Comparison of biologic and genomic phenotypes in *in vivo* selected clones of gastric orthotopic animal model

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The Master's Thesis submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medicine

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감사의 글

항상 저를 지켜주시며 바른 길로 인도해주시는 하나님께 감사드립니다.

오직 기도와 정성으로 보살펴 주신 할머니, 부족한 저이지만 항상 용서하시고 가장 큰 사랑으로 보살펴주신 부모님께 존경과 사랑을 드리며 이 논문을 바칩니다.

미숙한 저의 논문이 완성되기까지 세심한 지도와 열성으로 이끌어 주신 노 성훈 교수님께 진심으로 감사드립니다. 또한 의학뿐만 아니라 삶에 있어서도 중요한 가르침을 주시고 현재 암투병 중이신 민 진식 교수님께 감사드리며 하루속히 쾌유하시기를 기원합니다.

바쁘신 와중에도 학문적 충고와 자세한 자문을 주신 정 재복 교수님, 라 선영 교수님께 깊은 감사를 드립니다.

실험과정에 많은 조언을 주신 정 현철 교수님, 김 용일 선생님과 암전이 연구센터의 여러 선생님들께 감사의 말씀을 전하고 싶습니다.

소중한 처 정혜와 쌍둥이 예흠, 찬흠, 기승이 내외와 조카 성흠, 성은이의 따뜻한 배려와 사랑도 기억하겠습니다.

제가 힘들고 지쳤을 때 격려와 채찍이 되어 주셨던 모든 분들께 마음을 다하여 다시 한번 감사드립니다.

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ABSTRACT

Comparison of biologic and genomic phenotypes in *in vivo* selected clones of gastric orthotopic animal model

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(Directed by Professor Sung Hoon Noh)

Gastric cancer is the most common type of cancer in Korea with an unfavorable prognosis. Therefore, it is important to understand the mechanisms of gastric cancer development, invasion and metastasis. Even though enormous efforts have been made to solve these problems, the currently used *in vitro* and *in vivo* model systems have many limitations. Specifically, it is essential to choose the proper *in vivo* model system according to the aims of the particular study. The orthotopic animal model system is suitable for examining the pathophysiology of tumor cells as well as their interaction with the host microenvironment reflecting the nature of the human tumor. Based on an orthotopic gastric cancer animal model system established in the Department of Surgery,

Yonsei University College of Medicine, this study evaluated the biological and genomic characterization of the cell lines established from the *in vivo* selected clones, which reflects the primary gastric cancer properties for understanding the pathophysiology of gastric cancer development.

A piece of a subcutaneous xenograft tumor formed from YCC-3 gastric cancer cells was inoculated in the gastric mucosa of nu/nu mouse. When the primary tumor was developed in the gastric mucosa, the tumor was procured and a cell line was established from the primary gastric tumor. The same procedure was repeated for 7 passages and the resulting cell lines were called YCC-3-P1 - P7. When observing the *in vivo* patterns of tumor development, 94% developed primary tumors with various lymph node (LN) metastases. However, a systemic metastasis to liver or spleen decreased as the number of passages increased. Even though the cell lines established from the *in vivo* selected clones showed the same morphology under a microscope, the extent of *in vivo* tumorigenesis is increased with increasing number of passages to YCC-3-P7. The tumor doubling time, the motility and the anchorage independent tumor growth decreased with the increasing number of passages. The genome wide expression showed discrete patterns between the parent YCC-3 cells and other 7 daughter cell lines. When the patterns were evaluated with 317 cell growth related genes, the YCC-3-P1-4 and P5-7 cells showed the

different genomic patterns.

In conclusion, this study suggests that combining the *ex vivo* cell line with an orthotopic model system may be a useful tool for investigating the pathophysiology of gastric cancer. According to the aim of the study, the best model system might be chosen based on the biological and genomic characterization. In addition, the current results might be helpful for understanding the pathophysiology of human gastric cancer.

Key words : gastric cancer, orthotopic, genomic, biological, cDNA microarray

< 본 문 >

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I. INTRODUCTION

To use of a proper animal model system is essentially for understanding the novel pathophysiology of cancer, to validate the discovered molecules from the *in vitro* experiments, and to evaluate the efficacy and the toxicity of newly developed drugs to facilitate drug development[1,2,3]. Currently, human xenograft is the most commonly used model system in cancer research. In general, a tumor is established under the skin after a subcutaneous(SC) tumor cell injection. The reasons for using this model system are 1) tumor can be developed easily, 2) it is easy to observe the

changes in the tumor including tumor size and blood vessel formation, and 3) it is easy to access the tumor for intratumoral drug treatment.

One of the most significant characteristics of the tumor is cross-talk between tumor cells and the host microenvironment[3,4]. In case of frequent tumor metastases to specific organs, it can be assumed that the tumor cells have the specific microenvironment support with specific growth factors based on the seed and soil theory proposed by Paget[5]. Considering the tumor cell characteristics, it can be assumed that the usual SC xenograft model might not reflect the natural tumor biology. In order to overcome this problem, there have been many efforts to develop the orthotopic animal model system, which has the tumors in the organ of origin[6,7,8].

Gastric cancer is the most common fatal cancer in Korea. Regardless of the improved diagnosis and treatment modalities, more than 1/3 of advanced gastric cancer patients die as a result of their disease[9,10]. As the biological phenomenon is related to multiple complex genetic interactions, particularly in cancer, there is a need to evaluate many genes simultaneously, and characterize the genome, according to their biological phenotype. Due to improved biotechnology, a microarray enables thousands of molecules to be evaluated simultaneously[11]. There have been many

efforts aimed at identifying the significant genes related to the development and prognosis of gastric cancer using a microarray, which is useful in both understanding the pathophysiology and identifying the potential biomarker candidates[12,13]. In order to investigate the characteristics of a primary gastric cancer, a gastric orthotopic animal model system combined with the establishment of an *ex vivo* cell line was used for the biological and genomic characterization.

II. MATERIALS AND METHODS

Cell line and cell culture

The YCC-3 gastric cancer cell line, which was established at the Cancer Metastasis Research Center (CMRC), Yonsei University College of Medicine, Seoul, Korea, from the ascites of an advanced gastric cancer patient in Yonsei Cancer Center, Yonsei University College of Medicine, was used in this study. The cells were cultured and maintained in minimum essential media (MEM, GibcoBRL, USA) with 10% fetal bovine serum (GibcoBRL, USA), in 100 units/ml of penicillin and 0.1mg/ml of streptomycin (GibcoBRL, USA) at 37°C, in a 5% CO2 incubator.

Orthotopic animal model

An orthotopic gastric cancer model was developed based on a previously established method[6]. Athymic nude mice (BALB/c-nu, 7-8 weeks old, and approximately 20 grams in body weight were used. The mice were kept in a laminar-flow cage in specific pathogen free conditions with sterilized water and food (PicoLab, PMI, USA). The maintenance and the manipulation of the mice were performed using the standardized environmental conditions following the regulation of the Yonsei Medical Research Center,

Yonsei University College of Medicine, Seoul, Korea.

When the mice developed a subcutaneous tumor about 1cm after 1 X 10^7 cells were injected, the tumor was harvested and minced to 3mm fragments after removing the surrounding normal tissues. The tumor fragments were kept in 1:1 diluted Penicillin-Steptomycin (GibcoBRL, USA) with a phosphate buffered saline solution to prevent dehydration until use. The mice was anesthetized with 5-16mg/Kg of ketamine hydrochloride (Parke-Davis Co. USA) and 2.5mg/Kg of xylazine hydrochloride (Bayer Korea Co.). After sterilizing the abdominal wall, a 1cm sized incision was performed on the left upper abdomen and the stomach was exposed to the outside. The serosa of the greater curvature was incised with a 26 gauge needle and the prepared tumor tissue fragment was located on the subserosa layer and stitched with 6-0 vicryl. The fixed fragments were reinserted into the abdominal cavity and the abdomen was then closed by single-stitch sutures using 4-0 vicryl.

The tumors that developed in the primary gastric wall were divided into 3 pieces, one for formalin fixation, one for cell line establishment, and one for the consecutive gastric orthotopic tumor formation. The tumor pieces were reintroduced into gastric wall of new batches of mice for the *in vivo* selection (Figure 1).

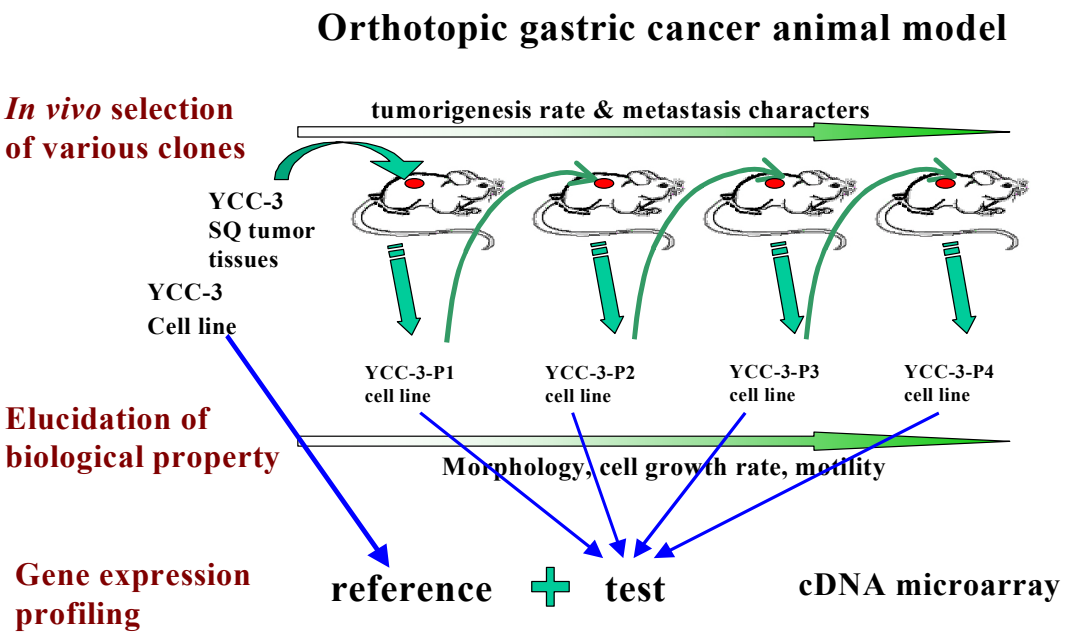


Figure 1. Scheme of the study

The mice were evaluated for the tumor size, body weight, general appearance and performance including an abdominal distension or the skin changes every 2 days. Following 8-12 weeks observation, the mice were sacrificed and a careful inspection of the gross tumor growth in the stomach, lymph node enlargement and liver or other intraabdominal organ metastasis was performed. When tumors were observed, the tumor tissues were fixed in formalin for a pathological evaluation.

Consecutive *ex vivo* cell line establishment from *in vivo* selected clones of primary tumor

One third of the primary gastric tumor tissues were minced in MEM into single cells. After the cells were passed through the mesh, they were plated in a 25mm^2 flask. The attached cells were cultured using the standard procedure of the CMRC, Yonsei University College of Medicine, (Seoul, Korea)[3]. The cell line was called YCC-3-P1 to P7 according to the passage number.

In vivo tumor tumorigenecity

In order to evaluate the *in vivo* tumorigenecity, the nude mice were re-injected subcutaneously with 1 X 10^7 cells of the established cell lines. The body weight and tumor size were

measured twice a week.

Cell culture and cell growth curve

In order to evaluate the cell growth rate[14], the cells were trypsinized with Trypsin-EDTA (GibcoBRL, U.S.A) into single cells and washed three times with MEM with 10% fetal calf serum. The cells were plated in 24 well plate with 10^6/ml cells in triplicate and cultured in a 37^0C incubator with 5% CO2 for 5 days. The cells were counted every day with a hemocytometer and the mean number of the cells with the standard deviation of the triplicate cultures was calculated. The tumor doubling time was then calculated.

Anchorage independent growth pattern

An anchorage dependent soft agar assay was carried out as previously described[3]. Briefly, 20,000 cells in 0.35% agar (Bactoagar, GibcoBRL, USA) were layered on top of a semi-solidified 0.6% agar layer in a 35 mm dish. Colonies more than 50μm in diameter were counted from the 14th to 21st days of incubation. Colonies from 5 dishes were counted and the mean number was calculated.

Cell motility using Boyden chamber

A Boyden chamber assay was performed to evaluate the cell motility as described without any chemo-attractant[3]. Briefly, matrigel was dried onto polycarbonated membrane filters (Poretics Corp, CA, USA). The cells were then added to the top chamber and incubated for 8 hours. The number of cells on the lower surface of the filter was counted. Three membranes of the 3 chambers were counted and mean number if cells was calculated.

Microarray experiment

1) RNA extraction and probe preparation

The total RNA was extracted from the cell lines when the cells reached 95% confluence according to the manufacturer's protocol. The quality and quantity of RNA was evaluated using gel electrophoresis (Gel Documentation-Photo system, Vilber Lourmat, France), spectrophotometry (Gene Spec III, Hitachi, Japan). Only good quality RNA that was not degraded was used for microarray hybridization.

2) Probe preparation

Fifty g of the total RNA was directly labeled and transcribed to cDNA. This study combined the total RNA from the following 11 cancer cell lines of the various tissue origins in equal quantities in order to prepare the Yonsei reference RNA(CMRC, Yonsei University College of Medicine, Korea): stomach, breast, colon, liver, lung, lymphoid and myeloid system, cervix, fibrous tissue, kidney, and brain cancer cell line. The cell lines were selected from the various organs to ensure that the pooled RNA contained as many transcripts as possible. Yonsei reference RNA was labeled with Cy3-dUTP (NEN Co., USA) and the test samples were labeled with Cy5-dUTP (NEN Co., USA). The labeling was performed at 42°C for 2h in a total volume of 30ul containing 400 units of SuperScript II (GIBCO, USA); 3ml Cy5-dUTP (or Cy-3 dUTP), 1.5 ml of each of dATP, dCTP, and dGTP, and 0.6 ml dTTP, 300mM, 6ul of the 5X first-strand buffer, and 4ug of the modified Oligo-dT primer. The unincorporated nucleotide was removed by using a PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. The eluted probes were then mixed and supplemented with 20ul of 1ug/ul Human Cot1 DNA (GIBCO, USA), 2ul of 10ug/ul polyA RNA (Sigma, USA), 2ul of 10ug/ul, and 288ul of 1M TE buffer. This probe mixture was concentrated using a Microcon-30 tube (Millipore, USA), and the 48 ul of the labeled mixture was mixed with 10.2

ul of 20 X SSC and 1.8ul of 10% SDS for hybridization.

3) Hybridization of fluorescence-labeled cDNA

The 17K human cDNA microarray (Yonsei CMRC-GenomicTree Co., Korea) was used in this study. Before hybridization, the slides were pre-blocked in a 10mg/ml BSA, 3.5 X SSC, 0.1% SDS solution to prevent nonspecific hybridization, and the probe mixtures were heated at 95°C for 2min and then centrifuged for 2min at 13,000r.p.m. After applying the probe to the slides, the slides were hybridized in hybridization chambers (GenomicTree Co., Korea) at 65°C for 16h. After hybridization, the slides were washed in 2×SSC for 10 min, and then transferred to 0.1×SSC and 0.1% SDS for 10 min, and rinsed twice with 0.1×SSC for 10 min. After washing, the slides were spun at 600 r.p.m. for 5min (Hanil Science Industrial Co., Korea). The hybridized slides were scanned using a GenePix 4000B (Axon Ins., USA) and the images were analyzed using GenePix Pro3.0 (Axon Ins., USA).

4) Data analysis

After filtering based on the flagging of the scanner, the intensity dependent, within print-tip normalization using the Lowess method was performed to normalize the data to the original microarray data based on M-A plot[15]. Genes with 20% missing values were

selected among the twenty-seven experiments, leaving 13757 genes for further analysis. Using Pearson correlation analysis, a two-way hierarchical clustering of the selected genes and samples was performed. TREEVIEW was used to visualize the results using the GeneSpring program (Silicon Genetics Inc., USA). The expression patterns of the cell lines were evaluated using 317 cell growth related genes selected using GeneSpring program (Silicon Genetics Inc., USA). In order to identify the differentially expressed genes among the cell lines, the t statistics with family-wise error rate adjustment[16] was used. A self-organized map was used for unsupervised clustering. Annotation of the selected genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID).

III. RESULTS

Orthotopic gastric cancer model

As reported previously, the tumor developed on the gastric wall after approximately 8-12 weeks after the tumor piece inoculation with the various metastatic manifestations (Fig. 2), including perigastric LN, mesenteric LN, sacral LN, and multiple metastases to the spleen and liver. The *in vivo* metastatic patterns of the YCC-3 derived clones are summarized in table 1. Tumorigenesis in the primary stomach was excellent (94 ± 10.5%) without changes with increasing passage number. However, the rate of a systemic metastasis such as to the liver reduced as the passage number was increased. In addition, the rate of LN metastases increased up to 82% in YCC-3-P6. We also observed a diaphragm invasion rate of 41-44% in YCC-3-P3 and P4.

In order to evaluate the biological phenotype, 4 cell lines were chosen, 1) the YCC-3 parent cell line, 2) YCC-3-P1, the first daughter cell line after the interaction with the mouse host with the original parent cell, 3) YCC-3-P3, from the gastric tumor when the most frequent liver metastasis developed, and 4)

YCC-3-P7, the last passage with no liver or spleen metastasis.

Cell morphology and in vivo tumorigenecity

When the morphology of the YCC-3 parent cells and other 7 daughter cell lines were evaluated under a microscope, there were no significant changes among the cell lines (Figure 3). Meanwhile, the *in vivo* tumorigenecity, tumor development in mice, of 4 selected cell lines increased as the passage number increased as shown in figure 3.

In vitro biological characterization of the cell lines

This study compared several *in vitro* biological characteristics of the cell lines (Figure 4). The tumor doubling time became shorter as the passage number increased with 36 hours for the YCC-3 parent cells and 27 hours for YCC-3-P7 cells. However, the anchorage independent growth using a soft agar assay showed more colonies in the parent YCC-3 cells compared to the other daughter cells. In addition, the motility of the cells appeared to be somewhat lower in the YCC-3-P7 cells.

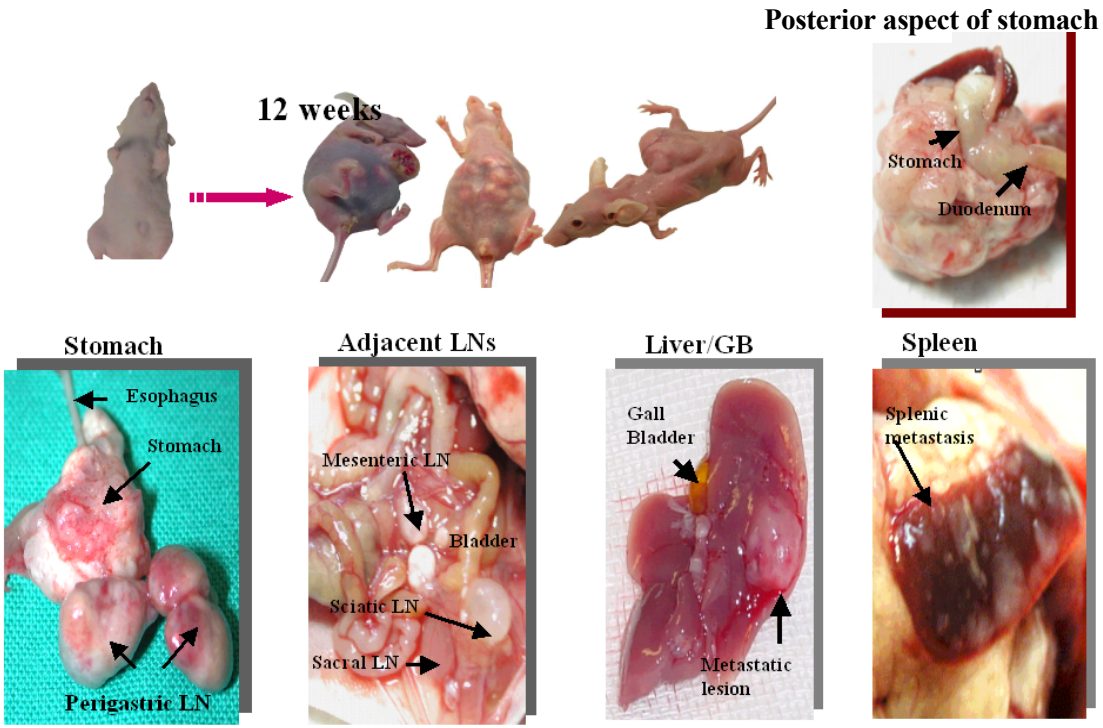
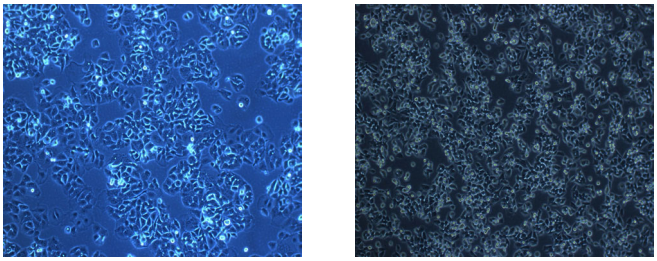


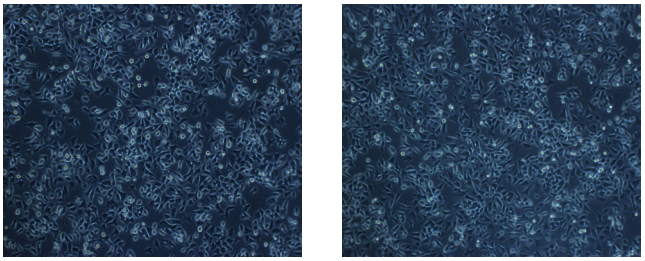
Figure 2. Results of orthotopic gastric cancer model

Table 1. *In vivo* metastatic patterns of YCC-3 derived clones in orthotopic animal model

	Imp. No.	Stomach	Liver	LN	Spleen	Diaphragm	Other
YCC-3-P1	2	100%	50%	0	0	0	0
YCC-3-P2	6	83%	83%	16%	16%	0	pancreas invasion(1)
YCC-3-P3	9	100%	12%	33%	0	44%	bleeding(2)
YCC-3-P4	12	100%	8%	50%	0	41%	bleeding(5)
YCC-3-P5	10	100%	10%	60%	0	37%	
YCC-3-P6	11	100%	0	82%	0	18%	
YCC-3-P7	8	75%	25%	25%	0	12%	



YCC-3 YCC-3-P1



YCC-3-P3 YCC-3-P7

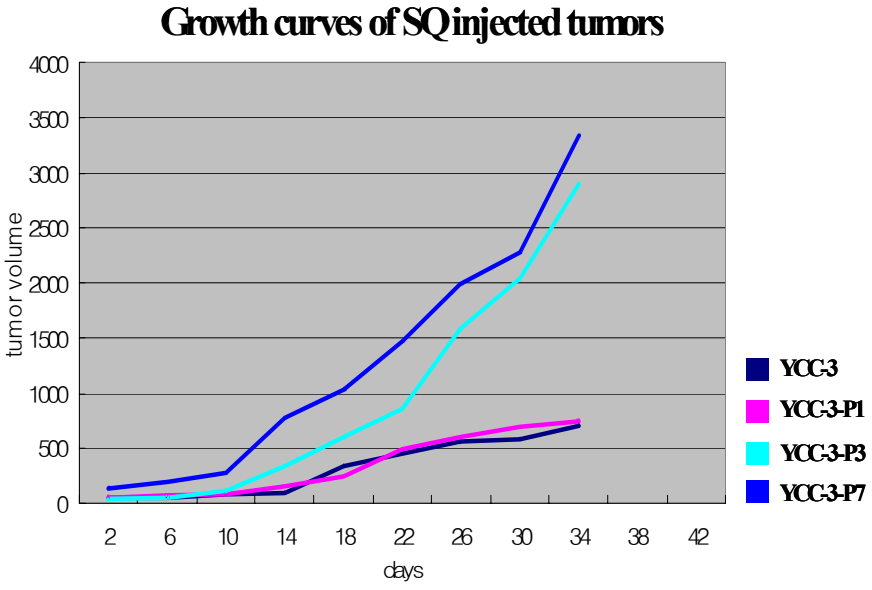


Figure 3. Cell morphology and *in vivo* tumorigenecity

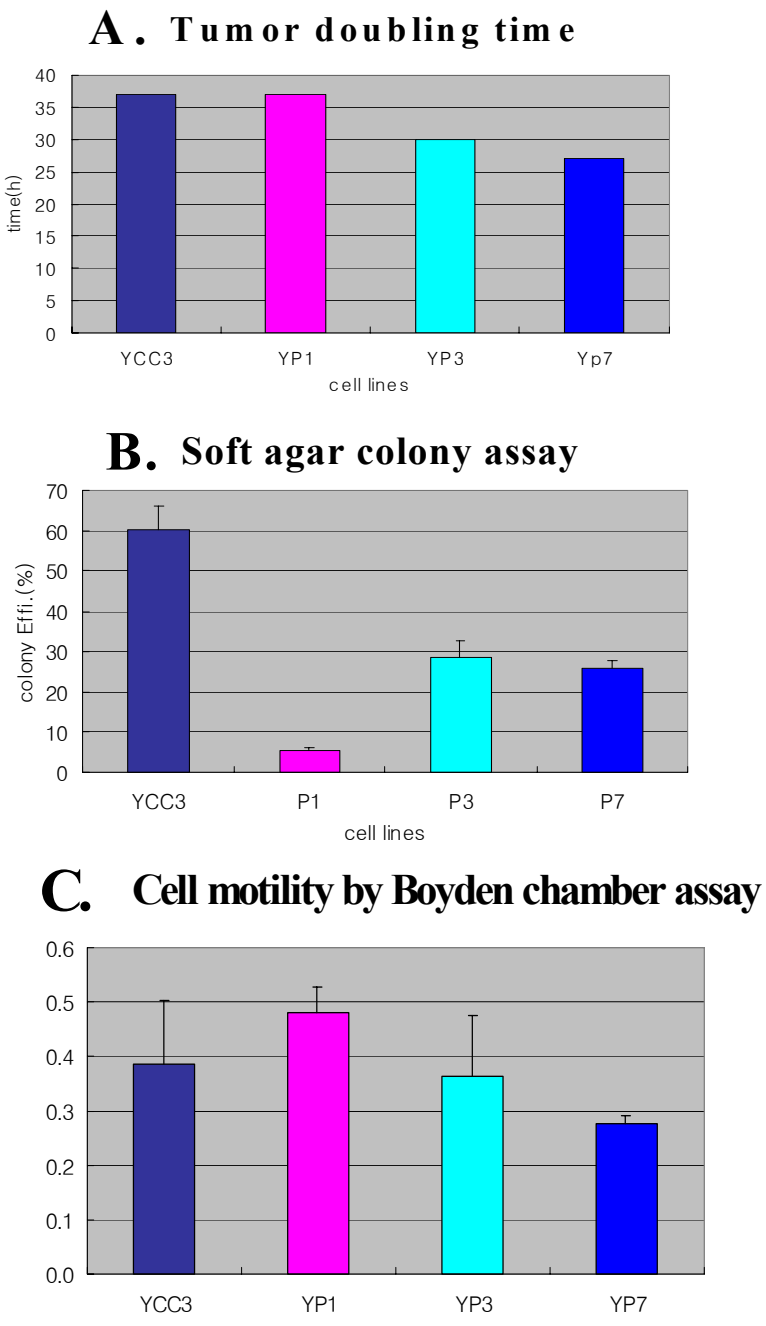


Figure 4. ***In vitro*** **biological characterization of cell lines**

Gene expression analysis using high-density microarray

1) Genome wide analysis of all cell lines

Using the reference design, this study evaluated the RNA expression pattern for each cell line using a high-density microarray. Due to the good quality RNA and an improved technique, the microarray results showed less variation, which can be observed as a small data adjustment after normalizations as shown in figure 5. After filtering and normalization, the noise of the data reduced with the missing data of more than 20% of the samples, resulting in 13757 genes. We performed an unsupervised 2-way hierarchical clustering of all the cell lines with the 13757 genes. As shown in figure 6-A, the YCC-3 parent cell and other 7 daughter cell lines were discretely separated in gene expression patterns. Among the genes, 317 cell growth related genes were chosen using GeneSpring software based on the known gene functions in the public databases. When a 2-way hierarchical clustering of all cell lines was performed with the selected 317 genes, it was again observed that the parent cell and daughter cells had different expression patterns of growth related genes (Fig. 6-B). Interestingly, the YCC-3-P1-4 and YCC-3-P5-P7 were observed to divide in two discrete branches. In addition, all cell lines were clustered using self-organizing map to identify the changing patterns as the number of passages increased (Fig. 7).

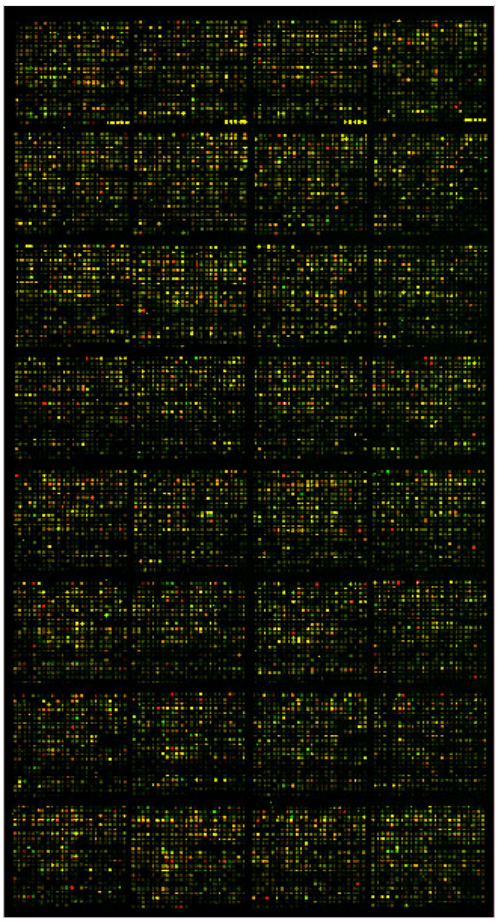


Figure 5. Representative images of high-density cDNA microarray (human 17K)

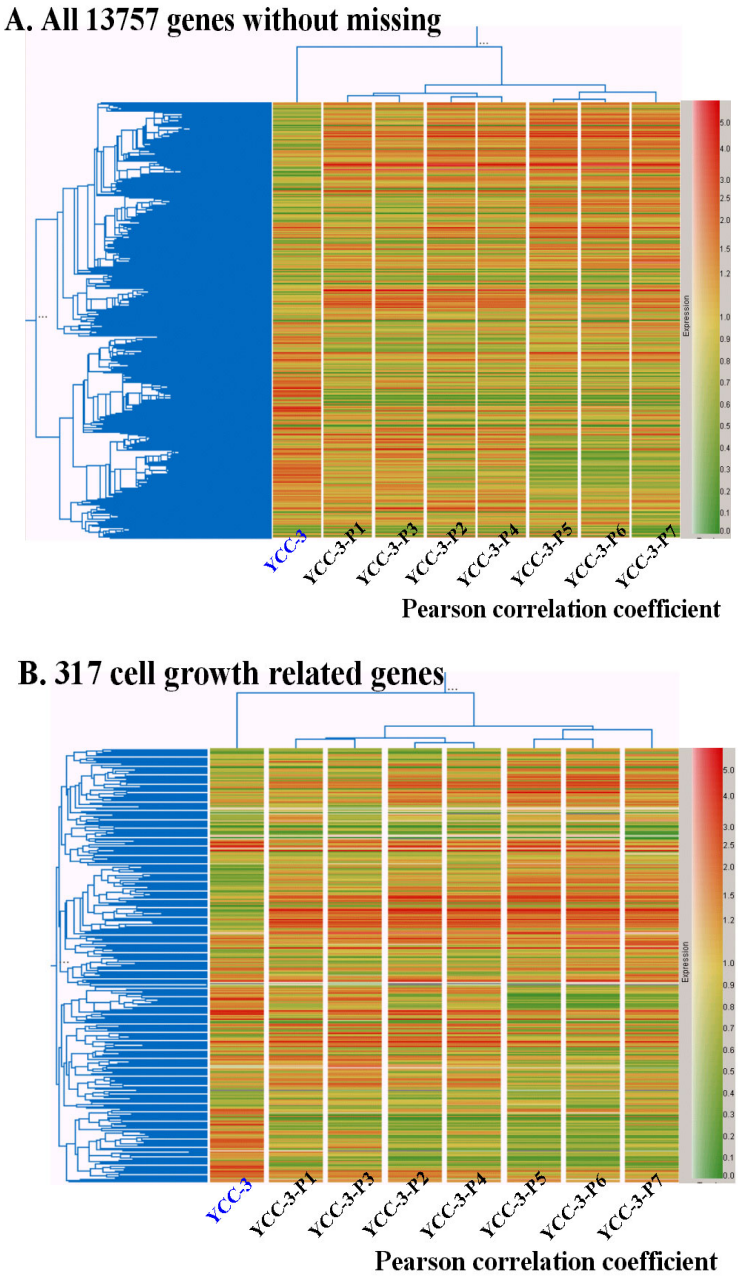


Figure 6. Two-way hierarchical clustering of all cell lines

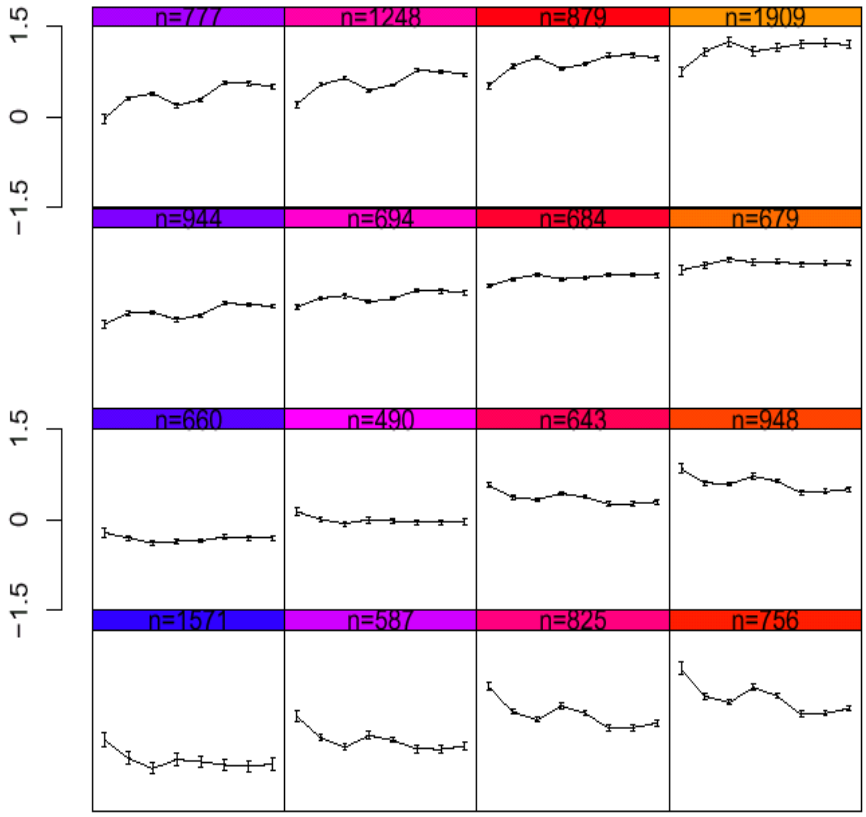


Figure 7. Changing patterns of genes using self-organizing map

It was confirmed that the parent cell and daughter cells clustered separately. There were 4 clusters of increasing expressions with increasing passage number, 6 clusters without changes, and 6 clusters with decreasing expressions through the passages(Fig. 7).

2) Significant gene selection

The specific genes related to the YCC-3 parent cells compared to the other daughter cells were identified using a statistical method based on the t-statistics and family-wise error rate correction. As a result, 70 genes were identified, 34 down regulated and 36 up-regulated genes in the YCC-3 cell line (Table 2).

When the differentially expressed genes between YCC-3-P1-4 and YCC-3-P5-7 were identified, 60 genes, 29 down regulated and 31 up-regulated genes, were chosen at a false discovery rate of 5%, (Table 3). Interestingly, the difference in the mean expression ratio of these genes between the two groups was small compared to the above YCC-3 specific genes.

Table 2. Differntially expressed genes between YCC-3 parent and daughter cells

Down-regulated in YCC-3 cells

Gene ID	Gene Name	YCC-3-P1-P7*	YCC-3
AI986336	adaptor-related protein complex 1, gamma 1 subunit	-0.10	-6.38
AA927761	DKFZP727G051 protein	-0.49	-5.57
AA872383	metallothionein 1E (functional)	0.71	-5.42
H72723	ESTs	-0.14	-4.95
AA456439	MAD (mothers against decapentaplegic, Drosophila) homolog 4	-0.17	-4.35
AI005521	DNAJ domain-containing	1.93	-4.20
AA293571	TNF receptor superfamily, member 6	0.15	-4.05
R66101	neuritin	0.70	-3.94
AA465193	Homo sapiens testis protein mRNA, partial cds	1.33	-3.58
T64469	p8 protein (candidate of metastasis 1)	1.65	-3.36
AA902815	ESTs	1.64	-2.72
AA521384	up-regulated by BCG-CWS	1.51	-2.69
AI688769	beta-parvin	1.54	-2.66
AA465521	SMC (mouse) homolog, Y chromosome	2.34	-2.48
AI364029	Homo sapiens mRNA; cDNA DKFZp434N1728 (from clone DKFZp434N1728)	2.61	-2.47
H79047	insulin-like growth factor binding protein 2 (36kD)	2.81	-2.19
AI623173	galanin	3.78	-1.71
R42433	protein tyrosine phosphatase, receptor type, O	5.12	-1.06
W73473	bone morphogenetic protein 7 (osteogenic protein 1)	5.52	-1.00
AI635989	kynureninase (L-kynurenine hydrolase)	-5.86	-0.81
AW071162	catenin (cadherin-associated protein), alpha 1 (102kD)	-4.87	-0.34
AA455911	ATP-binding cassette, sub-family B (MDR/TAP), member 1	4.93	-0.30
R63543	p75NTR-associated cell death executor; ovarian granulosa cell protein (13kD)	-6.42	-0.28
AA775616	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	-8.55	-0.27
AW009320	caudal type homeo box transcription factor 1	6.05	-0.19
AI336626	Homo sapiens cDNA: FLJ21763 fis, clone COLF6967	5.98	0.00

Up-regulated in YCC-3 cells

Gene ID	Gene Name	YCC-3 -P1-P7*	YCC-3
AA676957	catenin (cadherin-associated protein), alpha 1 (102kD)	-4.85	0.02
AA292410	accessory proteins BAP31/BAP29	-3.60	1.01
R61229	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	-2.66	1.53
AW072780	eukaryotic translation initiation factor 4 gamma, 1	-3.13	1.61
R62817	erythrocyte membrane protein band 7.2 (stomatin)	-2.86	1.74
AA994825	RAB38, member RAS oncogene family	-2.41	1.82
AA405668	neuronal cell adhesion molecule	-4.54	2.05
W70234	dipeptidylpeptidase IV (CD26, adenosine deaminase complexing protein 2)	-2.50	2.19
H86554	ceruloplasmin (ferroxidase)	-3.25	2.62
N33920	diubiquitin	-1.45	2.78
AI769855	defensin, beta 1	-3.68	2.94
AI364899	EST	-1.73	3.18
AA680136	coagulation factor V (proaccelerin, labile factor)	-1.82	3.23
R63065	glutathione S-transferase M3 (brain)	-3.16	3.58
AA704242	alpha-1-antichymotrypsin	-0.12	4.16
AA876375	Human DNA sequence from clone RP11-16L21 on chromosome 9.	-0.16	4.17
AI656811	transcriptional activator of the c-fos promoter	-0.20	4.26
AI380663	hypothetical protein FLJ22800	-0.45	4.42
AA146773	2′,5′-oligoadenylate synthetase 1	-0.06	4.45
R73909	pregnancy specific beta-1-glycoprotein 11	-2.33	4.95
AI261360	retinoic acid receptor responder(tazarotene induced) 1	-0.39	5.01
AA058357	carcinoembryonic antigen-related cell adhesion molecule 7	-0.92	5.44
AI865749	myosin-binding protein C, slow-type	-0.16	5.92
AA130584	carcinoembryonic antigen-related cell adhesion molecule 5	-0.92	6.30
T73468	glutathione S-transferase A2	0.85	7.23

*Mean expression ratio(log R/G) of YCC-3-P1-P7

Table 3. Differentially expressed genes between YCC-3-P1-P4 and YCC-3-P5-P7

Down-regulated genes in late passages

Gene ID	Gene name	P1-P4*	P5-P7*
AI890760	MHC class II, DR beta 1	-1.94	-2.96
T53298	insulin-like growth factor binding protein 7	-3.58	-2.85
N93686	aldehyde dehydrogenase 7	-2.30	-1.77
R56046	guanine nucleotide binding protein (G protein), alpha z polypeptide	-0.30	-1.17
AA188797	IDN3 protein	-0.60	-1.07
R93185	sialyltransferase 9 (CMP-NeuAc:lactosylceramide alpha-2,3-sialyltransferase; GM3 synthase)	-2.12	-1.07
AI359781	thyroid hormone receptor interactor 12	-1.36	-1.02
H73714	replication factor C (activator 1) 1 (145kD)	-0.62	-1.01
AI364119	DNA-dependent protein kinase catalytic subunit-interacting protein 2	-0.01	-1.00
AI310256	lipopolysaccharide specific response-68 protein	0.04	-0.96
AI318575	myeloma overexpressed gene(in a subset of t(11;14) positive multiple myelomas)	-2.71	-0.86
AI206032	ESTs	-1.35	-0.83
AA490981	CGI-28 protein	0.71	-0.67
AW029010	nuclear protein	-1.21	-0.64
AI955332	DOM-3 (C. elegans) homolog Z	0.20	-0.51
AA251418	ESTs	-1.03	-0.41
W90116	nuclear mitotic apparatus protein 1	0.29	-0.40
AA459266	postmeiotic segregation increased 2-like 9	0.28	-0.39
AA504356	ESTs	-1.37	-0.37
AA504327	protein tyrosine phosphatase type IVA, member 2	-0.74	-0.32
AA633811	nuclear factor, interleukin 3 regulated	0.65	-0.31
AA917483	MAWD binding protein	-0.65	-0.28
AI344474	CD33 antigen (gp67)	-1.05	-0.23
AA598582	oxidase (cytochrome c) assembly 1-like	0.44	-0.19
AA863093	ESTs	0.32	-0.16
AA485084	hypothetical protein FLJ22584	0.65	-0.11
AA481256	EST	-0.63	-0.02
AI361479	mRNA for FLJ00023 protein	0.96	-0.01
AA521017	EST	-0.36	0.00
AA459945	phosphatidylserine receptor	1.04	0.00

Up-regulated genes in late passages

Gene ID	Gene name	P1-P4*	P5-P7*
AI347128	ESTs	-0.68	0.01
R26186	protein phosphatase 1, catalytic subunit, βisoform	-0.30	0.03
AA486261	signal sequence receptor, delta (translocon-associated protein delta)	0.90	0.04
AA071518	proteasome (prosome, macropain) subunit, α-type, 2	-0.37	0.12
AA489678	RAD23 (S. cerevisiae) homolog B	-0.26	0.12
AA251129	ESTs	-0.87	0.13
AI300634	i-beta-1,3-N-acetylglucosaminyltransferase	0.91	0.21
AI339155	toll-like receptor 1	-0.53	0.24
AA903389	Homo sapiens tripartite motif protein TRIM5 isoform epsilon, complete cds; alternatively spliced	-1.03	0.32
AA910197	pre-mRNA splicing factor/S. cerevisiae Prp18	-0.15	0.33
AA504202	hypothetical protein MGC5508	1.09	0.41
AA991437	ESTs	-0.54	0.42
AI017416	hypothetical protein FLJ11159	-0.21	0.44
AI054096	ESTs	0.04	0.45
AI097258	ESTs	-0.05	0.48
AA173225	dihydroorotate dehydrogenase	1.68	0.49
AI306503	ESTs	0.11	0.50
AI143816	growth differentiation factor 5 (cartilage-derived morphogenetic protein-1)	-0.88	0.51
AA629808	ribosomal protein L6	0.91	0.56
AA521038	lysophospholipase II	0.36	0.65
AI394646	ectonucleoside triphosphate diphosphohydrolase 5	0.26	0.76
AI015690	Homo sapiens cDNA FLJ10004	0.20	0.82
AA486556	CD81 antigen (target of antiproliferative antibody 1)	1.39	0.86
AI341428	Homo sapiens mRNA; cDNA DKFZp586P1622	-1.10	0.87
R99515	E74-like factor 1 (ets domain transcription factor)	0.29	0.87
AI360484	calcium channel, voltage-dependent, alpha 2/delta subunit 1	0.41	0.88
R44739	grancalcin	1.47	0.89
AA669689	malic enzyme 1, NADP(+)-dependent, cytosolic	0.52	1.22
AA872020	protease, serine, 8 (prostasin)	2.04	1.54
R32848	S100 calcium-binding protein P	0.70	2.51

*Mean expression ratio(log R/G)

IV. DISCUSSION

Regardless of the improved diagnostic and treatment options, advanced gastric cancer is still almost incurable. With the improved biotechnologies, efforts to understand the tumor biology had been introduced in cancer research field. With the progressive advances in the cancer biology, biological targeted therapy is a reasonable approach for anti-cancer drug development. However, recent phase III clinical trial results using matrix metalloproteinase inhibitors or angiogenesis inhibitors, which are the most widely studied biological modifiers, failed to prove the preclinical potentials. One of the reasons for this was the improper clinical trial design and the misuse of the *in vivo* animal model systems in preclinical research. It is important to use a proper model system according to the study purposes, particularly the *in vivo* model system. The major limitations of the *in vivo* model system in cancer research are the reproducibility and the reflection of human disease nature.

One way to mimic human cancer is to use the orthotopic animal model system, where tumors develop in the organ of origin in the animal[17-20]. One of the significant tumor physiologies is cross-talk between cancer cells and the microenvironment of the host. This means that the tumor can not develop, invade or metastasize without a sufficient host microenvironment. Hence, the orthotopic

model system is the best system for mimicking the human nature of cancer development and progression.

Among the many orthotopic animal model systems, the gastrointestinal tract cancer orthotopic model system is more difficult. This is because 1) the organ is buried in the abdominal cavity and it is difficult to identify tumor formation and tumor size measurement, 2) its movement is unpredictable and is influenced by many factors, and 3) its mucosa is very thin and difficult to handle[6]. With improved surgical techniques and equipments, these problems were solved, which enabled this system to be used in the research field. However, regardless of the many advantages of the *in vivo* model system, there are still many limitations in terms of reproducibility, the inability to control the environment and the host effect, as well as the low throughput from cost-effectiveness[7,8].

This study used the gastric orthotopic model system combined with an *ex vivo* cell line established from each primary tumor, which may possess the consistent characteristics of each tumor. Using these cell lines *in vitro*, reproducible results of biological characters including cell morphology, cell growth and motility could be obtained (data not shown).

As the cell lines were established from each tumor that

consecutively developed in the gastric mucosa, each cell line became more tumorigenic in the gastric micro-environment than in a metastasis, which suggests the *in vivo* selection of the primary tumor clones. In addition, this study found that the tumors metastasized less to the distant organs and there were more intraabdominal metastasis patterns. There are various applications for orthotopic animal model systems, such as understanding carcinogenesis and the underlying pathogenesis of a primary tumor, the mechanism of invasion and metastasis, and facilitating the tumor specific drug development. This study may work as a proof of concept project for using various applications of the orthotopic model system. For example, if the consecutive cell lines are harvested and established from the metastatic foci in the liver or lymph nodes, they might serve as a significant model system for understanding the mechanism of a distant metastasis and lymph node metastasis.

One of the recent advances in biotechnology is the high-throughput technology of a cDNA microarray, which enabled the expression of thousands of genes to be evaluated simultaneously. As no single biological phenotype is the result of a single genetic event, particularly in cancer cells, the observation of multiple genetic interactions is very helpful for cancer research[11,21-24]. This study used a cDNA microarray to evaluate the genomic information in primary gastric cancer development.

As expected, the parent YCC-3 cell line was significantly different in terms of the genomic expression pattern from the other 7 daughter cell lines established from the orthotopic model system. This might be explained by the fact that the YCC-3 cell was obtained from a human cancer patient and the other cells were the result of an interaction between the human cancer cells and the mouse host microenvironment such as growth factors, cytokines and proteases. Tumor cell and host microenvironment cross-talk is significant and the effect appears to be transferred to the next generation even in an *in vitro* system. It could be confirmed that the significant role of the interaction between the tumor cell and the host cells. This is another reason that the orthotopic model system should be used for better understanding. This study showed that the biological phenotype and genotypes are not the same as expected. However, the correlation and reflection from the genotypes into the phenotypes is considerable. In addition, this study confirmed that the microarray is a useful tool for examining the complex genetic interactions with useful genetic sources for further meticulous research into the underlying mechanism, even though the major problem is data mining and validation.

In this study, by combining the orthotopic model system with a microarray, the gastric cancer biology and the possible mechanism of gastric tumor formation could be determined. Based on the biological and genomic characteristics of each cell line, this

technique may be extended to use each cell line based on its specific characteristics such as YCC-3-P7 for primary gastric tumor research and YCC-3-P1 for multiple systemic metastases.

V. CONCLUSION

Using orthotopic animal model system combined with an *ex vivo* cell line establishment, this study aimed to overcome the limitation of an animal model system to understand the biology of primary gastric cancer. As this study used the orthotopic model system, which more closely reflects the natural courses of human cancer, and the *ex vivo* cell line establishment from *in vivo* selected clones, which enabled the use of cell lines with consistent characteristics, this system and the database of genomic information based on the current model system will help in understanding the pathophysiology of primary gastric cancer development.

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국문초록

위암 동소이식모델에서 유래한 위암세포주의 생물학적 및 유전학적 성상 비교

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라 기 혁

우리나라에서 가장 많이 발생하는 위암은 조기에 발견하게 되면 완치가 가능하나, 침윤과 전이가 일어나게 되면 현존하는 치료방법으로 완치가 불가능하다. 이와 같은 한계점을 해결하기 위하여 많은 연구자들이 암의 침윤 및 전이가 일어나는 원인에 대한 분자 생물학적 및 유전학적 연구를 노력을 기울이고 있다. 이러한 연구를 하기 위해서 적절한 생체내 동물모델을 이용한 실험이 필요로 한다. 현재 가장 많이 사용되는 암 관련 연구 모델은 대개 피하에 종양세포를 투여하여 종양이 형성된 xenograft model을 사용하고 있다. 그러나 원발암의 미세환경과 다른 피하에서의 종양형성능이나 전이능은 실제 환자에서의 암의 성상과 다르다는 것을 쉽게 생각할 수 있다. 즉, 원발암의 원발 장기에 암세포를 투여하여 그 장기에 암이 생성되고 이후 침윤과 전이 과정을 나타내는 동물모델이야말로 종양생리를 정확하게 이해하고 그에 관한 약제 개발에 적절하게 응용될 수 있다. 특히 위암의 경

우는 원발 장기인 위에 암을 유발하는 것이 기술적으로 많은 제한점이 있었으나, 최근 본 외과학교실에서 위암의 동소이식모델을 완성할 수 있게 되어 이를 이용하여 원발 위암의 특성을 갖는 *in vivo* selected clone에서 유래한 cell line을 이용하여 그 생물학적 및 유전학적 특성을 비교하고자 하였다.

본 연세의료원, 연세암전이연구센터에서 확립한 YCC-3 위암 세포주를 이용하여 누누생쥐의 피하에 종양을 형성한 후 그 종양 조각을 위점막에 이식하여 종양이 형성되는 동소이식모델을 이용하였다. 매 원발암을 다시 확보하여 그 종양조각을 다음 생쥐의 위점막에 이식하는 계대배양을 시행하며 매번 세포주를 확립하였다. 먼저 각 계대마다 종양형성 및 전이 양상을 관찰하였다. 이후 형성된 세포주들의 생물학적 특성을 확인하기 위하여 먼저 세포주의 형태를 관찰하고, 생쥐의 피하에 주사하여 생체내 종양형성능을 확인하였다. 생체외 특성으로 세포성장속도를 tumor doubling time으로 측정하였고, 생체외 종양형성능과 세포의 이동능을 anchorage independent soft agar assay와 Boyden chamber assay를 이용하여 각각 관찰하였다. 각 세포주의 유전체학적인 성상을 평가하기 위하여 각 세포주의 total RNA를 추출한 후 17K 인간유전자가 점적되어있는 cDNA microarray에 보합하여 발현을 관찰하였다.

8-12주 경과 관찰하여 위암 동소이식모델이 형성된 후, 그 위암 조직을 다시 계대 배양을 하였을 때, 세대가 증가함에 따라 원발위암의 형성을 잘 되었고 주변 림프절의 전이 양상이 다양하게 관찰되었다. 그러나 초기 3계대 까지는 간과 비장 전이가 유발되었으나 그 이후에는 간전이가 감소함을

관찰하였다. 계대배양한 원발성 위암 조직을 이용하여 확립한 세포주들은 현미경적 성상은 유사하였으나 생체외 종양형성능은 YCC-3 세포주에 비하여 점차 계대가 늘어날수록 종양 형성능이 증가하는 것을 확인하였다. 생체외 생물학적성상을 관찰한 결과, YCC-P-7 세포주로 갈수록 YCC-3 세포주에 비하여 세포의 성장 속도는 빨라지고 이동능이 감소하는 경향을 관찰하였고, 생체외 종양형성능은 감소하였다. cDNA microarray를 이용한 유전자 발현결과 parent YCC-3 세포주와 기타 7개 계대배양세포주는 확연히 다른 양상을 가지고 있음을 확인하였고, 7개 세포주간에는 초기 4개 세포주와 후기 3 세포주간의 유사성을 확인하였다. 이와 같은 양상은 특히 세포 성장에 관련되는 유전자들을 선정하여 비교하였을 때도 동일한 결과를 확인할 수 있었다.

암 연구에서 가장 중요한 동물 모델 중 실제 암의 자연경과를 가장 잘 반영하는 동소이식 모델을 이용하여, 그 한계점인 재현성을 극복하고자 동소이식 모델에서 유래된 세포주를 확립하였다. 특히 본 세포주들은 원발 위암 조직의 계대배양을 하면서 확립되어서 원발 위암의 특성 및 발암기전을 잘 반영하리라고 생각되고, 본 세포주들의 생물학적 특성 및 유전체학적 변화 자료는 원발 위암의 발생 및 진행과 관련된 기초 자료로 유용하며 이를 이용한 연구에 유용하게 사용될 수 있으리라 생각한다.

핵심되는 말 :위암, 동소이식 모델, 생물학 특성, 유전체학적, microarray