processed for biotin labeled target preparation and hybridization to Affymetrix Gene 1.0 ST array according to the manufacturer's protocol to perform gene expression profiling experiments (Affymetrix, Santa Clara, CA, USA). After hybridization for 16 hours at 45°C with rotating at 60RPM, arrays were washed and stained on a GeneChip® Fluidics Station (Affymetrix, Inc.) and scanned using the GeneChip® Scanner 3000.

The CEL intensity data extracted by GCOS (Gene Chip Operating Software, Affymetrix, Inc.) were used for data analysis. Raw data were processed using Affymetrix® Expression Console software using default RMA parameters. Functional categorization of expression-based clusters based on gene ontology (GO) was performed using DAVID Functional Annotation Bioinformatics Microarray Analysis (http://david.abcc.ncifcrf.gov/home.jsp). After compared the signal

intensity level between samples, dChip searches all branches with at least four functionally annotated genes to assess whether a local cluster is enriched by genes having a particular function with GO term. Classification was performed using GeneCluster 2.0 software (http://broadinstitutes.org/cancer/software/genecluster2/gc2.html).

Reverse transcription-PCR analysis

RT-PCR was carried out using specific primers for *Calponin 3* mRNA. The primers used to amplify the *Calponin 3* cDNA were as follows: sense, 5- AGTCAAGAACAAGATTGCTTCC-3 and reverse 5-TCATACCATAAGCCTGAATAGC-3. The PCR conditions consisted of 5 min at 94 ^{o}C for initial denaturation, followed by 35 cycles of 94 ^{o}C (45 sec), 55 ^{o}C (30 sec) and 72 ^{o}C (1 min), and a final elongation of 7 min at 72 ^{o}C. Primers for β-actin were used to confirm RNA integrity. Amplified DNA fragments were fractionated in 2% (w/v)

agarose gel and stained with ethidium bromide.

Transient transfection of Calponin 3

pCMV-Myc-Calponin 3 transient vector for *Calponin 3* overexpression was provided by Dr. Jochen Haag who used this plasmid for his previous study (Haag,. et al, 2007). 2 x 10^4 ea/ml of HT-29 cells were seeded on 6-well plates 24 hours before transfection. Cell counting and western blotting were performed after 24, 48, 72 and 96 hours.

Stable transfection of Calponin 3

pcDNA3.1(+) control vector was obtained from Clontech(Clontech Laboratories, Inc., San Jose, CA, USA). *Calponin* 3 gene in pCMV-Myc plasmid which has restriction enzyme sites including EcoRI and NotI was cut with these two enzymes and isolated from agarose gel by

electrophoresis. pcDNA3.1(+) control vector was also cut with EcoRI and NotI enzymes and ligated to the isolated *Calponin 3* gene and transformed into *E.coli* DH5a competent cells (Intron biotechnology). *Calponin 3* recombinant DNA plasmid was obtained with Plasmid DNA Purification Kit (Intron biotechnology) and the sequence was confirmed by sequencing with ABI3730 sequencer (Applied Biosystems, Foster, CA, USA).

SW-480 cell line was selected by expressing a relatively lower level of *Calponin 3* than other colon cancer cell lines and was stably transfected with the *Calponin 3* recombinant DNA. 2 x 10^5 cells per well were seeded in 6-well plates and cultured with RPMI1640 media (Gibco) for 24 hours. The following day, the *Calponin 3* recombinant DNA plasmid was transfected with lipofectamine 2000 (Invitrogen) according to the manuscript in Opti-MEM I media for 24 hours. The media was replaced with equal volumes of RPMI1640 media

supplemented with 10% FBS (Thermo scientific, State, USA) without antibiotics after 24 hours. 48 hours after exchanging the media and 72 hours after the transfection was performed, half of the cells were harvested to confirm mRNA expression level of *Calponin 3*. The other half of the cells was transferred to T75 cm^2 flasks for selection.

Knockdown of Calponin 3 by siRNA transfection

SNU-C4 was selected by expressing a higher level of *Calponin 3* gene than other colon cancer cell lines and was transfected with control siRNA and Calponin 3 siRNA (No#: 1033350) (Bioneer). Calponin 3 siRNA was transfected with lipofectamine 2000 (Invitrogen) at a final concentration of 40nM, in Opti-MEM I for 6 hours and the media was replaced with an equal volume of RPMI1640 media (Gibco). Cell proliferation assay and cell cycle analysis were performed. siRNA transfected cells were harvested for confirming expression levels of

mRNA and protein of Calponin 3.

Cell counting

4.0 x 10^5 cells per well were seeded on 6-well plates and cultured for 48 hours. For *Calponin 3* knockdown, the cells were transfected with control siRNA and Calponin 3 siRNA in OPTI-MEM media and collected every 24 hours from 0 to 96 hours. For *Calponin 3* overexpression, the cells were transfected with pCMV-Myc-control and pCMV-Myc-Calponin3 overexpressed transient vector and pcDNA3.1(+) control and pcDNA3.1(+)-Calponin 3 vector. The cells were then stained with 0.4% trypan blue depending on each selected time. Cell counting was performed using Countess™ cell counting chamber slide and Countess™ automated cell counter (Invitrogen). This process was repeated three times.

Cell cycle analysis

For performing cell cycle analysis, the cells were collected and fixed with 70% EtOH and incubated at 4℃ for 48 hours. The cells were then washed with cold PBS and stained with propidium iodide (PI) (100 μg/ml) (Sigma Chemical Co. St. Louis, MO, USA) and RNase A (10 mg/ml) (Intron biotechnology) for 30 minutes in ice. After staining, the cells were introduced to a fluorescence-activated cell sorter (FACS CantoII™, BD, USA) to determine proportion of cell cycle phases.

Protein isolation and western blotting

Cells were rinsed three times with phosphate-buffered saline at room temperature, and lysed in PROPREP™ Protein Extraction Solution (Invitrogen) and placed on ice for 30 minutes. The lysates were centrifuged at 13,000 x g for 20 minutes at 4℃ and then the

supernatant was collected. The protein concentration was determined by SMART^{TM} micro BCA protein assay kit (Intron biotechnology). 12 μl of protein were resolved by 4x SDS sample buffer and was boiled at 95℃ for 5 minutes. The protein was loaded on 4-12% Bis-Tris gel (Invitrogen) at 100 volt for about 2 hours and transferred to PVDF membrane (Invitrogen) by electro-blotting at 270 mAmp constant current for 1.5 hours at 4℃. For blocking the membrane, it was incubated in 1.5% non-fat dry milk and 0.5% Tween 20-TBS buffer containing 1mM of $MgCl_2$ for 1 hour at room temperature. Primary antibodies against Calponin 3 (Santa Cruz Biotechnology, Inc. CA, USA) (1:2,000) and β-actin (Applied Biological Materials Inc. Richmond, BC, Canada) (1:5,000) were introduced to the membrane and incubated at room temperature for 1 hour. Peroxidase conjugated mouse or rabbit IgG antibody (Jackson Immunoresearch, West Grove, PA, USA) (1:5000) was added as a secondary antibody and incubated

at room temperature for 1 hour. Chemiluminescent working solution, WESTZOL^{TM} (Intron biotechnology) was decanted to membrane. The membrane was exposed to Fuji RX film for 1-5 minutes.

Immunocytochemistry of cell lines

To find out the location of Calponin 3 protein and to compare the difference of expression levels between SNU-2414A, the primary colon cancer cell line and SNU-2414B, the metastatic colon cancer cell line, immunocytochemistry was performed. The cells of each cell line were seeded on 12-well plates coated with cover slides. 24 hours after SNU-2414B cells were seeded, SNU-2414A cells were also seeded and they were cultured for another 48 hours. Then the cells were washed with 1 ml of PBS three times. 500 μl of 3.7% formaldehyde (Sigma) per each well were added for fixation. After washing twice with 1 ml of PBS, 500 μl of 0.25% Triton-100 (Merck,

Darmstadt, Germany) solution was added to the cells and they were incubated for 15 minutes at room temperature without shaking. The cells were washed twice with 1 ml of PBS. Blocking solution that included 1% Bovine Serum Albumin (BSA) (Sigma) in PBST buffer was introduced to the cells, and cells were incubated for 1 hour on a rotator. The cells were washed three times with PBS. Primary antibody, Calponin 3 was diluted with PBST (1:500) and introduced to the cells and they were incubated for overnight in a 4℃ cold room on a rotator. After being washed twice with PBST, the secondary antibody, Alexa Fluor® 488 (Invitrogen) was diluted with PBST (1:500) and added to the cells. They were then incubated for 1.5 hours at room temperature. The cells were washed three times with PBST and stained with 4', 6-diamidino-2-phenylindole (DAPI) (1:500) (Sigma) and incubated for 1.5 hours at room temperature. After being washed three times with PBST, the cover slides were collected from

the 12-well plates and were mounted with mounting medium (Dako, Cytomation, Glostrup, Denmark) on slides. To keep them preserved, the edges of cover slides were coated with clear nail polish and dried well. Finally, the slides were introduced to Confocal Laser Microscope LSM5 (Carl Zeiss, Jena, Germany).

Immunocytochemistry of paired tissues

To compare the expression level of Calponin 3 protein in paired CRC tissues, list of twenty-four patients who were underwent palliative colon resection despite of histologically proven peritoneal metastasis at Seoul National University Hospital from 2005 to 2008 was obtained. But only six paired (primary CRC and peritoneal metastatic cancer of same patient) tissue blocks were available. Matched peritoneal metastasis samples were all confirmed to metastatic adenocarcinoma.

Core tissue biopsies were obtained from individual paraffin-embedded CRCs (donor blocks) and arranged in a tissue microarray block using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea).

The staining intensity was subclassified as follows: 0, absent; 1, weak; 2, moderate; 3, strong. Two researchers identified the results without knowledge of clinical informations.

In vitro invasion assay

SNU-C4 cells were transfected with siRNA specific for the Calponin3, and cell invasiveness was determined. An *in vitro* invasion assay was carried out using a kit (Chemicon, Temecula, CA, USA), according to the manufacturer's instructions. Briefly, the assay was carried out in an invasion chamber consisting of a 24-well tissue culture plate with 12-cell culture inserts. A cell suspension of SNU-C4 in serum-free RPMI-1640 medium (Invitrogen) containing 2 mg/mL sodium

bicarbonate, 100 U/mL penicillin and 0.1 mg/mL streptomycin was added to the inserts. Each insert was placed in the lower chamber containing RPMI-1640 medium + 10% bovine calf serum. After 72 and 96 h incubation in a cell culture incubator, invasiveness was evaluated by staining cells that migrated through the extracellular matrix layer and adhered to the polycarbonate membrane at the bottom of the insert. Under 200× magnification, random fields were selected for each cell lines, and the number of cells adhering to the bottom of the insert were counted.

Results

General characteristics of cell lines and cell line culture

CRC specimens for cell line culture were collected from patients during surgeries conducted at Seoul National University (SNU) Hospital. The 6 colorectal carcinoma cell lines (SNU-2404A, SNU-2404B, SNU-2414A, SNU-2414B, SNU-2335A, SNU-2335D) were established in AR5 medium and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. SNU-2404A, SNU-2414A, and SNU-2335A originated from primary CRCs; SNU-2404B, SNU-2414B, and SNU-2335D originated from CRC peritoneal metastasis. The clinicopathologic characteristics of three paired colon cancer cell lines are summarized in Table 1. The *in vitro* characteristics of these cell lines are shown in Figure 1.

None of the cell lines were contaminated with Mycoplasma or bacteria (Figure 2). All cell lines showed high viability with relatively long

doubling times. MSI was not found in all six cell lines.

Table 1. The clinicopathologic characteristics of three paired cell lines.

Cell line	Original site of cell line	Sex/ Age	Pathology	TNM stage	MSI status
SNU-2335A	Sigmoid colon mass	F/55	M/D Adenocarcinoma	T3N2M1	MSS
SNU-2335D	Omental seeding nodule				
SNU-2404A	Ascending colon mass	F/62	M/D Adenocarcinoma	T4bN2M1	MSS
SNU-2404B	Omental seeding nodule				
SNU-2414A	Sigmoid colon mass	M/76	P/D Adenocarcinoma	T4bN2M1	MSS
SNU-2414B	Omental seeding nodule				

MSI status of the tumors were evaluated by fragmentation assay using

ABI-310.

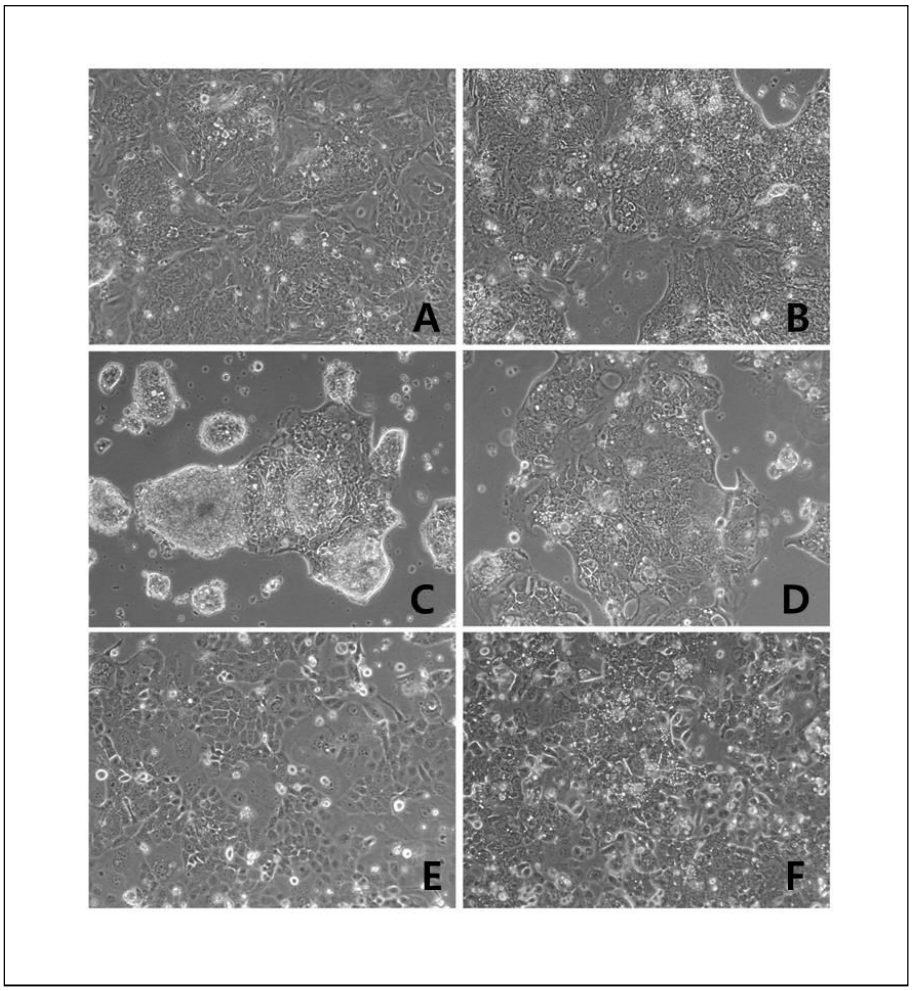

Figure 1. Phase-contrast microscopy of paired colon cancer cell lines.

(A) SNU-2335A, (B) SNU-2335D, (C) SNU-2404A, (D) SNU-2404B,

(E) SNU-2414A, (F) SNU-2414B.

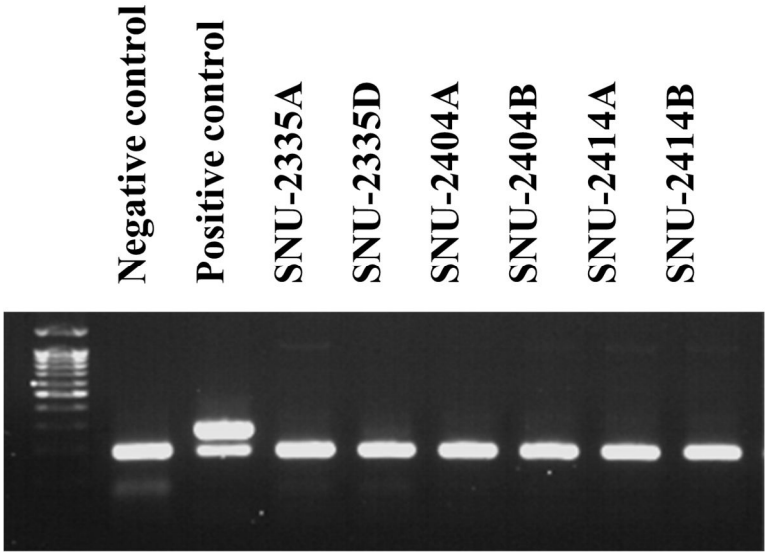

Figure 2. Mycoplasma test of 6 established cell lines

DNA profiles

DNA fingerprinting revealed that the 6 cell lines were unique (Table 2). These results definitely excluded the possibility of cell line cross-contamination.

Table 2 . DNA fingerprinting analysis using 16 STR loci

	SNU -2335A	*SNU -2335D*	*SNU -2404A*	*SNU -2404B*	*SNU -2414A*	*SNU -2414B*
D8S1179	10,15	10,15	11,14	11,14	13,15	13,15
D21S11	-	-	29	29,32.2	29	29
D7S820	12	12	10,12	10,12	9,11	9,11
CSF1P0	11,12	11,12	10,12	10,12	11	11
D3S1358	16,18	16,18	17	17	15,17	15,17
TH01	6,9	6,9	9	9	7	7
D13S317	11	11	10,11	10,11	8,12	12
D16S539	9	9	9	9	11	11
D2S1338	19,22	19,22	18,21	18,21	18,23	18,23
D19S433	14,16.2	14,16.2	13,14	13,14	14,14.2	14,14.2
vWA	14,17	14,17	14,18	14,18	16,17	16,17
TPOX	8,11	8,11	8,9	8,9	8,9	8,9
D18S51	8,16	8,16	8,14	8,14	10,14	10,14
Amelogenin	X, X	X, X	X, X	X, X	X, Y	X, Y
D5S818	10,13	10,13	11	11	13	13
FGA	26	26	23	23	20,22	20,22

Gene selection from microarray data analysis.

Because the high-density oligonucleotide microarray contains a large number of probes, 2 fold-change was used in parallel to select genes that were differentially expressed in metastatic omental seeding cell lines (SNU-2335D, SNU-2404A, SNU-2414A) compared to primary CRC cell lines (SNU-2335A, SNU-2404). In SNU-2335D and SNU-2335A cell lines, a total of 743 genes were selected as having 2 fold-changes. 399 genes were up-regulated and 344 were down-regulated in SNU-2335D cell lines. In SNU-2404B and SNU-2404A cell lines, we found 721 differentially expressed genes, 272 of which were up-regulated and 449 of which were down-regulated. In SNU-2414B and SNU-2414A cell lines, we identified 1254 differentially expressed genes, 586 of which were up-regulated and 668 of which were down-regulated.

After the individual gene selection in each cell lines, we next screened

for genes that were differentially expressed in more than two of the metastatic cell lines (Table 3 and 4). We identified 128 genes that were up-regulated and 539 genes that were down-regulated in more than two metastatic cell lines; only 5 up-regulated (Table 3, Figure 3) and 2 down-regulated (Table 4, Figure 3) genes were differentially expressed in all three metastatic cell lines. Differentially expressed gene in more than two metastatic cell lines was about 3 times more in down-regulated than up-regulated genes. In contrast, up-regulated genes were more than down-regulated genes in all three metastatic cell lines. Many of the up-regulated genes in all three metastatic cell lines are associated with actin binding (*CNN3*, *SORBS1*, *KLHL5*). Among differentially expressed genes in two metastatic cell lines, *CALD1* and *PPP1R9A* were up-regulated, *IQGAP2*, *TNS4* and *TMSB4X* were down-regulated. In addition, *EPSTI1* and *BST2* were average 3.2 and 5.4 fold up-regulated, respectively. *TRY6* (2.9 fold-change) and *SYTL5*

(4.9 fold-change) were down-regulated in all three metastatic cell lines.

Gene ontology analysis

To gene ontology analysis, we selected terms that were *p*-value <0.05 and involved at least 5 genes. We found functional terms using differentially expressed genes by each cell line set (SNU-2335A vs D, SNU-2404A vs B and SNU-2414A vs B) and selected terms that were common in three cell line sets. In biological process, we found 128, 157 and 69 terms in SNU-2335, SNU-2404, SNU-2414, respectively (Figure 4). Common terms in all three cell line sets were three, negative regulation of cell proliferation, positive regulation of cell communication and response to estrogen stimulus (Figure 4B). In negative regulation of cell proliferation and positive regulation of cell communication, > 20 genes were involved terms in all metastatic cell line sets. Terms in molecular function, cellular component and

pathway were no common.

Classification in primary colon cancer cell lines and metastatic omental seeding cell lines

To classify primary cell lines and metastatic cell lines, first we selected 156 probe sets that were *p*-value <0.05 using paired T-test. We identified per 15 genes that were the highest score genes in primary and metastatic classes. Total 30 genes were classified two groups (Figure 5). In addition, *CNN3*, *SORBS1* and *TRY6* among top 30 genes were the genes that showed significantly differential expression in all three paired cell lines (Table 3 and 4).

Table 3. Up-regulated genes in metastatic cancer cell lines

Probe Set ID	Symbol	Gene Description	Function	Fold change		
				SNU-2335D	SNU-2404B	SNU-2414B
Up-regulated genes in all three cell lines						
7917885	CNN3	calponin 3, acidic	actin binding	2.5	10.6	4.2
7935188	SORBS1	sorbin and SH3 domain containing 1	actin binding	3.5	5.3	4.6
7971296	EPSTI1	epithelial stromal interaction 1 (breast)		2.5	3.1	3.9
8035304	BST2	bone marrow stromal cell antigen 2	signal transducer activity	2.5	5.5	8.2
8094625	KLHL5	kelch-like 5 (Drosophila)	actin binding	2.3	2.1	4.0
Up-regulated genes in SNU-2335D and SNU-2404B cell lines						
7917283	MCOLN2	mucolipin 2	ion channel activity	2.3	2.8	1.3
7939559	TSPAN18	tetraspanin 18		3.1	2.6	-1.1
7951271	MMP1	matrix metallopeptidase 1 (interstitial collagenase)	interstitial collagenase activity	5.0	5.0	-1.1
8001197	NETO2	neuropilin (NRP) and tolloid (TLL)-like 2	receptor activity	2.8	2.2	-1.7
8043945	MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4	nucleotide binding	5.8	2.2	1.7
8062844	C20orf121	chromosome 20 open reading frame 121	transporter activity	2.1	2.1	1.3
8092095	TNIK	TRAF2 and NCK interacting kinase	nucleotide binding	2.4	2.3	1.3
8120402	BAG2	BCL2-associated athanogene 2	protein binding	2.3	2.2	1.1
8121277	AIM1	absent in melanoma 1	molecular_function	2.3	2.3	-1.4
Up-regulated genes in SNU-2335 and SNU-2414 cell lines						
7899627	TINAGL1	tubulointerstitial nephritis antigen-like 1	cysteine-type endopeptidase activity	2.0	-1.3	2.2
7902541	IFI44L	interferon-induced protein 44-like		5.2	1.2	94.1
7902553	IFI44	interferon-induced protein 44		5.5	-1.5	51.5
7906400	IFI16	interferon, gamma-inducible protein 16	DNA binding	6.7	1.3	5.7
7907893	MR1	major histocompatibility complex, class I-related	MHC class I receptor activity	3.2	1.5	2.4
7909789	TGFB2	transforming growth factor, beta 2	beta-amyloid binding	22.5	1.3	8.7
7914127	IFI6	interferon, alpha-inducible protein 6	protein binding	7.2	-1.4	16.4
7916584	TACSTD2	tumor-associated calcium signal transducer 2	receptor activity	2.0	-1.8	2.3
7918936	VTCN1	V-set domain containing T cell activation inhibitor 1		2.8	1.0	4.0
7927681	BICC1	bicaudal C homolog 1 (Drosophila)	RNA binding	3.0	1.9	8.2
7929047	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	binding	12.4	-1.3	9.6
7929052	IFIT3	interferon-induced protein with tetratricopeptide repeats 3	binding	7.8	-1.1	13.7
7929065	IFIT1	interferon-induced protein with tetratricopeptide repeats 1	binding	15.6	-1.6	39.2
7929072	IFIT5	interferon-induced protein with tetratricopeptide repeats 5	binding	2.5	1.2	3.2
7938035	TRIM22	tripartite motif-containing 22	transcription factor activity	5.8	1.2	10.9
7940775	RARRES3	retinoic acid receptor responder (tazarotene induced) 3		4.9	-1.3	4.7
7941505	CST6	cystatin E/M	cysteine protease inhibitor activity	3.9	1.3	2.3
7943413	BIRC3	baculoviral IAP repeat-containing 3	ubiquitin-protein ligase activity	2.8	-4.0	2.7

Table 3. Continued

Table 3. Continued

Probe Set ID	Symbol	Gene Description	Function	Fold change		
				SNU-2335D	SNU-2404B	SNU-2414B
7944722	STS-1	Cbl-interacting protein Sts-1		3.9	-1.4	2.6
7946983	SAA2	serum amyloid A2		2.4	1.3	2.9
7958884	OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	RNA binding	2.7	-3.3	16.0
7958895	OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	RNA binding	4.8	-2.3	10.9
7958913	OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	RNA binding	12.1	1.1	38.7
7962250	CPNE8	copine VIII		2.0	-1.3	4.2
7964119	STAT2	signal transducer and activator of transcription 2, 113kDa	transcription factor activity	2.5	-1.2	2.9
7965573	NTN4	netrin 4		2.0	1.0	2.0
7967117	OASL	2'-5'-oligoadenylate synthetase-like	DNA binding	5.8	-1.1	2.4
7974316	FRMD6	FERM domain containing 6	binding	4.3	1.2	3.5
7974366	PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa	rhodopsin-like receptor activity	3.8	-1.1	5.0
7976443	IFI27	interferon, alpha-inducible protein 27	molecular_function	2.5	-1.1	12.3
7981068	SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	serine-type endopeptidase inhibitor activity	3.5	-1.5	2.7
7982597	THBS1	thrombospondin 1	endopeptidase inhibitor activity	2.1	-4.1	6.6
7995783	MT2A	metallothionein 2A	copper ion binding	3.8	-1.5	2.1
8004184	XAF1	XIAP associated factor-1	zinc ion binding	3.5	-1.0	22.4
8019622	TMEM106A	transmembrane protein 106A		2.4	1.2	2.2
8020110	RAB31	RAB31, member RAS oncogene family	nucleotide binding	3.1	1.1	4.5
8022692	DSC3	desmocollin 3	calcium ion binding	2.2	-1.3	2.1
8033257	C3	complement component 3	endopeptidase inhibitor activity	5.0	1.3	20.5
8037750	IGFL4	IGF-like family member 4		2.4	-2.5	2.7
8040080	RSAD2	radical S-adenosyl methionine domain containing 2	catalytic activity	9.3	1.2	5.1
8041179	CLIP4	CAP-GLY domain containing linker protein family, member 4		2.7	-1.2	2.7
8041206	LBH	limb bud and heart development homolog (mouse)		8.9	1.1	3.1
8041422	RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	signal transducer activity	5.4	1.3	6.5
8047784	AK127271			2.4	1.3	2.1
8048940	SP100	SP100 nuclear antigen	DNA binding	2.2	-1.7	4.2
8049123	ALPP	alkaline phosphatase, placental (Regan isozyme)	magnesium ion binding	3.5	1.7	2.8
8056285	IFIH1	interferon induced with helicase C domain 1	nucleotide binding	3.6	-1.4	6.5
8057744	STAT1	signal transducer and activator of transcription 1, 91kDa	transcription factor activity	2.9	-1.0	5.2
8059650	SP110	SP110 nuclear body protein	DNA binding	3.6	1.3	17.5
8059854	ARL4C	ADP-ribosylation factor-like 4C	nucleotide binding	2.4	-2.7	3.1
8065136	RRBP1	ribosome binding protein 1 homolog 180kDa (dog)	receptor activity	2.0	-1.7	2.0
8066117	SAMHD1	SAM domain and HD domain 1	catalytic activity	3.2	-1.2	2.8
8068697	MX2	myxovirus (influenza virus) resistance 2 (mouse)	nucleotide binding	6.3	1.2	37.2
8068713	MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	nucleotide binding	4.8	1.1	24.8

Table 3. Continued

Table 3. Continued

Probe Set ID	Symbol	Gene Description	Function	Fold change		
				SNU-2335D	SNU-2404B	SNU-2414B
8070489	RIPK4	receptor-interacting serine-threonine kinase 4	nucleotide binding	2.0	-1.1	2.3
8071155	USP18	ubiquitin specific peptidase 18	ubiquitin thiolesterase activity	3.0	1.4	3.3
8073068	APOBEC3C	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3C	molecular_function	2.5	1.6	3.2
8082100	PARP14	poly (ADP-ribose) polymerase family, member 14	NAD+ ADP-ribosyltransferase activity	2.1	1.1	5.0
8084732	RTP4	receptor (chemosensory) transporter protein 4	protein binding	2.7	1.1	4.3
8090018	PARP9	poly (ADP-ribose) polymerase family, member 9	molecular_function	2.7	1.0	5.2
8092348	LAMP3	lysosomal-associated membrane protein 3		2.5	-1.0	5.8
8096335	HERC6	hect domain and RLD 6	ubiquitin-protein ligase activity	2.5	-1.1	4.0
8096361	HERC5	hect domain and RLD 5	ubiquitin-protein ligase activity	4.7	-1.0	3.2
8096602	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	protein tyrosine phosphatase activity	2.9	-1.5	3.3
8101126	CXCL10	chemokine (C-X-C motif) ligand 10	chemokine activity	3.2	-1.1	2.1
8101701	PPM1K	protein phosphatase 1K (PP2C domain containing)	magnesium ion binding	2.1	1.2	2.3
8103563	FLJ20035	hypothetical protein FLJ20035	nucleic acid binding	5.9	-3.3	8.6
8103601	DKFZp781D1175			3.1	-3.1	4.2
8107044	LRAP	leukocyte-derived arginine aminopeptidase	aminopeptidase activity	3.0	1.8	3.2
8114010	IRF1	interferon regulatory factor 1	transcription factor activity	2.1	1.2	2.7
8115147	CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	cytokine binding	2.1	1.3	8.3
8125512	TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	nucleotide binding	2.2	-1.3	2.0
8136347	CALD1	caldesmon 1	actin binding	2.4	1.1	2.1
8138553	FAM126A	family with sequence similarity 126, member A	signal transducer activity	3.1	-1.2	2.8
8138805	CPVL	carboxypeptidase, vitellogenic-like	serine carboxypeptidase activity	2.8	-2.3	4.7
8140967	SAMD9	sterile alpha motif domain containing 9		7.1	-1.2	4.2
8140971	SAMD9L	sterile alpha motif domain containing 9-like		12.4	-1.2	12.1
8143327	PARP12	poly (ADP-ribose) polymerase family, member 12	nucleic acid binding	2.4	-1.2	3.2
8148572	LY6E	lymphocyte antigen 6 complex, locus E	GPI anchor binding	2.1	-1.3	2.5
8150509	PLAT	plasminogen activator, tissue	peptidase activity	2.2	-1.0	2.6
8154233	CD274	CD274 molecule	receptor activity	2.7	-1.2	2.4
8154245	PDCD1LG2	programmed cell death 1 ligand 2	molecular_function	3.5	1.4	3.4
8160559	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	nucleotide binding	5.7	-1.1	5.7
8168749	SRPX2	sushi-repeat-containing protein, X-linked 2		2.1	-1.2	2.3
8175871	L1CAM	L1 cell adhesion molecule	protein binding	6.8	-1.3	7.8
8178867	TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	nucleotide binding	2.2	-1.3	2.0

Table 3. Continued

Table 3. Continued

Probe Set ID	Symbol	Gene Description	Function	Fold change		
				SNU-2335D	SNU-2404B	SNU-2414B
Up-regulated genes in SNU-2404 and SNU-2414 cell lines						
7901748	FLJ10986	hypothetical protein FLJ10986		-1.0	2.1	2.0
7902518	GIPC2	GIPC PDZ domain containing family, member 2	protein binding	-1.9	3.3	3.7
7918857	TSPAN2	tetraspanin 2		1.5	5.8	3.5
7929032	FAS	Fas (TNF receptor superfamily, member 6)	transmembrane receptor activity	1.4	2.1	2.4
7935337	PIK3AP1	phosphoinositide-3-kinase adaptor protein 1	kinase activity	1.1	2.6	5.7
7942453	PLEKHB1	pleckstrin homology domain containing, family B (evectins) member 1	signal transducer activity	1.0	2.3	2.4
7957452	CART1	cartilage paired-class homeoprotein 1	transcription factor activity	-1.6	2.2	2.6
7959856	PIWIL1	piwi-like 1 (Drosophila)	single-stranded RNA binding	-1.3	7.7	6.1
7983828	TEX9	testis expressed 9		1.3	6.3	2.9
8019334	NOTUM	notum pectinacetylesterase homolog (Drosophila)		-1.1	2.1	2.9
8020352	C21orf15	chromosome 21 open reading frame 15		-1.3	3.8	2.3
8020762	DSG3	desmoglein 3 (pemphigus vulgaris antigen)	calcium ion binding	1.8	3.6	2.6
8055992	ACVR1C	activin A receptor, type IC	nucleotide binding	1.3	2.1	2.1
8072710	APOL6	apolipoprotein L, 6	lipid transporter activity	1.5	2.3	3.0
8083779	SERPINI1	serpin peptidase inhibitor, clade I (neuroserpin), member 1	serine-type endopeptidase inhibitor activity	-2.8	2.9	2.6
8092169	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	signal transducer activity	1.8	3.5	3.5
8102532	PDE5A	phosphodiesterase 5A, cGMP-specific	nucleotide binding	1.9	2.8	2.3
8102912	TBC1D9	TBC1 domain family, member 9 (with GRAM domain)	Rab GTPase activator activity	1.0	2.2	3.9
8106448	PDE8B	phosphodiesterase 8B	two-component response regulator activity	1.0	5.8	2.3
8113369	SLCO4C1	solute carrier organic anion transporter family, member 4C1	transporter activity	-1.1	3.1	4.0
8117343	HFE	hemochromatosis	iron ion binding	-1.0	3.7	2.3
8131666	ITGB8	integrin, beta 8	receptor activity	1.9	3.1	5.1
8131957	SNX10	sorting nexin 10	protein binding	1.2	3.3	4.0
8134351	PPP1R9A	protein phosphatase 1, regulatory (inhibitor) subunit 9A	actin binding	-1.2	2.3	2.4
8161755	ALDH1A1	aldehyde dehydrogenase 1 family, member A1	retinal dehydrogenase activity	-10.6	2.6	8.6
8168557	SH3BGRL	SH3 domain binding glutamic acid-rich protein like	SH3	1.0	13.6	2.1
8173955	SYTL4	synaptotagmin-like 4 (granuphilin-a)	transporter activity	1.0	2.1	2.6

Table 4. Down-regulated genes in metastatic cancer cell lines

Probe Set ID	Symbol	Gene Description	Function	Fold change		
				SNU-2335D	SNU-2404B	SNU-2414B
Down-regulated genes in all three cell lines						
8136790	TRY6	trypsinogen C		-2.2	-2.6	-4.0
8166747	SYTL5	synaptotagmin-like 5	protein binding	-8.5	-2.7	-3.6
Dowun-regulated genes in SNU-2335D and SNU-2404B cell						
7898623	UBXD3	UBX domain containing 3		-2.6	-2.3	1.5
7962559	SLC38A4	solute carrier family 38, member 4	amino acid transporter activity	-4.0	-3.5	1.2
7985317	KIAA1199	KIAA1199		-4.2	-2.3	-1.2
8005733	LOC729490	similar to nuclear receptor co-repressor 1		-2.8	-2.5	-1.2
8020141	APCDD1	adenomatosis polyposis coli down-regulated 1		-6.7	-2.8	-1.0
8026490	LOC729642	hypothetical protein LOC729642		-5.4	-5.6	-1.2
8028955	CYP2B7P1	cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1	iron ion binding	-2.1	-2.8	-1.0
8062728	SGK2	serum/glucocorticoid regulated kinase 2	nucleotide binding	-2.0	-2.4	-1.5
8086540	SLC6A20	solute carrier family 6 (proline IMINO transporter), member 20	transporter activity	-2.1	-2.8	-1.9
8088491	CADPS	Ca2+-dependent secretion activator	calcium ion binding	-2.3	-3.2	1.1
8091306	PLSCR4	phospholipid scramblase 4	calcium ion binding	-2.5	-2.5	2.4
8095819	GENX-3414	genethonin 1	catalytic activity	-2.2	-4.0	1.3
8099467	FGFBP1	fibroblast growth factor binding protein 1	heparin binding	-2.0	-3.0	1.1
8099633	PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	nucleotide binding	-2.4	-2.7	1.7
8103094	NR3C2	nuclear receptor subfamily 3, group C, member 2	transcription factor activity	-2.1	-2.1	-1.1
8103951	ACSL1	acyl-CoA synthetase long-chain family member 1	magnesium ion binding	-2.1	-2.3	1.5
8118242	LY6G6D	lymphocyte antigen 6 complex, locus G6D		-2.2	-3.2	-1.5
8133233	AUTS2	autism susceptibility candidate 2	molecular_function	-2.1	-2.5	-1.6
8135661	CFTR	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	nucleotide binding	-3.5	-2.2	-1.7
8139033	AOAH	acyloxyacyl hydrolase (neutrophil)	lipoprotein lipase activity	-2.3	-3.2	3.3
8148315	POU5F1	POU domain, class 5, transcription factor 1	transcription factor activity	-2.7	-3.8	-1.2
8151042	TTPA	tocopherol (alpha) transfer protein (ataxia (Friedreich-like) with vitamin E deficiency)	transporter activity	-2.6	-5.5	1.2
8152617	HAS2	hyaluronan synthase 2	transferase activity, transferring glycosyl groups	-3.0	-21.1	2.7
8154295	IL33	interleukin 33	cytokine activity	-6.7	-3.8	-1.2
8178070	LY6G6D	lymphocyte antigen 6 complex, locus G6D		-2.2	-3.6	-1.4
8179309	LY6G6D	lymphocyte antigen 6 complex, locus G6D		-2.2	-3.6	-1.4

Table 4. Continued

Table 4. Continued

Probe Set ID	Symbol	Gene Description	Function	Fold change		
				SNU-2335D	SNU-2404B	SNU-2414B
Dowun-reulated genes in SNU-2335 and SNU-2414 cell lines						
7901788	NFIA	nuclear factor I/A	transcription factor activity	-2.8	1.1	-3.8
7925691	ZNF124	zinc finger protein 124	DNA binding	-2.5	1.0	-2.3
7937016	CLRN3	clarin 3		-3.1	-1.0	-3.2
7944164	TMPRSS4	transmembrane protease, serine 4	serine-type endopeptidase activity	-2.1	1.0	-2.4
7951077	SESN3	sestrin 3		-2.3	-1.9	-7.4
7953965	EIF2S3	eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	nucleotide binding	-2.3	-1.1	-2.7
7969171	DLEU1	deleted in lymphocytic leukemia, 1		-2.0	1.1	-4.7
7969916	ERCC5	excision repair cross-complementing rodent repair deficiency, complementation group 5 (xeroderma pigmentosum, complementation group G (Cockayne syndrome))	DNA binding	-2.1	1.8	-2.7
7989146	MNS1	meiosis-specific nuclear structural 1		-2.1	4.2	-2.2
8013015	PRR6	proline rich 6	carbon-sulfur lyase activity	-2.1	-1.6	-2.1
8072587	SLC5A1	solute carrier family 5 (sodium/glucose cotransporter), member 1	transporter activity	-3.7	11.4	-2.7
8081431	ALCAM	activated leukocyte cell adhesion molecule	receptor binding	-2.6	-1.5	-2.0
8081548	PVRL3	poliovirus receptor-related 3	receptor activity	-4.0	1.7	-2.6
8104570	FAM105A	family with sequence similarity 105, member A		-4.7	2.8	-2.6
8106354	IQGAP2	IQ motif containing GTPase activating protein 2	actin binding	-2.4	5.0	-2.7
8123246	SLC22A3	solute carrier family 22 (extraneuronal monoamine transporter), member 3	ion transporter activity	-2.0	-1.0	-6.9
8124388	HIST1H3B	histone cluster 1, H3b		-2.1	1.5	-2.4
8141328	CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5	monooxygenase activity	-2.2	-1.6	-5.0
8151795	CDH17	cadherin 17, LI cadherin (liver-intestine)	transporter activity	-2.1	-1.1	-2.3
8155673	PIP5K1B	phosphatidylinositol-4-phosphate 5-kinase, type I, beta	protein binding	-2.8	-1.1	-2.1
8160531	C9orf72	chromosome 9 open reading frame 72		-2.1	-1.1	-2.6
8162006	GKAP1	G kinase anchoring protein 1	protein binding	-2.2	1.3	-2.4
8166219	SYAP1	synapse associated protein 1, SAP47 homolog (Drosophila)		-2.1	-1.2	-2.0
8166278	SCML1	sex comb on midleg-like 1 (Drosophila)	transcription factor activity	-4.1	1.3	-2.6
8166335	PDHA1	pyruvate dehydrogenase (lipoamide) alpha 1	pyruvate dehydrogenase (acetyl-transferring) activity	-2.6	1.0	-2.3
8166455	PRDX4	peroxiredoxin 4	thioredoxin peroxidase activity	-2.0	1.1	-3.2
8166525	POLA1	polymerase (DNA directed), alpha 1	nucleotide binding	-2.7	1.2	-2.4
8166826	USP9X	ubiquitin specific peptidase 9, X-linked	cysteine-type endopeptidase activity	-2.1	-1.4	-2.5
8167163	CXorf24	chromosome X open reading frame 24		-3.0	-1.3	-2.1
8167305	EBP	emopamil binding protein (sterol isomerase)	C-8 sterol isomerase activity	-2.0	1.1	-2.0
8167654	GSPT2	G1 to S phase transition 2	nucleotide binding	-2.9	1.1	-4.3

Table 4. Continued

Table 4. Continued

Probe Set ID	Symbol	Gene Description	Function	Fold change		
				SNU-2335D	SNU-2404B	SNU-2414B
8171352	TRAPPC2	trafficking protein particle complex 2	intracellular transporter activity	-3.6	-1.4	-2.6
8171381	FANCB	Fanconi anemia, complementation group B		-2.9	1.4	-3.3
8171561	SCML2	sex comb on midleg-like 2 (Drosophila)	transcription factor activity	-3.3	1.1	-4.5
8171758	SCARNA9	small Cajal body-specific RNA 9		-2.6	-1.3	-5.4
8171762	RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 3	nucleotide binding	-2.1	-1.1	-3.1
8172158	CASK	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	nucleotide binding	-2.8	-1.2	-2.6
8172358	UXT	ubiquitously-expressed transcript	microtubule binding	-2.7	1.0	-2.4
8172425	SLC38A5	solute carrier family 38, member 5		-2.2	7.8	-3.0
8174985	SMARCA1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1	nucleotide binding	-4.8	1.1	-2.6
8177003	SLC25A6	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6	transporter activity	-2.6	-1.3	-2.0
Down-regulated genes in SNU-2404 and SNU-2414 cell lines						
7909332	CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	GPI anchor binding	1.3	-2.0	-2.3
7936144	COL17A1	collagen, type XVII, alpha 1	structural molecule activity	-1.2	-2.1	-2.1
7942064	GAL	galanin	neuropeptide hormone activity	2.0	-2.2	-2.7
7950810	SYTL2	synaptotagmin-like 2	transporter activity	-1.8	-3.0	-6.7
7952335	LOC85391	RNA, small nucleolar		1.1	-2.1	-2.4
7972297	ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	nucleotide binding	3.1	-6.1	-2.6
7972713	EFNB2	ephrin-B2	ephrin receptor binding	2.1	-5.1	-4.1
7977786	SLC7A7	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	basic amino acid permease activity	3.3	-4.5	-2.7
8011415	TAX1BP3	Tax1 (human T-cell leukemia virus type I) binding protein 3	receptor activity	1.3	-2.6	-2.5
8013399	ULK2	unc-51-like kinase 2 (C. elegans)	nucleotide binding	-1.3	-2.1	-3.5
8015016	TNS4	tensin 4	actin binding	1.5	-2.2	-3.1
8025984	FLJ14959	hypothetical protein FLJ14959	nucleic acid binding	-1.4	-2.3	-5.3
8029098	CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	GPI anchor binding	-1.2	-15.1	-4.7
8066493	SLPI	secretory leukocyte peptidase inhibitor	serine-type endopeptidase inhibitor activity	1.8	-9.6	-2.8
8067007	TMSB4X	thymosin, beta 4, X-linked	actin binding	-1.7	-2.1	-2.5
8071420	SERPIND1	serpin peptidase inhibitor, clade D (heparin cofactor), member 1	serine-type endopeptidase inhibitor activity	1.1	-2.2	-5.2
8081645	C3orf52	chromosome 3 open reading frame 52		1.8	-3.3	-2.2
8084717	ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	beta-galactoside alpha-2,6-sialyltransferase activity	1.3	-4.2	-4.5
8087530	CAMKV	CaM kinase-like vesicle-associated	protein serine	1.1	-3.4	-2.1
8100464	NMU	neuromedin U	receptor binding	1.1	-2.7	-2.5
8102232	LEF1	lymphoid enhancer-binding factor 1	DNA binding	1.1	-4.5	-2.7
8103769	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	prostaglandin E receptor activity	-1.2	-3.7	-4.0

Table 4. Continued

Table 4. Continued

Probe Set ID	Symbol	Gene Description	Function	Fold change		
				SNU-2335D	SNU-2404B	SNU-2414B
8110932	SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	receptor activity	1.0	-2.1	-2.7
8113717	MGC32805	hypothetical protein MGC32805		1.2	-7.1	-2.2
8114468	SNORD63	small nucleolar RNA, C/D box 63		-1.4	-2.0	-2.3
8120967	NT5E	5'-nucleotidase, ecto (CD73)	nucleotide binding	2.2	-7.8	-3.5
8126729	CLIC5	chloride intracellular channel 5	voltage-gated chloride channel activity	1.3	-2.1	-9.4
8136801	TRY6	trypsinogen C		-1.8	-3.0	-2.6
8140668	SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	chemorepellant activity	1.3	-7.0	-2.2
8151730	CALB1	calbindin 1, 28kDa	vitamin D binding	-1.1	-6.7	-10.2
8158240	TMSB4X	thymosin, beta 4, X-linked	actin binding	-1.8	-2.4	-2.9
8161964	FRMD3	FERM domain containing 3	binding	-1.0	-3.8	-3.1
8163618	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	cytokine activity	1.3	-3.0	-2.2
8171802	ACOT9	acyl-CoA thioesterase 9	acetyl-CoA hydrolase activity	-1.9	-2.1	-2.3

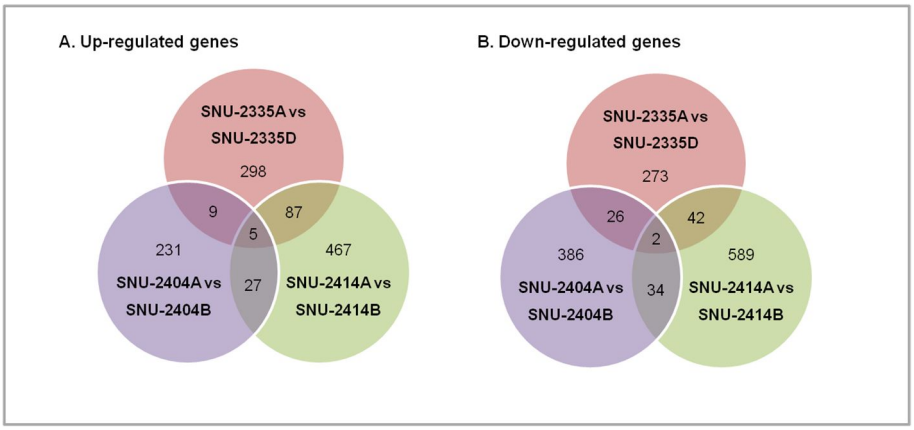

Figure 3. Numbers represent gene identified as differentially expressed between primary CRC versus metastatic omental seeding cell lines. Venn diagram shows the overlap of differentially expressed genes from 2 fold change. (A) Up-regulated genes, (B) Down-regulated genes

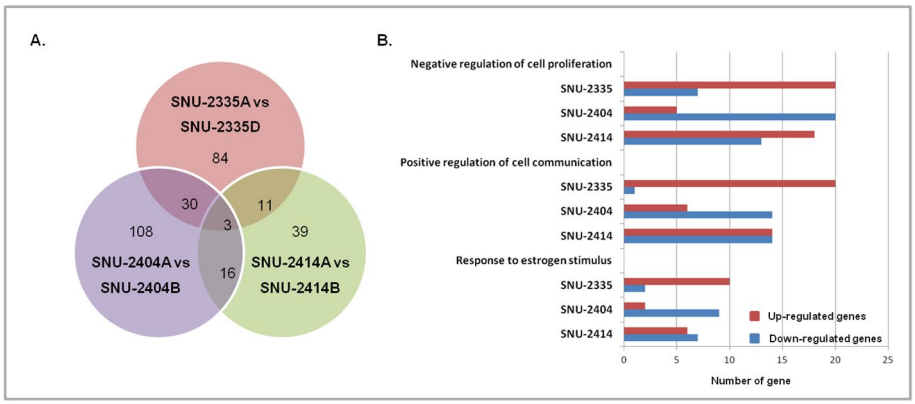

Figure 4. Functional analysis. (A) Venn diagram of biological process. (B) Differentially expressed genes of 3 common biological processes in three metastatic cell lines.

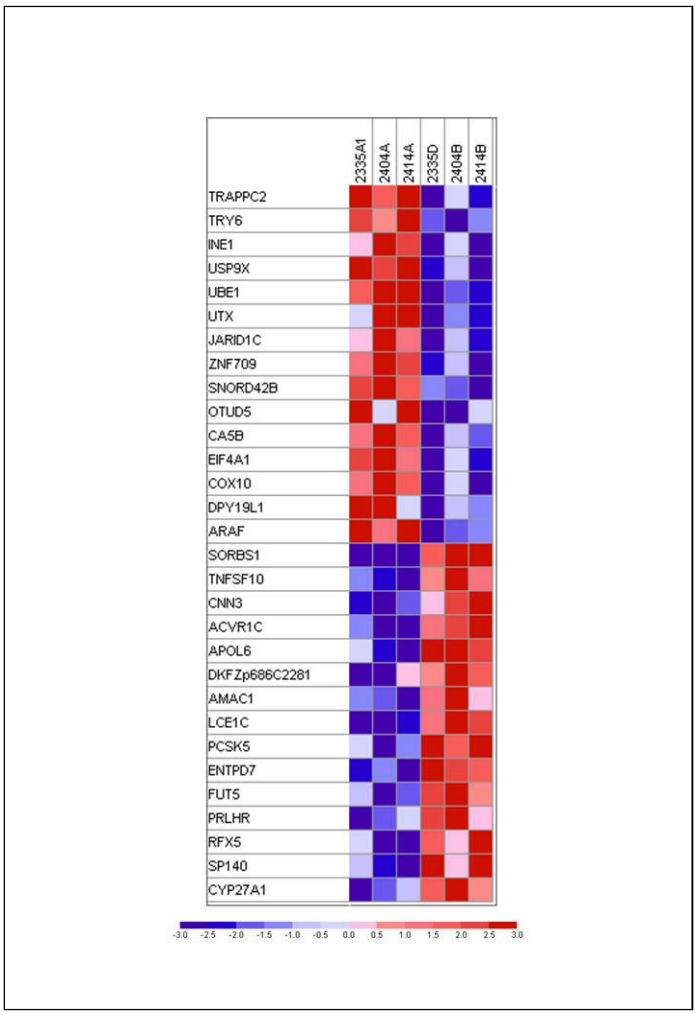

Figure 5. Classification in primary CRC cell lines vs metastatic cell lines. Genes selected for the highest score in primary CRC class (SNU-2335A, SNU-2404A, SNU-2414B) and metastatic class (SNU-2335D, SNU-2404B, SNU-2414B).

Calponin 3 expression analysis in colorectal cancer cell lines by RT-PCR

Calponin 3 mRNA expression levels were examined in paired there CRC cell lines. *Calponin* 3 gene was weakly expressed in parent cell lines, SNU-2335A, SNU-2404A and SNU-2414A cell lines, whereas this gene was upregulated in metastatic cell lines, SNU-2335D, SNU-2404B and SNU-2414B (Figure 6). *Calponin 3* expression levels were also examined in 32 colorectal cancer cell lines. In agarose gel electrophoresis stained with ethidium bromide, amplified DNA fragments were absent in 3 of the 32 CRC cell lines, i.e., in SNU-175, Caco-2 and SW-403. Of the remaining 29 cell lines, 10 (SNU-61, SNU-81, SNU-283, SNU-407, SNU-769B, SNU-C1, Colo-201, HT-29, SW-480 and WiDr) exhibited weak expression, whereas 19 of the CRC cell lines (SNU-503, SNU-769A, SNU-1033, SNU-1040, SNU-1047, SNU-1197, SNU-C2A, SNU-C4, SNU-C5, Colo-205, Colo-320,

DLD1, HCT-8, HCT-15, HCT-116, LoVo, LS174T, NCI-H716 and SW-1116) exhibited strong expression (Figure 7).

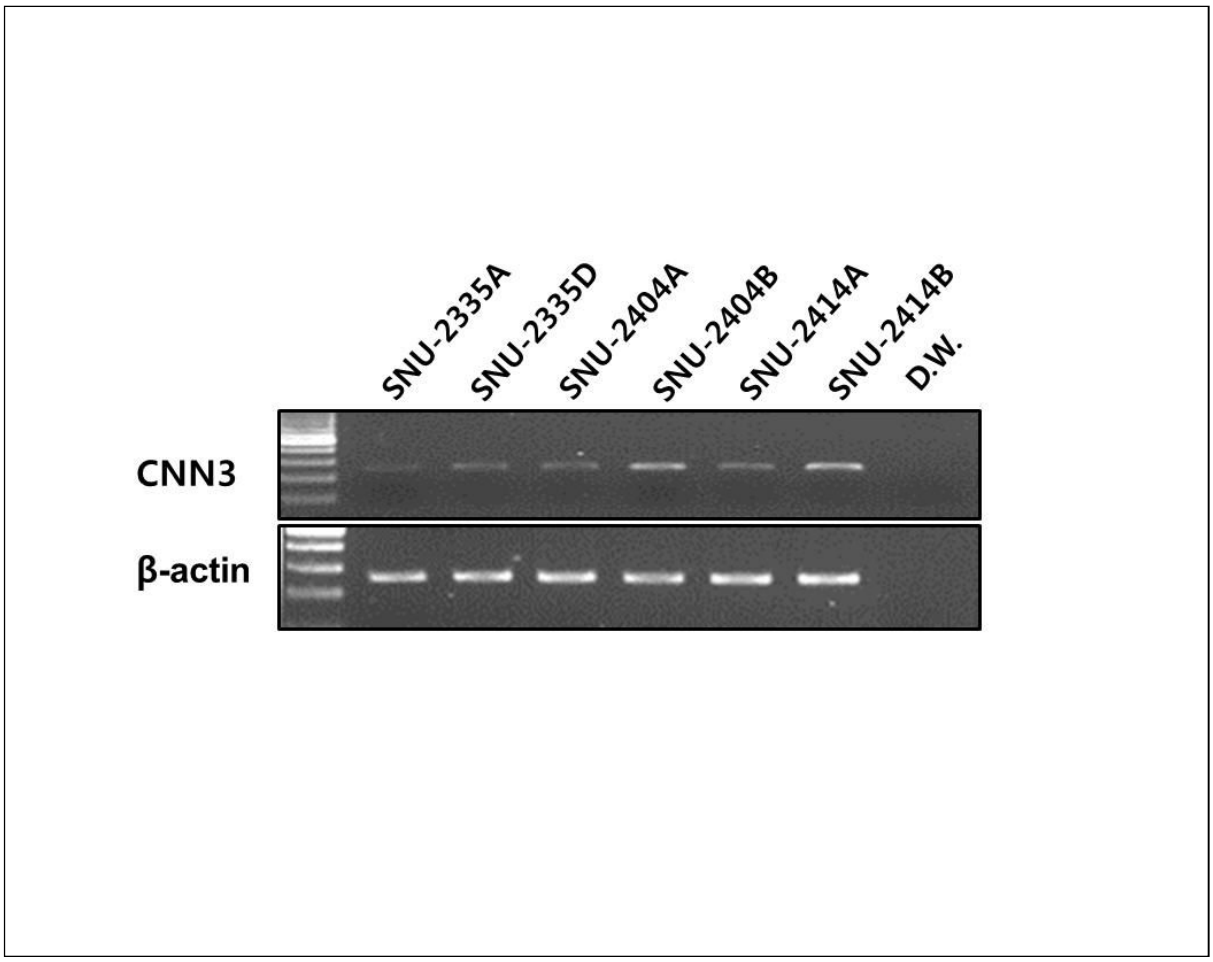

Figure 6. *Calponin 3* expression analysis in paired three colon cancer cell lines.

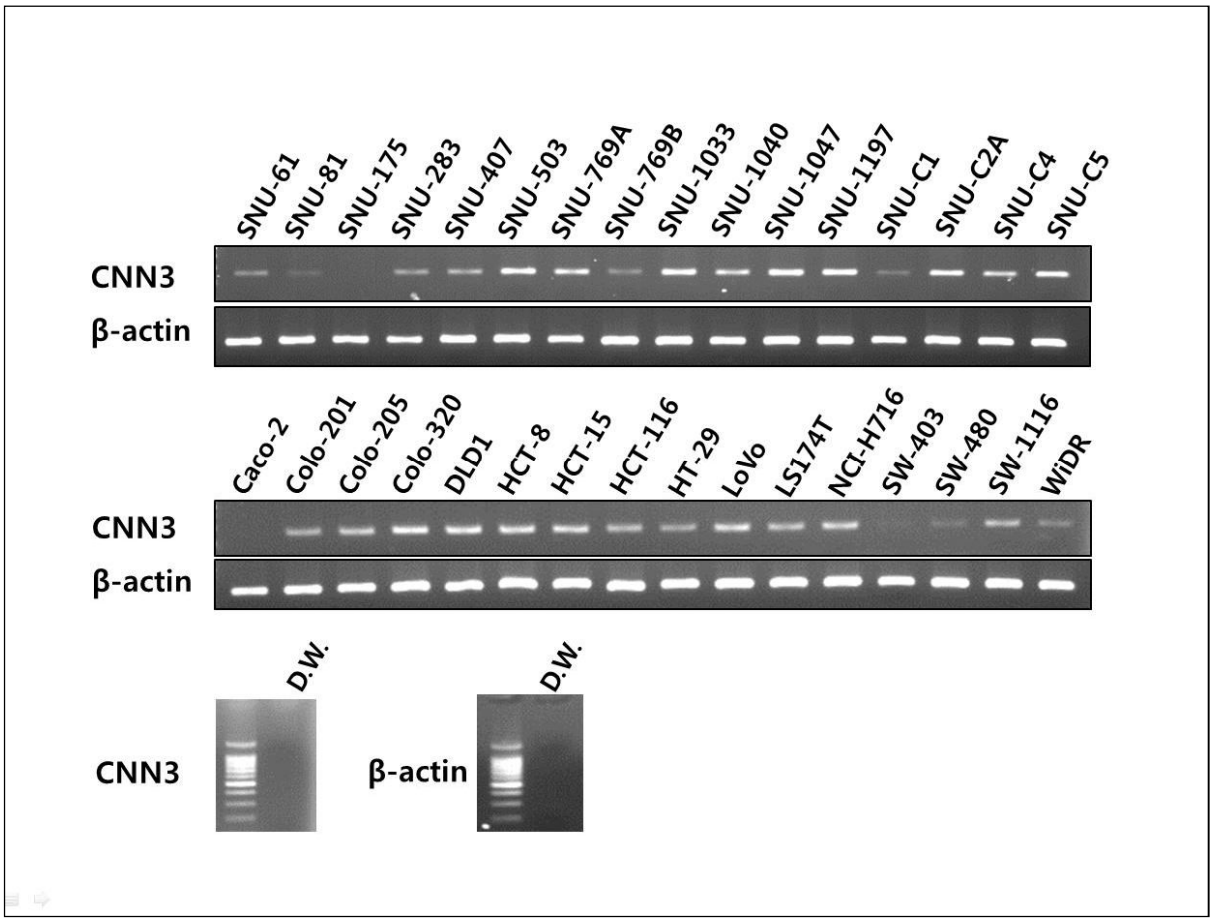

Figure 7. *Calponin 3* mRNA expression analysis in 32 CRC cell lines.

Calponin 3 expression analysis in CRC cell lines by western blotting

Calponin 3 protein expression levels were examined in paired three CRC cell lines. *Calponin* 3 gene was absent or weakly expressed in parent cell lines, SNU-2335A, SNU-2404A and SNU-2414A cell lines, whereas this gene was upregulated in metastatic cell lines, SNU-2335D, SNU-2404B and SNU-2414B (Figure 8). Calponin 3 protein expression levels were also examined in other CRC cell lines, SNU-C5, HCT-8, HCT-15, HCT-116, HT-29, Colo-205, LoVo, SW480, SW620, KM12C, KM12SM and KM12L4. Calponin 3 protein were absent or weakly expressed in HT-29, Colo-205, LoVo, SW-480 and KM12C cell lines, whereas SNU-C5, HCT-8, HCT-15, HCT-116, SW-620, KM12SM and KM12L4 CRC cell lines exhibited strong expression (Figure 8).

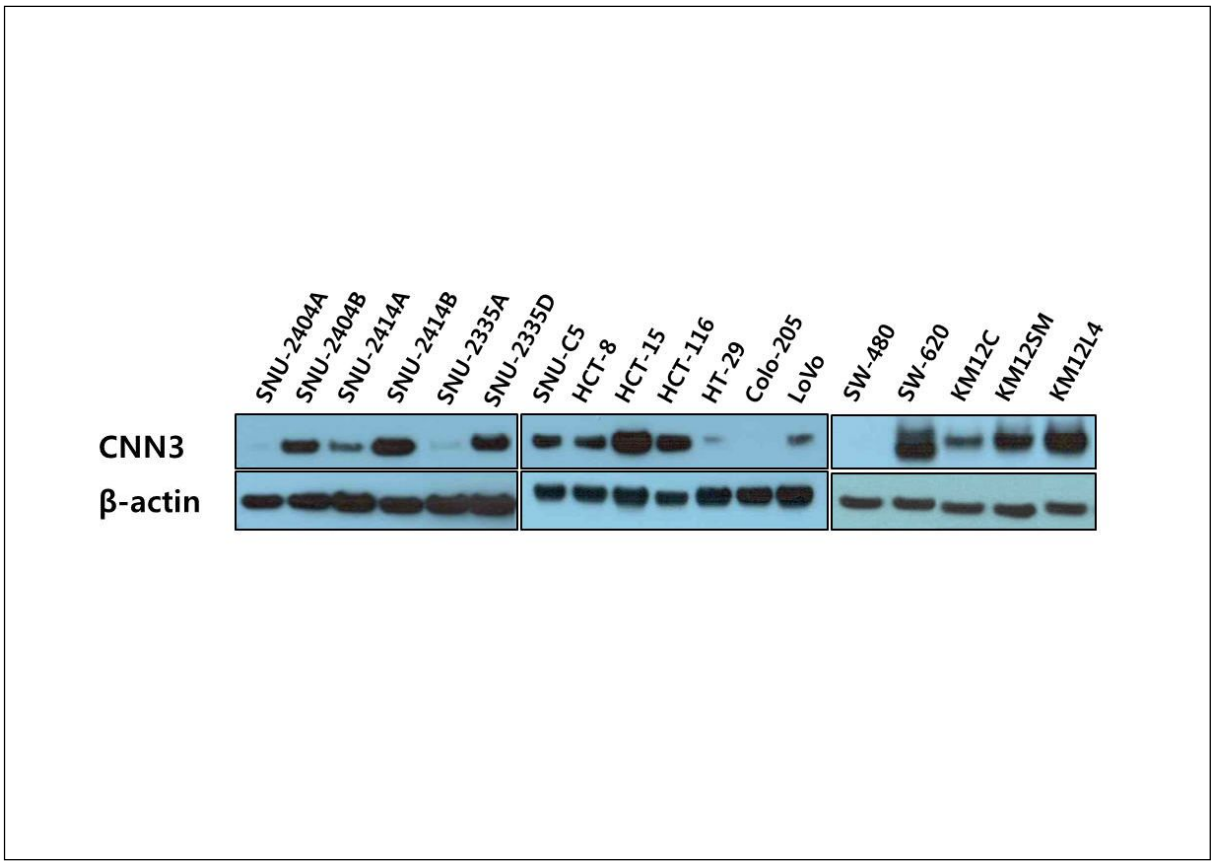

Figure 8. Calponin 3 protein expression analysis in CRC cell lines.

Increased cell proliferation by overexpression of Calponin 3 gene

HT-29 colorectal cancer cell line was selected by expressing lower level of *Calponin 3* gene and protein in RT-PCR and western blotting results. To examine the proliferation ability of pCMV-Myc control vector and pCMV-Myc-Calponin 3 vector transfected HT-29 cells, cell counting was performed. We demonstrated that pCMV-Myc control vector transfected HT-29 cells grow faster comparing to pCMV-Myc-Calponin 3 tranfected HT-29 cells. The cell numbers of pCMV-Myc-Calponin 3 transfected HT-29 cell line started increasing 24 hours after transfection and was maintained with higher growth rate than pCMV-Myc control vector transfected HT-29 cells (Figure 9).

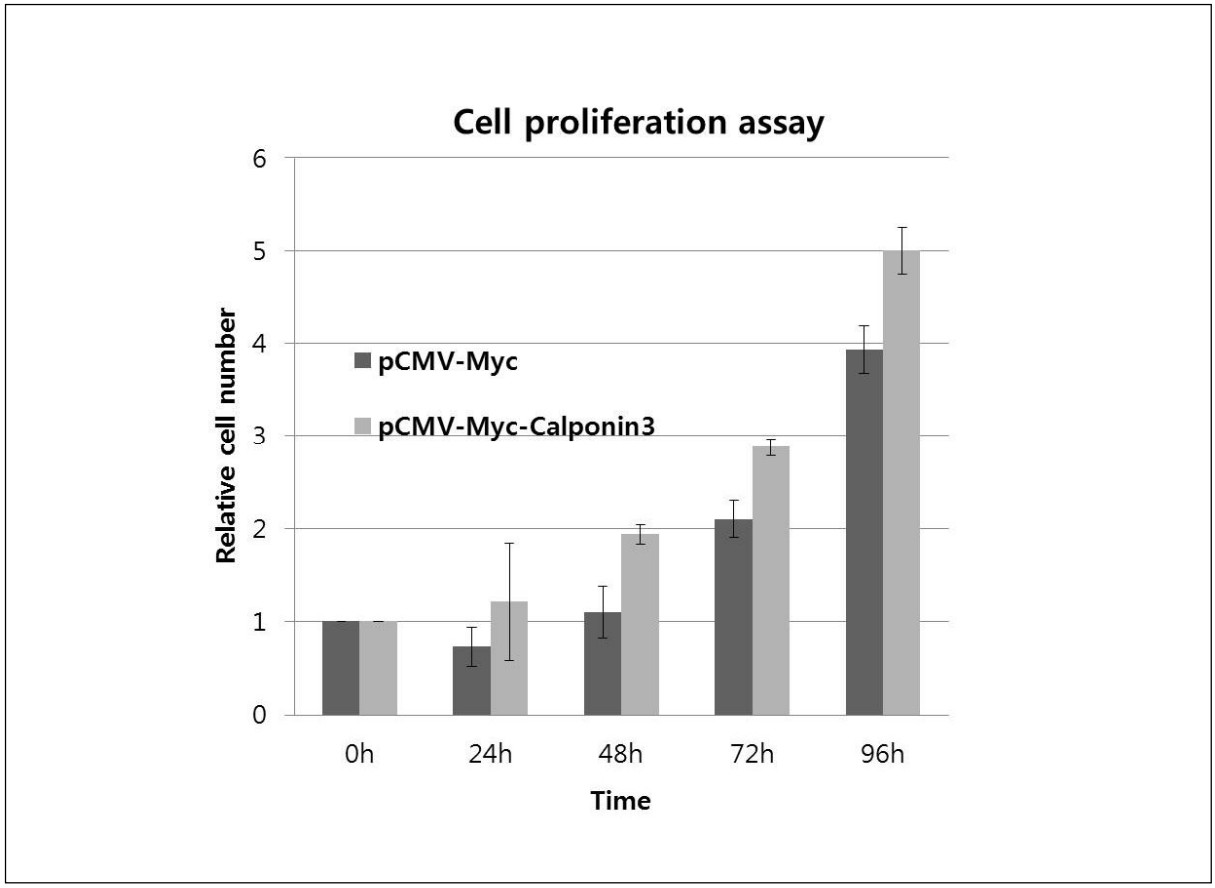

Figure 9. Cell proliferation assay of HT-29 cell line transfected with pCMV-Myc control and pCMV-Myc-Calponin 3 overexpressed vectors. Cell counting was performed after 0, 24, 48, 72 and 96 hours after transfection and repeated 3 times.

Decreased cell proliferation by overexpression of Calponin 3 gene?

SW-480 colorectal cancer cell line was selected by expressing lower level of *Calponin 3* gene and protein in RT-PCR and western blotting results. To examine the proliferation ability of *Calponin* 3 gene transfected cell, pcDNA3.1(+) control vector and *Calponin 3* recombinant DNA tansfected SW-480 cells, cell counting was performed. We demonstrated that *Calponin 3* recombinant DNA tansfected SW-480 cells growth slower comparing to pcDNA3.1(+) control vector SW-480 cells. But differential expression of Calponin 3 protein was not seen in western blot analysis. Thus, this result of MTT assay could not be adopted.

Expression of Calponin 3 protein in paired colon cancer cell lines

To compare the location of Calponin 3 protein in paired CRC cells, SNU-2414A and SNU-2414B were selected as a primary CRC cell

line and a metastatic CRC cell line. We confirmed that Calponin 3 protein was located in cytoplasm surrounding nucleus. Furthermore, significantly high expression level of Calponin 3 was detected in SNU-2414B cells (Figure 10).

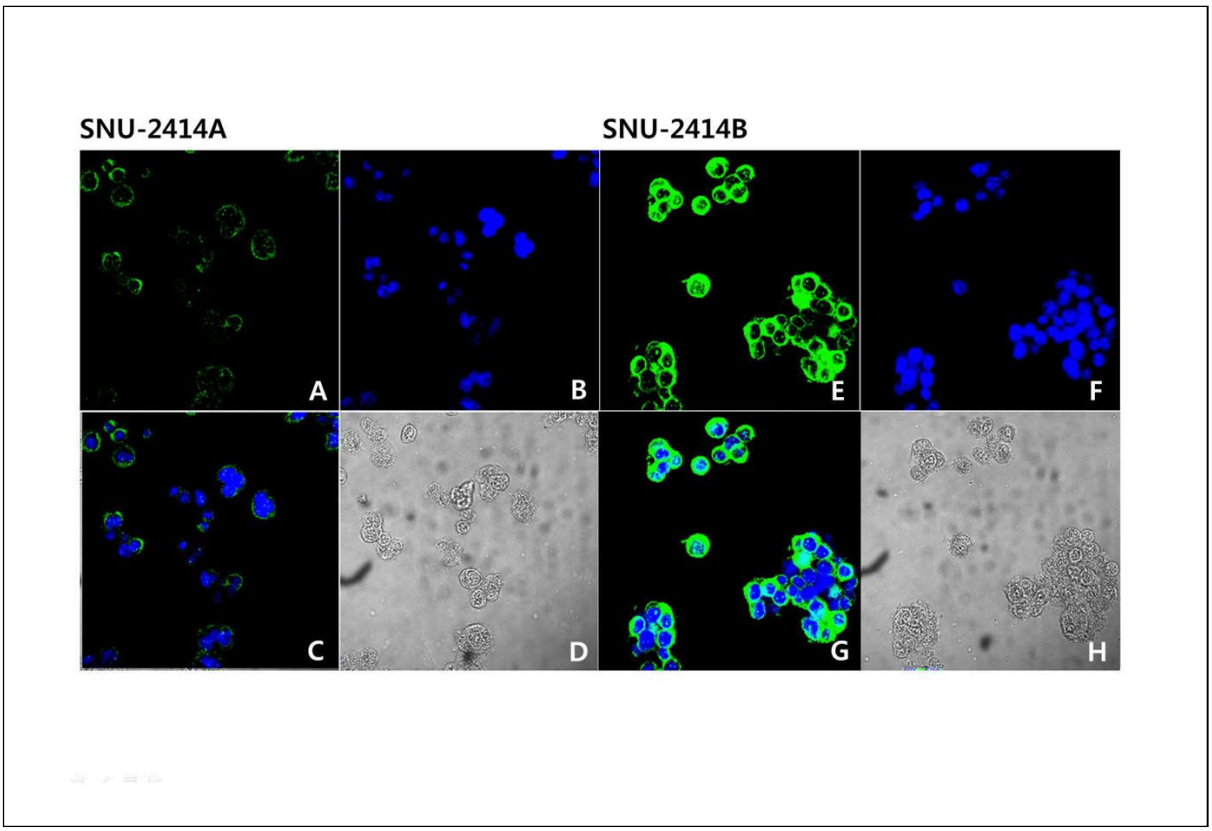

Figure 10. Localization of Calponin 3 protein in CRC cell by immunocytochemistry. Each cell line was incubated with PBST including Calponin 3 antibody (1:500), Alexa Fluor® 488 and stained with DAPI (1:500). (400x). (A) SNU-2414A stained with Calponin 3, (B) SNU-2414A, DAPI stained, (C) SNU-2414A, merged, (D) SNU-2414A, bright field, (E) SNU-2414B stained with Calponin 3, (F) SNU-2414B, DAPI stained, (G) SNU-2414B, merged, (H) SNU-2414B, merged.

Expression of Calponin 3 protein in paired colon cancer tissues

Primary sites of tumors were ascending colon (n=4), descending colon (n=1), sigmoid colon (n=1). In six primary CRC cases, Calponin 3 staining was absent in four cases, weak in one case, and moderate in one case. In six peritoneal metastasis cases, Calponin 3 staining was weak in four cases, and absent in two cases. (Photograph is not shown.)

In vitro invasion assay

Local invasion of cancer cells is the early and major steps of metastasis. *Calponin 3* gene was highly expressed in cell lines originated from peritoneal metastatic CRC, and Calponin 3 was highly expressed in other highly metastatic CRCs in western blot analysis, thus we had an idea that Calponin3 act a positive function in invasion of CRC cells.

Calponin 3 knockout cell lines showed reduced invasion and slightly

reduced proliferation than that of control cell lines (Figure 11), whereas three paired CRC cell lines did not show consistent results. Of the three paired cell lines, SNU-2404B and SNU-2414B (peritoneal metastasis CRC cell lines) showed decreased invasiveness compared with SNU-2404A and SNU-2414A (primary CRC cell lines), SNU-2335D showed no difference in invasiveness compaired with SNU-2335A cell line (Figure 12).

Although the result of invasion assay with three paired CRC cell lines was not consistent with the result of invasion assay with Calponin 3 siRNA transfected cell lines. This inconsistent result is maybe due to different characteristics of each cell lines.

From these results, we concluded that Calponin3 is a promoter of CRC cell invasion.

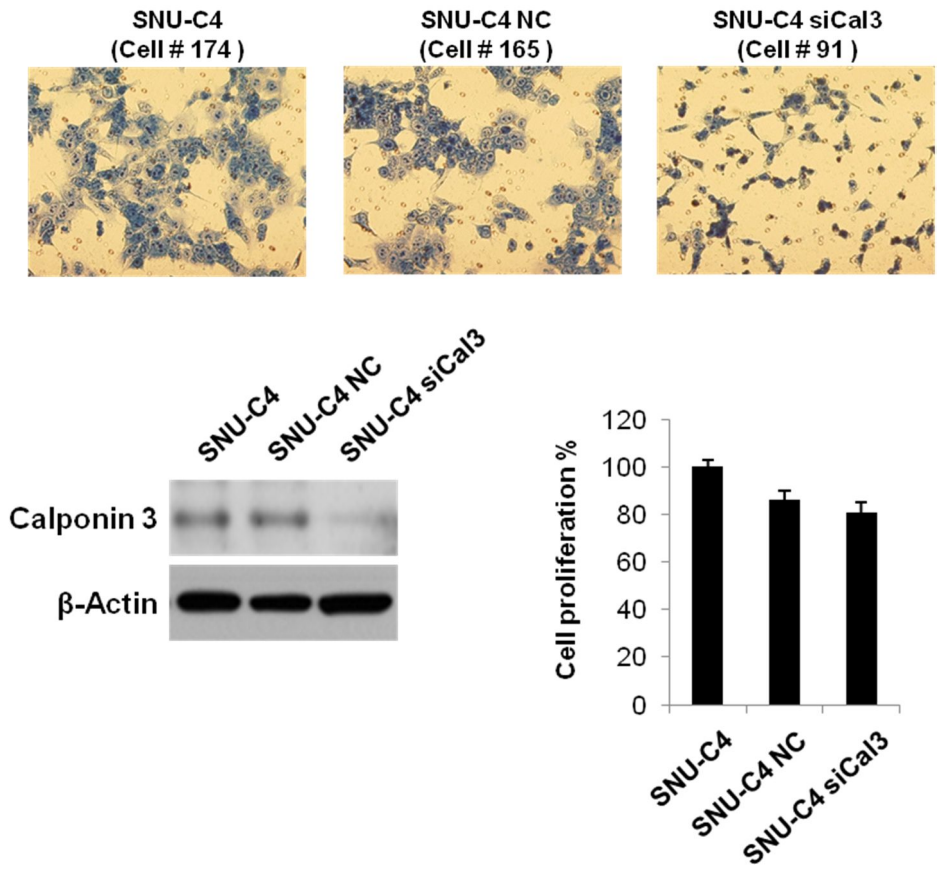

Figure 11. Invasion assay, western blot and MTT assay of Calponin 3 siRNA transfected cell line

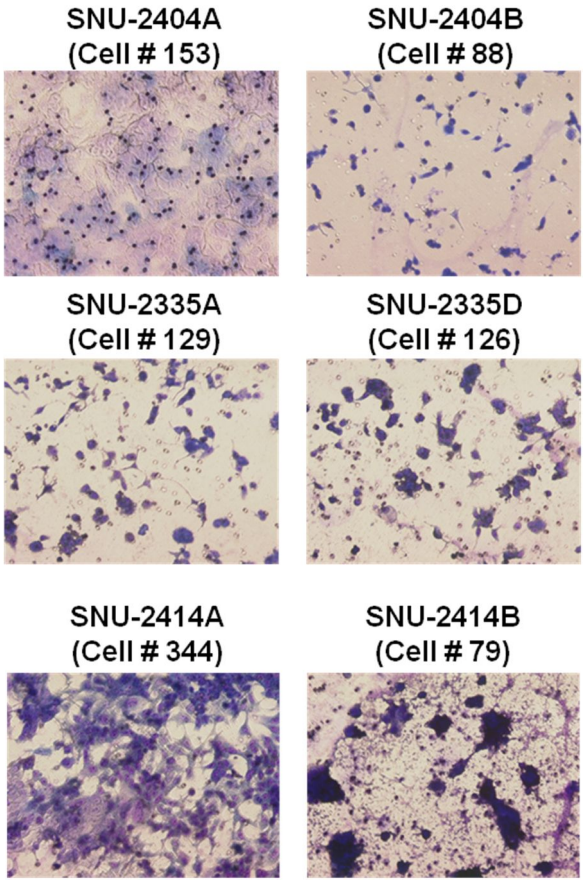

Figure 12. Invasion assay of three paired CRC cell lines

Discussion

Most of genetic studies of colorectal carcinogenesis have focused on the difference between normal colonic mucosa and primary carcinomas. Buckhaults et al. (2) reported several genes those were expressed at markedly higher levels in both benign and malignant tumors compared with normal colonic epithelium. And there have been many researches to find genes for diagnostic tools, chemotherapeutic targets, and for prediction of prognosis (3-7).

There were a few studies on the genetic alteration of primary carcinomas to peritoneal metastases. The first genomic profiling of local recurrences and carcinomatoses was reported by Diep et al. (8). They concluded that peritoneal carcinomatoses and liver metastases usually have more DNA copy number change than original lesion. Kleivi et al. reported that gains of chromosome arm 5p are common in

peritoneal carcinomatoses and twenty genes (including *PTGER4*, *SKP2*, and *ZNF622*) mapping to this region were overexpressed in the tumors. And by analyzing the gene expression profiles of three cell lines those were derived from a primary carcinoma, liver metastasis, and peritoneal carcinomatosis from same patient, they made a list of 600 genes associated with the different tumor stages (9). Analysis of gene expression profiling for identification of genes related to peritoneal carcinomatoses were conducted in other cancers. Motoori et al. reported that their prediction system using 18 genes identified by systematic analysis of gene expression profiling can predict peritoneal metastasis in patients with advanced gastric cancer after curative resection by 75% accuracy (10).

In our study, we identified 128 genes that were up-regulated and 539 genes that were down-regulated in more than two metastatic cell lines; only 5 up-regulated and 2 down-regulated genes were differentially

expressed in all three metastatic cell lines. And *CNN3*, *SORBS1* (up-regulated genes) and *TRY6* (down-regulated gene) were the genes that showed significantly differential expression in all three metastatic cell lines. Calponin 3 was selected to clarify the biologic characteristics of differentially expressed genes by performing functional study.

Calponin 3, also called acidic calponin, h3-calponin or CNN3, is encoded by Calponin 3 gene that is a calcium binding protein and located on Chr.1 *p21-p22* (10). Calponin 3 was first identified in 1995 (11). As one of the three isoforms(basic/h1-calponin, neutral/h2-calponin, acidic/h3-calponin) of calponin family that functions as calmodulin and F-actin binding partners, calponin3 is expressed in smooth muscle and non-muscle cells (12).

Whereas the role of calponin family in regulating smooth muscle contractility has been extensively investigated, the function and regulation of calponin family in nonmuscle cells is much less

understood. The recent studies have demonstrated that calponin plays a regulatory role in non-muscle cell motility. Therefore, calponin in non-muscle cell is an attractive target for the control of cell proliferation, metastases, and the cancer treatment (13).

In CRC, the expression of Calponin1 was significantly reduced in the peripheral region of CRC vascular tissues and the expression level was in association with tumor progression, lymphatic invasion, vascular invasion and recurrence (14). The down-regulation of Calponin1 in vessels in the peripheral region of CRC tissues was inversely associated with the expression of VEGF (vascular endothelial growth factor) (15). When Calponin 1 was introduced to the v-src-transformed rat fibroblast cell line SR-3Y1, Calponin 1 played a role as tumor suppressor mainly by decreasing VEGF expression and angiogenesis *in vitro* (15).

In other cancers, expression levels of Calponin 1 are decreased in

several tumors, including human fibrosarcoma, leiomyosarcoma, and osteosarcoma, and Calponin 1 expression is lost in malignant prostate tissue (16). High levels of Calponin 1 expression are important for the suppression of metastasis by inhibition of metastatic cell motility (17). Calponin 2 showed significant expression in the smooth muscle during early pregnancy (18). But, the expression of Calponin 2 decreased to low levels in adult smooth muscle cells, indicating its role in cell proliferation (19, 20). However, the roles Calponin 2 in smooth muscle remain to be investigated. In cancer tissue, Calponin 2 also inhibits the proliferation and migration of prostate cancer cells and its low expression in cancer cells correlates with the potency of metastasis (21).

In the central nervous system, Calponin 3 is found in the brain, and shows increased expression in epileptic patients (22) and ICF syndrome (23). The elevated Calponin 3 suggests that deregulation of

actin filament dynamics in axonal and dendritic outgrowth, and synaptic rearrangement may contribute to pathophysiology of epilepsy. Despite the fact that Calponin 3 is found in multiple non-muscle cells, its role in cancer cell biology has not been clear.

In non-muscle cells but not in cancer tissue, Calponin 3 facilitates ERK1/2 (extracellular signal-regulated kinase 1/2) mediated l-CaD (l-Caldesmon) phosphorylation and enhances cell motility, resulting in increased wound healing activity in REF52.2 fibroblasts *in vitro* (24). In CRC cells, l-CaD was reported that plays a role role for increasing metastatic property and decreasing chemoradiotherapy susceptibility (25).

Cytoplasmic protein, Calponin 3, related to the fusion of BeWo choriocarcinoma cells. Calponin 3 was expressed in cytotrophoblasts in human placenta. *Calponin 3* gene knockdown promoted actin cytoskeletal rearrangement and syncytium formation in BeWo cells,

suggesting *Calponin 3* to be a negative regulator of trophoblast fusion (26).

Little is known about biochemical function of Calponin 3. In functional analysis of rat Calponin 3 by Fujii et al., Calponin 3 is functionally distinct from Calponin 1. They reported that Calponin 3 protein is sensitive to the heat treatment in contrast with Calponin 1 (27).

In cancer tissue, *Calponin 3* was reported as a novel tanslocation gene in MALT lymphoma, but there was no more clinical information (28). In transcriptomic analysis of an in vitro murine model of ovarian carcinoma, *Calponin 3* had a positive correlation with 'time to death' that was one of the tumorigeneic parameters of mouse ovarian surface epithelial cell line (29).

In this study, *Calponin* 3 gene was absent or weakly expressed in parent cell lines (SNU-2335A, SNU-2404A and SNU-2414A),

whereas this gene was upregulated in peritoneal metastatic cell lines (SNU-2335D, SNU-2404B and SNU-2414B). Calponin 3 protein were strongly expressed in SNU-C5, HCT-8, HCT-15, HCT-116, SW-620, KM12SM and KM12L4 CRC cell lines whereas absent or weakly expressed in HT-29, Colo-205, LoVo, SW-480 and KM12C cell lines. SW-620 cell line is the metastatic cell line and SW-480 is the primary CRC cell line, and two cell lines were derived from same patient. KM12SM and KM12L4 cell line are the metastatic cell lines and KM12C is the primary CRC cell line, and three cell lines were derived from same patient. And Calponin 3 plasmid transfected cell lines tend to grow faster than the control plasmid transfected cell lines in proliferation study. The ability to induce cellular proliferation often correlates with the cancer progression.

Our present results imply that the higher expression of Calponin 3 plays a role for increasing cell proliferation and invasion of colorectal

cancer(CRC) cells, and suggest that Calponin 3 can be linked to positive function in peritoneal metastasis of CRC.

Moreover the established three paired CRC cell lines in this study should be useful in investigations of the biological characteristics of CRC, particularly for investigations related to gene alterations associated with primary CRC and peritoneal metastatic CRC.

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국문초록

대장암에 있어서 원발암의 유전자 변이나 다형성에 대한 연구는 많이 행하여져 왔다. 그러나 원발 대장암과 복막 전이암 사이의 유전자 차이에 대한 연구는 많지 않다. 그러므로 본 연구의 목적은 동일 환자 기원의 원발 대장암과 복막전이암 조직으로 세포주를 수립하고 수립된 세포주의 유전자 분석을 통하여 쌍으로 수립된 세포주 사이에 차별 발현되는 유전자를 찾고 그 유전자의 기능 실험을 통해 차별 발현된 유전자가 대장암의 복막전이와 어떤 관계가 있는지 규명하고자 함이다.

세 명의 한국인 대장암 환자의 원발 대장암, 각각의 복막전이암 조직을 가지고 세 쌍의 세포주를 수립하였다. 그리고 Affymetrix GeneChip® hybridization 방법으로 세 쌍 모두에서 원발 대장암 세포주에 비하여 복막전이암 세포주에서 액틴결합단백질인 칼포닌 3(Calponin 3)가

과발현되는 것을 확인하였다. 그리고 추가 실험을 통하여 칼포닌 3가 전이 성향이 강한 다른 대장암 세포주들에서도 과발현이 됨을 웨스턴블롯을 통해 알게 되었다. 흥미롭게도 칼포닌 3를 형질주입한 세포주가 대조군 세포주에 비해 성장이 더 빠름을 확인하였고, 반대로 칼포닌 3를 넉아웃시킨 세포주는 성장이 더디고 침윤 분석 상에서는 침윤이 낮아지는 것이 보였다.

결론적으로 칼포닌 3는 대장암 세포의 성장을 촉진하고 침윤능을 증가시키는 작용을 하는 것으로 보이며 이는 대장암의 복막전이에 긍정적인 역할을 할 가능성이 있어 보인다.

더구나 대장암 세포주에 있어서 동일 환자 기원의 원발 대장암 유래 세포주와 복막전이암 유래 세포주가 수립된 예가 드물어 향후 이번에 쌍으로 수립된 세포주들이 대장암의 복막전이와 관련된 연구에 유용하게 쓰일 것으로

생각된다.

핵심단어: 대장암, 칼포닌 3(Calponin 3), 세포주, 복막전이

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