Comparative genomic analysis of side population in epithelial ovarian cancer

Hyo Sup Shim

Department of Medicine

The Graduate School, Yonsei University

Comparative genomic analysis of side population in epithelial ovarian cancer

Directed by Professor Nam Hoon Cho

Doctoral Dissertation
submitted to the Department of Medicine

the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Hyo Sup Shim

June 2009

This certifies that the Doctoral Dissertation of Hyo Sup Shim is approved.

Thesis Supervisor : Nam Hoon Cho

[Dong Wook Kim: Thesis Committee Member#1)

[Jae Hoon Kim: Thesis Committee Member#2)

[Sun Young Rha: Thesis Committee Member#3)

[Dong Su Kim: Thesis Committee Member#4)

The Graduate School
Yonsei University

June 2009

ACKNOWLEDGEMENTS

I would like to express my gratitude to professor Nam Hoon Cho, who enabled me to write this thesis. I am also grateful to the committee members, professor Dong Wook Kim, professor Jae Hoon Kim, professor Sun Young Rha, and doctor Dong Su Kim, for critical review and advice. I am very indebted to Yoon Pyo Choi and Suki Kang for their excellence and faithfulness.

Finally, this thesis is dedicated to my family.

HS Shim

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

<ABSTRACT>

Comparative genomic analysis of side population in epithelial ovarian cancer

Hyo Sup Shim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Nam Hoon Cho)

Ovarian cancer is the fifth leading cause of cancer deaths and has the highest mortality rate among gynecologic cancers. It is more devastating in that it shows high response rate to initial therapy but most advanced diseases recur and become drug-resistant. Recent observations indicate that there is a small population of cancer cells, named as cancer stem cells (CSCs), which can self-renew and drive tumor growth. CSCs are thought to participate in the mechanisms of recurrence and drug resistance of ovarian cancer. Side population (SP) analysis is a valid marker-independent method to identify putative CSCs. SP has been reported to be enriched in tumorigenic and stem-like cells. In this study, to investigate the characteristics of ovarian CSCs, SP cells were isolated from cell lines primarily cultured from human epithelial ovarian cancer tissue, and then array comparative genomic hybridization (aCGH) was performed to analyze the specific genetic alteration for SP cells. Cell lines from a human ovarian cancer specimen showed heterogeneous populations with variation of surface marker expression, proliferation rate, cell cycle distribution, and SP fraction. There was SP fraction in range of less than 0.5% to 1% in the finally survived 3 clones. aCGH analysis revealed that 2383 genes were specifically altered in SP cells. These genes were involved in the networks, which top functions are cell cycle and cellular and embryonic development. Finally selected 64 genes were core molecules related to

canonical pathways, such as Notch signaling, Wnt/β-catenin signaling, PTEN signaling, G1/S and G2/M checkpoint regulation, PI3K/AKT signaling, and p53 signaling. In review of literatures, there were common genetic changes between present study and previous reports, such as gain of 19p13.1, which is thought to be related to recurrence of ovarian cancer. These results indicate that the origin of CSCs is to some extent related to chromosomal instability and specific alteration happens in the genes participating in cell cycle and embryonic development. Characterization of the ovarian CSC would provide advance in developing novel therapeutics in the management of ovarian cancer.

Key words : epithelial ovarian cancer, cancer stem cells, side population, comparative genomic analysis

Comparative genomic analysis of side population in epithelial ovarian cancer

Hyo Sup Shim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Nam Hoon Cho)

I. INTRODUCTION

Ovarian cancer is the fifth leading cause of cancer deaths and has the highest mortality rate among gynecologic cancers.[1] Epithelial ovarian cancer is the most common type of ovarian cancer and accounts for about 90%.[2] Its overall prognosis is poor: 5-year survival rates for women with advanced disease are only 30%.[1] This prognosis is related in part to the lack of early detection markers, as well as incomplete understanding of mechanisms involved in cancer recurrence and drug resistance. Current therapies are limited by emerging of drug resistance after high initial response rate. Aggressive surgery followed by chemotherapy results in complete clinical response in 50-80% of patients with advanced disease. However, the majority of patients will relapse and become drug-resistant.[3]

It has been increasingly evident that a small population of cancer cells, referred to as "cancer stem cells (CSCs)", is responsible for the aggressiveness of the disease, metastasis and resistance to therapy.[4, 5] Several observations have been suggested the existence of CSCs. Tumors are composed of a heterogeneous mixture of tumor cells at various levels of differentiation. It was observed that only a small fraction of cells within a tumor possess self-renewal capacity.[6] And it was demonstrated that cells

capable of establishing a human acute myelogenous leukemia (AML) phenotype in a recipient mouse were isolated only within the cell fraction containing the hematopoietic stem cells, defined by the CD34+CD38- phenotype.[7] Further, these cells could be passed from animal to animal and maintain the AML phenotype,[8] confirming the property of self-renewal. Similar approach has led to the identification of CSCs within breast tumors,[9] gliomas,[10-12] melanoma,[13, 14] prostate cancer,[15, 16] hepatocellular carcinoma,[17] pancreatic cancer,[18] and colorectal cancer.[19, 20] Some groups proposed terms such as "tumor-initiating cell" or "cancer-initiating cell",[19, 21-23] because in fact the extent to which CSCs share the characteristics of normal stem cells is unclear. Although all terms may be misleading and appropriate only in a limited sense, CSCs have been defined as "a small subset of cancer cells within a cancer that constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and to cause the heterogeneous lineage of cancer cells that comprise the tumor". [4, 24]

Improvements in cancer therapies have prolonged the lives of cancer patients. However, after successful initial therapy, there is often development of secondary tumors leading to the relapse of the disease. If the CSC model is correct and if such cells retain the hallmarks of some tissue stem cells in being rare and entering the cell cycle infrequently, they could constitute a population that is resistant to current therapies designed to kill cycling cells. The ATP-binding cassette (ABC) drug transporters have been shown to protect CSCs from chemotherapeutic agents.[25] If CSCs are relatively refractory to current therapies, then they are unlikely to be curative and relapse would be expected. The CSC hypothesis would require that we refocus on the minority stem cell population that drives tumor growth.[4]

There are many methods to identify CSCs. Side population (SP) fraction technique is one of these methods. Goodell et al. observed that when bone marrow–derived cells are incubated with Hoechst dye 33342 and then

analyzed by dual-wavelength flow cytometry, a small population of cells does not accumulate an appreciable amount of dye and is thus identified as a Hoechst SP.[26] Since its initial application in bone marrow hematopoietic stem cells, the SP technique has been adapted to identify putative stem cells and progenitors in multiple tissues and organs including mammary glnads,[27, 28] lung,[29, 30] liver,[31] epidermis,[32, 33] forebrain,[34] kidney, [35] and prostate.[36] The SP–enriched stem cells are rare (0.01-5%)[33, 35, 37] and heterogeneous, varying with tissue types, stages of development, and methods of preparation.[38-40] The SP phenotype is mediated by the ABC family of transporter proteins. One of the major mediators seems to be ABCG2 or BCRP,[41] which was initially identified in MCF7 breast cancer cells and later found to efflux multiple chemotherapeutic drugs.[42] On the other hand, it should be noted that only a fraction of the SP cells expresses ABCG2[43] and that both SP and known stem/progenitor cells also express other ABC transporters such as MDR-1 (i.e., ABCB1 or P-glycoprotein), MRP-1 (ABCC1), and ABCA2, suggesting that these latter molecules may also be involved in mediating the SP phenotype.[44-46]

SP is enriched in tumorigenic, stem-like cancer cells,[47] and several studies have shown that SP could explain the aggressive behavior of epithelial ovarian cancer.[48-50] Consequently, it is thought to be important to investigate the characteristics of SP cells in understanding the basic mechanisms involved in drug resistance and recurrence. The other reason to investigate the characteristics of SP cells is that current studies have focused on mainly CSC surface markers, most of which is known as normal stem cell markers. SP analysis is a valid marker-independent method to identify stem cell-like cells. To my knowledge, there have been no reports investigating thorough comparative genomic alteration of SP cells so far. In this study, the

SP cells were isolated from primary cell culture of human epithelial ovarian cancer, and then comparative genomic alteration of SP cells was analyzed.

II. MATERIALS AND METHODS

Cell lines and culture conditions

Ovarian cancer (NIH:OVCAR-3, SK-OV-3), breast cancer (MCF7), Lung cancer (A549), Prostate cancer (PC-3), bladder cancer (T24), and Cervix cancer (HeLa, SiHa) cell lines were obtained from the Korean Cell Line Bank (KCLB, Seoul, South Korea) and cultured in RPMI-1640 medium and DMEM containing 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100μg/mL streptomycin (all, Gibco BRL, Grand Island, NY, USA). And normal human foreskin keratinocytes (HFKs) were obtained from Cambrex Bio Science (Walkersville, USA) and grown in KGM Bulletkit (Cambrex). Cultures were maintained at 37℃ in a 5% CO_2 atmosphere at 100% humidity.

Primary cell culture and clone derivation

The study was approved by the ethics committee and informed consent was obtained from the patient. Tumor tissue specimen was obtained from a 48-year-old patient undergoing surgery for the removal of ovarian tumor diagnosed as mucinous adenocarcinoma and stored in HBSS, containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 2.5 μg/ml amphotericin B and 5% FBS (all, Gibco BRL). Then these tissues underwent washing, quantification and mincing, followed by incubation with extracellular matrixdegrading enzymes, such as dispase, proteinase and DNase (all, Sigma-Aldrich, St. Louis, MI, USA), at 37℃ for 12 to 16 hours.

Cells were harvested using a cell strainer with 100 μm nylon mesh (BD Falcon, Bedford, MA, USA). To eliminate normal cells, the cell suspension was subjected to ficoll (1.077g/ml) gradient centrifugation, at 400g for 15 min.

The cell pellet was collected and suspended in IMDM containing 100 IU/mL penicillin and 100 μg/mL streptomycin, 100 μg/mL gentamicin, 2.5 μg/mL amphotericin, and 20% FBS (all, Gibco BRL). In the following step, cell suspension was seeded in 24-well plates and maintained at 37℃ in a 5% CO_2 atmosphere at 100% humidity. To avoid fibroblast overgrowth, we attempted to remove the fibroblasts mechanically or transfer the tumor cells selectively by double trypsinization (trypsin 0.05% w/v, EDTA 0.02% w/v). Also, routine assays for Mycoplasma, fungi, and bacterial contamination were negative. Each cell population was periodically recloned by plating single cells in medium under direct vision and expanding fresh populations from a single clone. Once isolated and recloned, each clone was examined for expression of stem cell markers to determine potential surface markers for ovarian cancer stem cells (CSCs) and was collected at each passage by centrifugation, resuspended in fresh medium. The present culture gave rise to 20 clones designated C2, C3, C4, C4.2, C5, C5.2, C6, C6.2, C7, C7.2, C8, C9, C10, C11, C12, C13, C13.2, C14, C15, and C20. Among these clones, 3 clones, C2, C4 and C7.2, survived until the last 28^{th} passages.

Semiquantitative RT-PCR analysis

Potential ovarian CSC markers were selected after considering previous reports. They are ABCG2, CD24, CD117, CD44, and EpCAM/CD326. Total RNA was extracted from finally survived 3 clones by using an UltraspecTM-II RNA isolation kit (Biotecx, Houston, TX, USA) according to the manufacturer's protocol. cDNA was synthesized using 500ng of total

RNA with SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). Primers, product size, and PCR conditions are listed in table 1.

Table 1. Primer list of potential CSC markers

Molecules	Sense (5'-3')	Anti-Sense (5'-3')	Size	Tm	Conditions
ABCG2	GGGTTCTCTTCTTCCTGACGACC	TGGTTGTGAGATTGACCAACAGAC	389bp	62℃	95℃ 15min, 35 cycles : 94℃ 40sec, Tm 1min, 72℃ 1min, 72℃ 10min
CD24	ACCCAGCATCCTGCTAGAC	CTTAAGAGTAGAGATGCAGAA	287bp	57.5℃	95℃ 15min, 35 cycles : 94℃ 40sec, Tm 1min, 72℃ 1min, 72℃ 10min
CD117 (C-Kit)	GCCCACAATAGATTGGTATTT	AGCATCTTTACAGCGACAGTC	570bp	62℃	95℃ 15min, 35 cycles : 94℃ 40sec, Tm 1min, 72℃ 1min, 72℃ 10min
CD44	AACCTGCCGCTTTGCAGGTGT	ATCAAAGGCATTGGGCAG	342bp	57.5℃	95℃ 15min, 35 cycles : 94℃ 40sec, Tm 1min, 72℃ 1min, 72℃ 10min
EpCAM/ CD326	GCCAAGCAGCAGTACATTCTACACG	GCTGTTCTTCACGTGCTCAAAATC	342bp	62℃	95℃ 15min, 35 cycles : 94℃ 40sec, Tm 1min, 72℃ 1min, 72℃ 10min
GAPDH	ACAACTTTGGTATCGTGGAA	AAATTCGTTGTCATACCAGG	458bp	58℃	95℃ 15min, 35 cycles : 94℃ 40sec, Tm 1min, 72℃ 1min, 72℃ 10min

Cell proliferation assay

Cells were plated in triplicate into a 96-well flat-bottom microtiter plates (Corning-Costar) and cultured for an appropriate period of time. Cell proliferation was measured using a WelCount Cell Viability Assay Kit (WelGENE, Seoul, South Korea) at 0, 24, 48, 72, and 96hrs after seeding cells. After observing a change in color of the solution, we quantified by measuring the absorbance of the samples with using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 450 nm, and nonspecific readings were eliminated by using a wavelength of 690 nm. All experiments were performed in triplicate.

Cell cycle distribution observation

Cell cycle distribution was monitored by flow cytometry (FACSCalibur;

Becton Dickinson, San Jose, CA) using PI staining, according to the manufacturer's instructions. Briefly, Flow cytometry analysis of PI-stained cells was performed to demonstrate the progression of the cell cycle. Briefly, cells were harvested, washed and fixed in 70% ethanol overnight at 4°C. Prior to flow cytometry, cells were washed and stained with 1 ml PI (5mg/ml) containing 0.1 mg/ml RNaseA (all, Sigma-Aldrich). DNA content was determined with a FACScan flow cytometer (Becton Dickinson) and the proportion of cells in a particular phase of cell cycle was determined with CellQuest software. The list-mode files were analyzed with ModFit LT for Win32 version 3.1 software (Verity Software House, Topsham, ME, USA). All experiments were performed in triplicate.

Side population (SP) analysis

SP analysis was performed in the Flow Cytometry Laboratory Clinical Research Institute Seoul National University Hospital according to Goodell protocols.[51] Briefly, 3 X 10^6 cells/mL and a parallel aliquot as a negative and a isotype controls for flow cytometry analysis were incubated in HBSS containing 2% FBS, 10 mmol/L HEPES (all, Gibco BRL), and 40 μg/mL Hoechst 33342 dye (Sigma-Aldrich) for 90 min at 37℃, either alone or in the presence of 400 μM verapamil (Sigma-Aldrich). And cells were gently agitated every 15 minutes. Cells were then washed twice with ice-cold HBSS containing 2% FBS (Gibco BRL) for 5 min at 300×g, and then resuspended in ice-cold HBSS/2% FBS. FcR blocking reagent was added to prevent non-specific binding and incubated at 4℃ for 10 min, after which the sample was washed once with HBSS/2% FBS and resuspended with HBSS/2% FBS. After washed twice with HBSS/2% FBS, cells were washed and then incubated in ice-cold HBSS (Gibco BRL) supplemented with 2% FBS (Gibco BRL), 10 mmol/L HEPES (Gibco BRL), and 1 μg/mL 7-AAD

(Sigma-Aldrich), at 4℃ for 10 min, to discriminate dead cells. The suspension was filtered through a cell strainer with 70 μm nylon mesh. (BD Falcon, Franklin Lakes, NJ, USA). Samples were analyzed on a BD FACSAria™ cell sorter (BD Biosciences, San Jose, CA, USA) by using a dual wavelength analysis (blue, 424–444 nm; red, 675 nm) after excitation with 350-nm UVlight.

array Comparative Genomic Hybridization (aCGH) analysis

Human female reference genomic DNA (gDNA) was purchased from Promega (Madison, WI , USA). And the sample genomic DNAs (gDNAs) of SP and NSP cells were extracted using QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and were quantified by standard techniques. The isolated gDNAs from SP and NSP cells were amplified using the GenomePlex WGA kit (Sigma) and 50 ng of these in each WGA reaction was used to generate a representative amplification of gDNAs. After amplification, excess primers and dNTPs were removed by using QIAquick PCR Purification Kit (Qiagen). All procedures were carried out according to the manufacturer's instructions. Also, human female reference gDNA and non-amplified NSP DNA samples were digested with AluI and RsaI. Agilent's Genomic DNA Labeling Kit PLUS (Agilent Technologies, Santa Clara, CA, USA) was then used to label the amplified WGA gDNAs and restriction enzyme-digested gDNAs with Cy3 and Cy5, respectively. As recommended by Agilent, 2.0 μg of amplified DNA was used as the input starting material for each labeling reaction. Cy3- and Cy5-labeled samples were mixed and hybridized to Agilent microarrays from either the Human Genome CGH Microarray Kit 244A following Agilent's standard processing recommendations. Microarray scanning and data analysis Scanning and image analysis were conducted according to Agilent's

Oligonucleotide Array-based CGH for Genomic DNA analysis Protocol (version 5.0). Microarrays were scanned using an Agilent DNA Microarray Scanner (Agilent P/N G2565BA). Agilent's Feature Extraction software (v9.5.3.1) was used to extract data from raw microarray image files in preparation for analysis. Agilent CGH Analytics software (v4.0.73) was used to visualize, detect and analyze aberration patterns from CGH microarray profiles. All regions of statistically significant copy number changes are identified using the aberration detection module-1 (ADM-2) algorithm.[52-55]

Ingenuity Pathways Analysis (IPA analysis)

Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA) is a useful and up-to-date software application that enables researchers to model, analyze, and understand the complex biological, chemical, and functional systems at the core molecular network of life science research. Under the criteria combining Gene accession numbers, the fold change, and the t-test P value, the significantly altered 264 genes were selected from aCGH data and used for the network generation and pathway analysis. And the genes were categorized based on molecular functions using the IPA software. The identified genes also were mapped to genetic networks in the IPA database and ranked by score. The score reflects the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone. Scores of 3 or higher indicate a 99.9% confidence level that the network was not generated by chance alone. This score was used as the cut-off for identifying gene networks. Moreover, relationships between the network genes were examined with gene ontology analysis in IPA to find the related functions, and the known pathways associated with metabolism and signaling were investigated by Canonical pathway analysis. The genetic network analysis focuses on the functional relationships, among the genes that

are below the threshold, that are present in the literature to create a network of genes with similar functions and recorded interactions. Further analysis can be done by focusing on the functions themselves in which the genes are categorized in IPA, using gene ontology information taken from the literature. This provides insight into what processes were active when the samples were taken. To concentrate on any specific gene and its pathway, another tool, canonical pathway analysis was available.[56, 57]

Statistical analysis

The Student's *t*-test was used for statistical analysis. Statistical significance was accepted if the null hypothesis was rejected with $P<0.05$.

III. RESULTS

Derivation of finally survived 3 clones with different characteristics

The present culture system gave rise to 20 clones designated C2, C3, C4, C4.2, C5, C5.2, C6, C6.2, C7, C7.2, C8, C9, C10, C11, C12, C13, C13.2, C14, C15, and C20. Among these clones, 3 clones, C2, C4 and C7.2, survived until the last 28th passages. Consequently, this study focused on these 3 clones.

Three clones showed somewhat similar or different characteristics each other in basic analyses investigating the expression of surface markers, cell proliferation assay, and cell cycle distribution observation. Semiquantitative RT-PCR analyses for expression of potential ovarian CSC markers showed that ABCG2, CD24, CD44, and EpCAM/CD326 were similarly expressed in 3 clones, whereas CD117 was relatively highly expressed in C2 and C7.2 than in C4 (figure 1A). Cell proliferation assays showed somewhat different growth rate between C2, C7.2 and C4. Clone 4 shows the fastest growth rate

(figure 1B). Cell cycle distribution observation showed variable proportion of G0/G1, S, and G2/M among clones (figure 1C). For example, in C7.2, 66.5% of cells was in G0/G1 phase, 8.5% in S phase, and 25% in G2/M phase. C7.2 had the largest proportion for G2/M phase. There was also the most aneuploidy in C7.2. SP proportions were also different among 3 clones according to the following result.

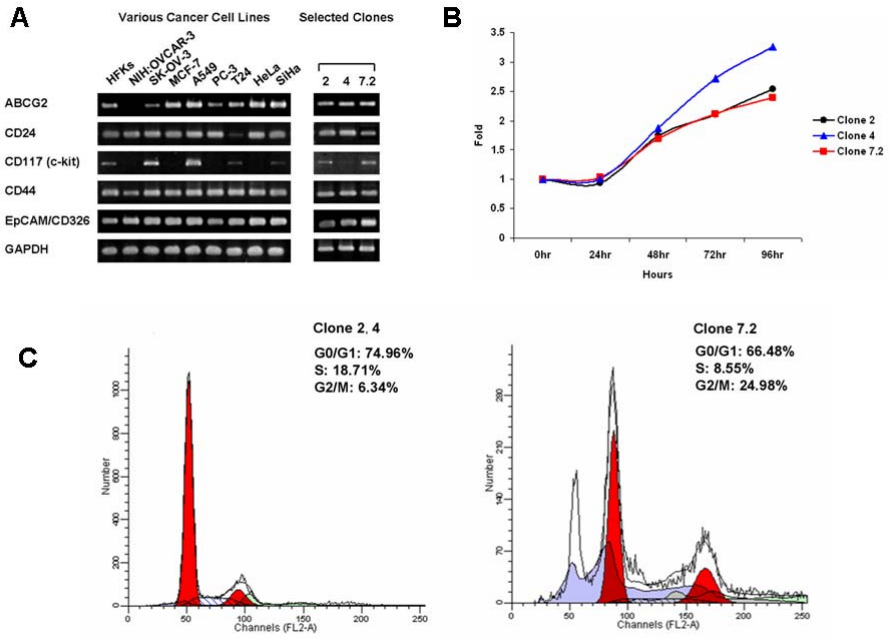

Figure 1. Different characteristics of finally survived 3 clones (C2, C4, and C7.2) from a human ovarian cancer specimen. A, Semiquantitative RT-PCR analyses for expression of potential ovarian CSC markers. ABCG2, CD24, CD44, and EpCAM/CD326 are similarly expressed in 3 clones, whereas CD117 is relatively highly expressed in C2 and C7.2 than in C4. GAPDH was used as an internal control. B, Cell proliferation assays shows somewhat different growth rate among clones. Clone 4 shows the fastest growth rate. C, Cell cycle distribution observation shows different proportions of G0/G1, S, and G2/M among clones. C7.2 has the largest proportion for G2/M phase (24.98%) and shows the most aneuploidy.

Result of SP analysis

Hoechst 33342 HSC staining followed by flow cytometry showed that there was side population of less than 0.5% in C2, 0.6-0.8% in C4, and more than 1% in C7.2 (figure 2A, E, and I). C7.2 had the largest proportion of SP. The treatment with verapamil confirmed the presence of SP cells (figure 2B, F, and J). SP and NSP cells were recovered in culture and observed with an inverted microscope. SP cells from 3 clones tightly formed colonies after 2 weeks in culture (figure 2C, G and K), whereas NSP cells were scattered and did not proliferate (figure 2D, H and L).

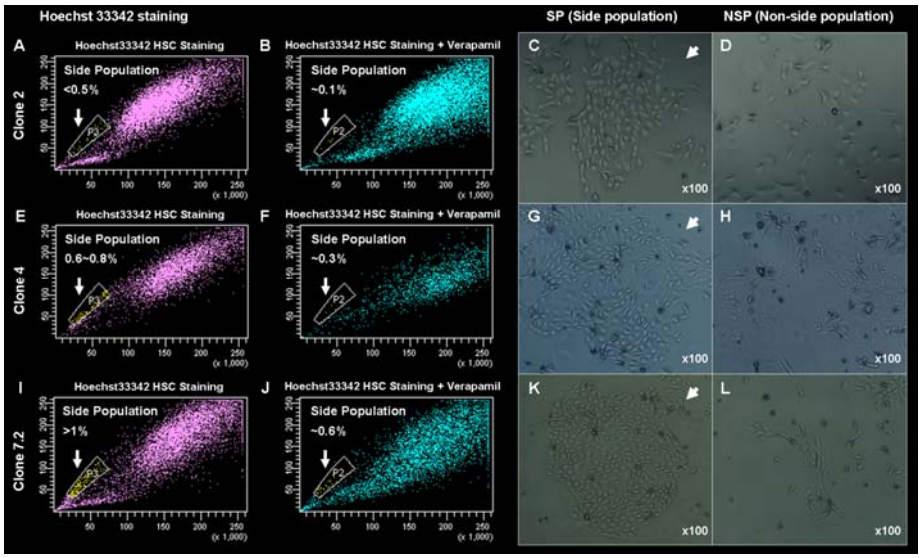

Figure 2. Identification and growth characteristics of SP cells in 3 clones. 3 clones were stained with Hoechst 33342 dye and analyzed by flow cytometry before (A, E and I) and after (B, F and J) treatment with verapamil. There is side population of less than 0.5% in C2, 0.6-0.8% in C4, and more than 1% in C7.2. SP (C, G and K) and NSP (D, H and L) cells were recovered in culture and photographed with an inverted microscope (x100). SP cells from 3 clones tightly form colonies after 2 weeks in culture, whereas NSP cells are scattered and do not proliferate.

Selection of a clone for aCGH analysis

C7.2 was finally selected for aCGH analysis because, first of all, it had the largest proportion of SP among 3 clones. In addition, C7.2 had the most aneuploidy cells and showed more different characteristics of cell cycle distribution than other clones.

Result of aCGH analysis for SP and NSP

aCGH analysis showed there were numerous genomic gains and losses in SP and NSP cells (figure 3). The apparent common regions with chromosomal gains were 6, 7, 17, 19, 20, and Xq, and the apparent common regions with chromosomal losses were 3p and 9p. To focus on the genomic alteration of SP cells, the common genomic changes between SP and NSP and the specific change for NSP were extracted. There remained 2383 genes, which were the specific changes for only SP cells, and 198 genes were increased or decreased at least 2-fold.

Result of IPA analysis

The significance of the specific genomic changes of SP cells was validated using IPA analysis. IPA analysis was performed for 198 genes with gains or losses at least 2-fold in aCGH results and identified 13 networks (figure 4A). Their top functions were as follows; cell cycle, hair and skin development and function, embryonic development, cellular growth and proliferation, cellular movement, and gene expression. Ten networks were merged through link molecules (figure 4B). Sixty four genes were finally selected by IPA analysis (figure 5, table 2). These 64 genes played a pivotal role as core molecules in

networks. Canonical pathways with molecules closely related to SP cells were Notch signaling, Wnt/β-catenin signaling, PTEN signaling, G1/S and G2/M checkpoint regulation, PI3K/AKT signaling, and p53 signaling. These networks were also related with potential ovarian CSC markers, such as CD44, KIT (CD117), and EpCAM.

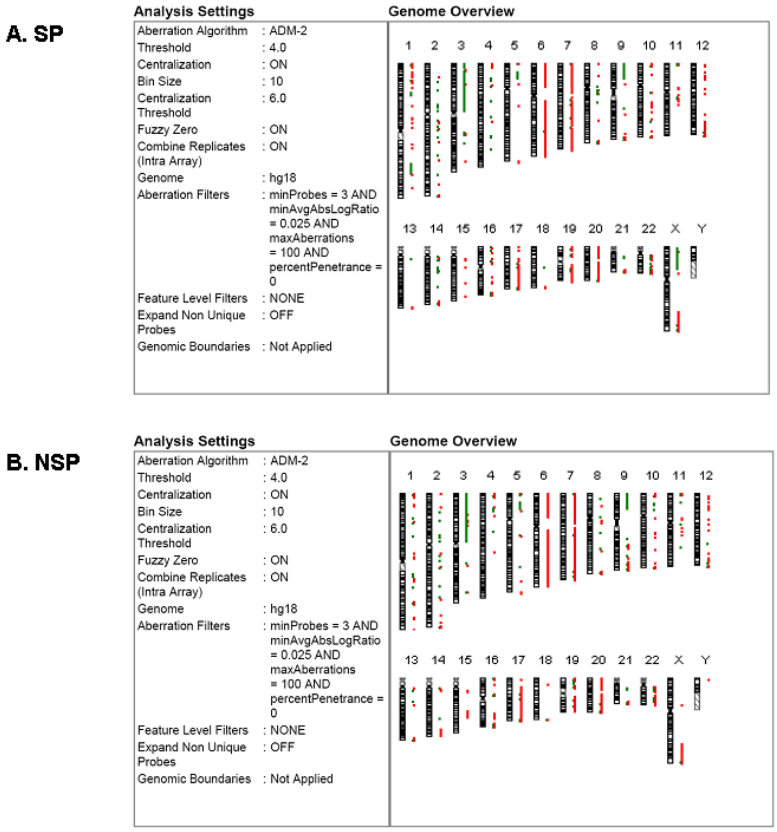

Figure 3. Chromosomal alterations in SP (A) and NSP (B) cells by aCGH analysis (red: gain, green: loss). The apparent common regions with chromosomal gains were 6, 7, 17, 19, 20, and Xq, and the apparent common regions with chromosomal losses were 3p and 9p.

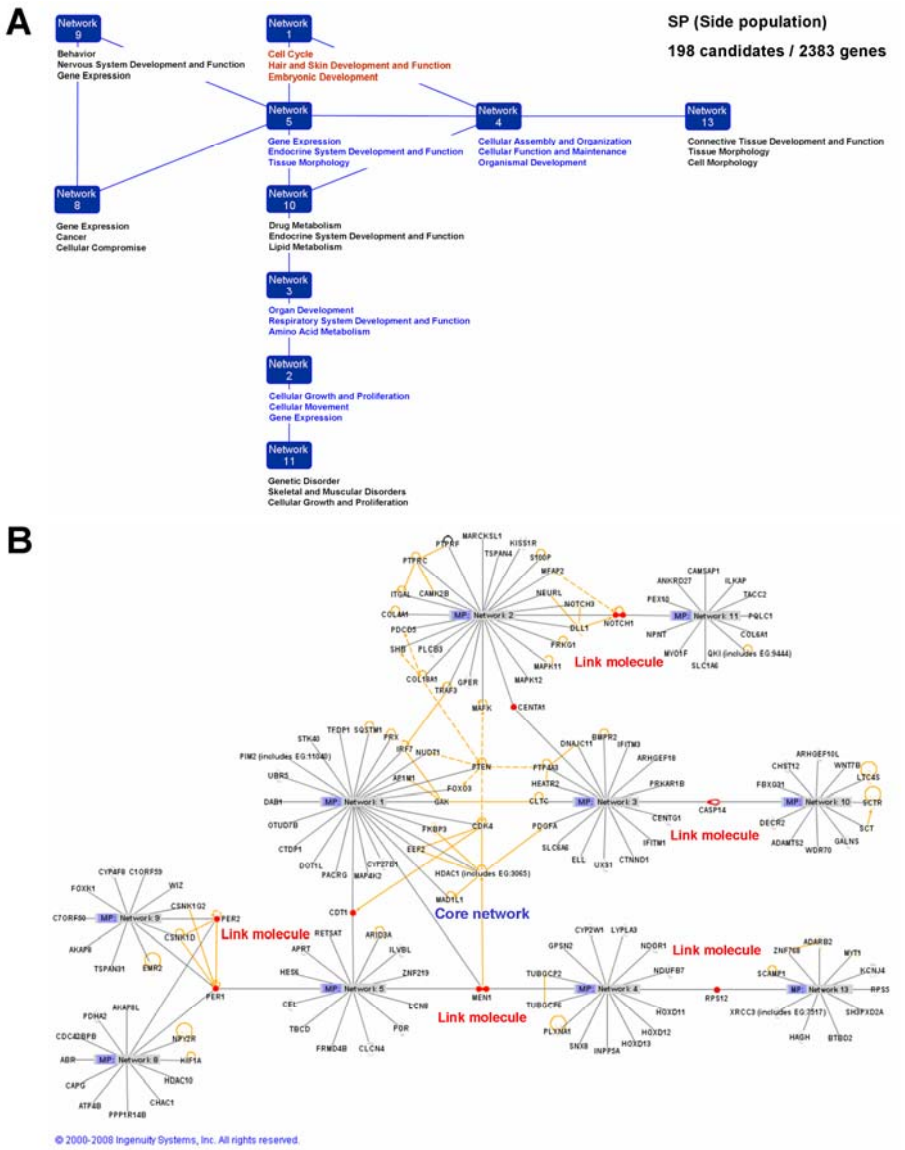

Figure 4. Overlapping networks of genes based on chromosomal alterations in SP by IPA analysis. IPA analysis was performed for 198 genes with gains or losses at least 2-fold in aCGH results and identified 13 networks. Finally, 10 networks were merged by overlapping genes by IPA analysis. A, Overlapping network for top functions. B, Show all of genes among each network.

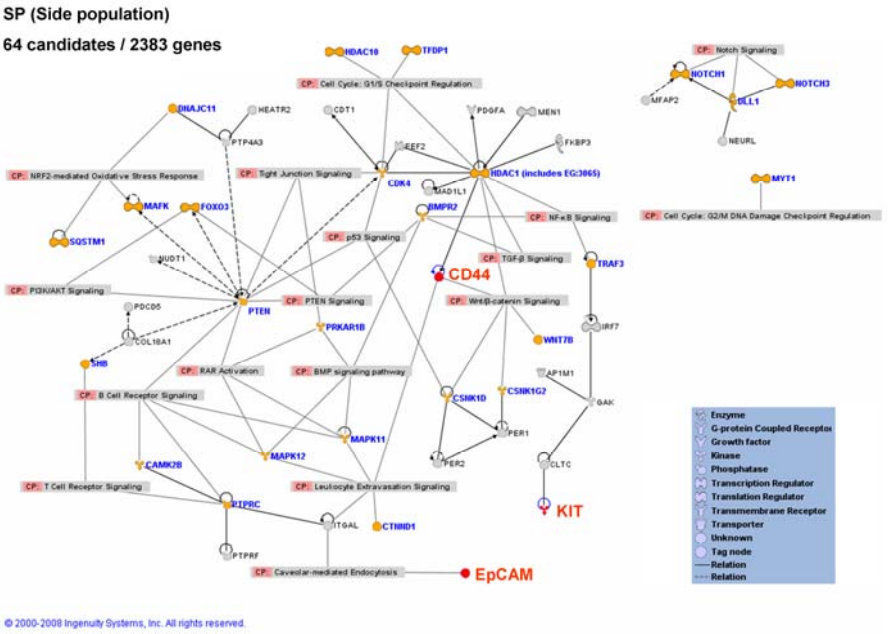

Figure 5. Core networks and canonical pathways of genes related to SP cells. The number of finally selected genes are 64 by IPA analysis. Novel core networks with molecules closely related to SP cells are Notch signaling, Wnt/β-catenin signaling, PTEN signaling, G1/S and G2/M checkpoint regulation, PI3K/AKT signaling, and p53 signaling. The diagram also shows relationship with potential ovarian CSC markers, such as CD44, KIT (CD117), and EpCAM.

Table 2. Genomic gains/losses of SP cells compared with NSP cells

Chromosome	Gains		Losses	
	Cytoband	Gene name	Cytoband	Gene name
chr1	p36.31, p36.13, p35.1, p34.2, p21.2	DNAJC11, MFAP2, HDAC1, PTPRF, CDC14A	q31.3	PTPRC
chr2	q14.2, q37.3	SCTR, PER2	q33.1, q37.3	BMPR2, KIF1A
chr3	q21.3	PLXNA1		
chr4	p16.3, p16.1	GAK, S100P	q32.1	NPY2R
chr5	q14.1, q35.3	SCAMP1, LTC4S, SQSTM1		
chr6	q21, q23.2, q26, q27	FOXO3, RPS12, QKI, DLL1		
chr7	p22.3 - p22.2, p22.3, p13	PDGFA, HEATR2, CEMTA1, MAFK, MAD1L1, NUDT1, EIF3B, MAFK, CAMK2B		
chr8	q24.3	PTP4A3		
chr9	p13.2, q34.3	SHB, NOTCH1		
chr10	p15.3, q11.23, q23.31, q26.3	ADARB2, PRKG1, PTEN, TUBGCP2	q24.33	NEURL
chr11	p15.5, q13.1	IRF7, SCT, MEN1		
chr12	q14.1	CDK4		
chr13	q34	COL4A1		
chr14	q12, q21.3, q32.32	NOVA1, FKBP3, TRAF3		
chr16	p11.2, q24.3	ITGAL, ZNF768, CDT1	p13.3	FAHD1
chr17	p13.1, q23.1, q25.3	PER1, CLTC, CSNK1D		
chr19	p13.3, p13.3, p13.3, p13.12, p13.11, q13.11	ARID3A, CSNK1G2, EEF2, EMR2, CASP14, NOTCH3, AP1M1, PDCD5	q13.2	PRX
chr21			q22.3	COL18A1
chr22	q13.33	MAPK11, TUBGCP6		

IV. DISCUSSION

A CSC hypothesis has proposed that tumors contain a small proportion of stem-like cancer cells with unique properties that would retain self-renewal and tumor-initiating potential.[4, 5] Recent studies have more and more indicated that aggressiveness of cancer may be related to these CSCs, which can resist and survive to current therapy.[25] Epithelial ovarian cancer is one of the most lethal malignancy among gynecologic cancers.[1] It is more devastating in that it shows high response rate to initial therapy but most advanced diseases recur and become drug-resistant.[3] The CSC hypothesis could explain the aggressiveness of epithelial ovarian cancer.

In this study, SP cells were the main focus to investigate how CSCs take

part in aggressiveness of ovarian cancer. SP cells can be isolated through unique property, that is efflux of Hoechst 33342 dye.[26] This ability is related to ABC transporters. For example, ABCG2, a representative of ABC transporters, was originally identified in mitoxantrone-resistant cells.[25] It was reported that SP was enriched in tumorigenic, stem-like cancer cells.[47] In addition, ovarian cancer SP defined cells with stem cell-like characteristics,[49] and the SP of ovarian cancer was suggested to be a primary target of antitumor therapy.[50] The SP approach represents a valid marker-independent method to identify stem-like cells. The other reason to focus on SP was that many reports mainly investigated CSC surface markers. Although research for surface markers is very important to isolate CSCs, more basic research is necessary for CSC characteristics and it is doubt that these surface markers are maintained through generations.

To obtain SP cells of epithelial ovarian cancer, a primary cell culture was performed from the surgically resected tissue obtained from a patient diagnosed as mucinous adenocarcinoma. This primary cell culture gave rise to 20 clones. All these clones could be successfully subcultured. However, clones had diverse characteristics, such as variable expression of surface markers, variable proliferation rate, and variable cell cycle distribution. It was no wonder that these primary clones were heterogeneous, based on the hierarchical theory of CSC model. However, this clonal heterogeneity became an obstacle to generalize the results of basic studies to evaluate clonal characteristics. Among 20 clones, finally survived 3 clones, C2, C4, and C7.2, were selected. This selection was based on that stem-like cells were identified in immortalized cell lines[46] and long-term cultured cancer cells.[45, 58] It was thought that SP cells might more exist in finally survived clones than in other clones. C7.2 was finally selected to investigate characteristics of SP cells, based on three reasons. First, as mentioned above, C7.2 was one of the finally survived 3 clones. Second, in cell cycle distribution observation, C7.2

was the most unique clone in that it had the most aneuploidy cells and the largest proportion of G2/M phase. Third, in fact first of all, C7.2 had the largest fraction of SP. The limitation of CSC study is that there are very few CSCs (less than 1%) in the tumor. So, it is not easy to investigate an amount of purified CSCs. In this study, C7.2 could not but be selected to obtain the maximum SP cells.

Cancer is a disease of genetic and epigenetic alterations. Various molecular changes participate in the tumorigenesis, invasion and metastasis in genomic, transcriptional, and translational levels. Likewise, diverse molecular mechanisms may be participated in SP cells. In this study, above all, comparative genomic analysis (CGH) was performed to investigate SP characteristics. CGH was applied because it is a powerful tool to screen the entire genome of a tumor for genetic changes by highlighting regions of altered DNA sequence copy numbers (deletions and amplifications). There is a reason that CGH was preferentially used rather than other methods, such as cDNA array and proteomics. It has been reported that cancer can also occur as a result of chromosomal gain, loss and/or rearrangement which are presumed to related to CSCs.[59, 60] Both mutations and chromosomal derangements might be involved in the establishment of CSCs,[59] and chromosome instability might be related to CSCs.[61]

The result of aCGH showed there were numerous genomic gains and losses in all SP and NSP cells. The apparent common regions with chromosomal gains were 6, 7, 17, 19, 20, and Xq, and the apparent common regions with chromosomal losses were 3p and 9p. When the genetic alteration of SP cells was compared with NSP, the remained genes, specific for SP cells, were related to several networks, which top functions were cell cycle, hair and skin development and function, embryonic development, cellular growth and proliferation, cellular movement, and gene expression. By IPA analysis, 64 genes were finally selected. These 64 genes played a pivotal role as core

molecules in canonical pathways, such as Notch signaling, Wnt/β-catenin signaling, PTEN signaling, G1/S and G2/M checkpoint regulation, PI3K/AKT signaling, and p53 signaling. These networks were also related with potential ovarian CSC markers, such as CD44, KIT (CD117), and EpCAM. These results indicate that the origin of CSCs is to some extent related to chromosomal instability and specific alteration happens in the genes participating in cell cycle and cellular and embryonic development.

CGH has been applied extensively to analyzing genomic changes in ovarian cancer. Several reports have been published on the correlation of CGH alterations and a variety of clinical parameters in ovarian cancer (table 3). Hu et al. reported that gains of 2p22p25, 19p12q13.1, and 20q12q13 and loss of 5q14q22 were associated with higher recurrence rates.[62] Kiechle et al found that undifferentiated (high grade) tumors correlated with gains in 8q and 7p as well as losses in 11p and 13q.[63] Makhija et al. found that patients with a loss of D6S1581 (located in 6q25.1) were more likely to be platinum-resistant.[64] Gain in 5p was significantly correlated with a higher risk of recurrence according to the report of Bruchim et al.[65] The CGH data from most of the previous studies were derived from a mixed tumor population of different histologic types as well as a mixed population of primary and recurrent tumors. So, it is difficult to directly compare this present result with previous reports. Furthermore, this present study was performed to SP cells from primary cell culture. However, there can be several indications in that SP cells can be related to cancer recurrence or aggressiveness. In comparison with chromosomal alteration of recurrent tumors, there were common genetic changes, such as gain of 19p13.1 between present study and previous reports. Further study is necessary to evaluate the significance of these altered genes.

To my knowledge, this study is the first report of aCGH result for SP cells. A reason to investigate the characteristics of SP cells is that this is a means to develop agents directed against CSCs. It is important that these agents

discriminate between CSCs and non-CSCs as well as between CSCs and normal stem cells. This will require the identification of realistic drug targets unique to CSCs. The identification of such targets and the development of anti-cancer agents will require a deeper understanding of normal stem cell biology as well as cancer biology. Characterization of the ovarian CSC would provide advance in developing novel therapeutics in the management of ovarian cancer.

Table 3. Comparative genomic hybridization studies in ovarian cancer (modified from reference 65)

Reference	Ovarian cancer, no. of cases	Gains	Losses
Iwabuchi et al.,[66] 1995	44 primary	3q, 8q	16q, 17
Suzuki et al.,[67] 2000	60 primary	3q26, 8q24, 20q13	4q
Kiechle et al.,[63] 2001	106 primary	8q, 1q, 20q, 3q, 19p	13q, 4q, 18q
Hu et al.,[62] 2003	10 primary +10 recurrent	1q, 8q, 19, 20q, 3q, 12p, 2p, 7p, 5p, 17q	Xp, 4q, 18q, 13q, 9p, 16q
Fishman et al.,[68] 2005	6 primary +6 metastatic	1q, 2p, 2q, 3q, 6q, 8q, 12p	18q, X
Helou et al.,[69] 2006	17 primary	1q21-31q, 2p, 3q, 5p, 7, 10p, 12p, 16p, 17, 19q, 20q, 22q	X, 3p, 8p, 9, 11p, 13, 14, 18
Bruchim et al.,[65] 2009	45 primary	2q, 3q, 8q, 1q	5q, 19, 22
Present study	Primary cell culture	6, 7, 17, 19, 20, Xq	3p, 9p

V. CONCLUSION

1. Primary cell lines from a human ovarian cancer specimen showed heterogeneous populations with variation of surface marker expression, proliferation rate, cell cycle distribution, and SP fraction.
2. There was SP fraction in range of less than 0.5% to 1% in the finally survived 3 clones.
3. There were 2383 altered genes, which are specific for SP cells compared with NSP cells by aCGH analysis.
4. Altered genes in SP cells were involved in the networks, which top functions are cell cycle, embryonic development, and hair and skin development and function.
5. Finally selected 64 genes were core molecules related to canonical pathways, such as Notch signaling, Wnt/β-catenin signaling, PTEN signaling, G1/S and G2/M checkpoint regulation, PI3K/AKT signaling, and p53 signaling.
6. In review of literatures, there were common genetic changes between present study and previous reports, such as gain of 19p13.1, which is thought to be related to recurrence of ovarian cancer.
7. These results indicate that the origin of CSCs is to some extent related to chromosomal instability and specific alteration happens in the genes participating in cell cycle and embryonic development.
8. Characterization of the ovarian CSC would provide advance in developing novel therapeutics in the management of ovarian cancer.

REFERENCES

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. CA: a cancer journal for clinicians 2008;58(2):71-96.

2. Seidman JD, Kurman RJ. Pathology of ovarian carcinoma. Hematol Oncol Clin North Am 2003;17(4):909-25, vii.

3. Cannistra SA. Cancer of the ovary. N Engl J Med 1993;329(21):1550-9.

4. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, et al. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res 2006;66(19):9339-44.

5. Houghton J, Morozov A, Smirnova I, Wang TC. Stem cells and cancer. Semin Cancer Biol 2007;17(3):191-203.

6. Harrington L. Does the reservoir for self-renewal stem from the ends? Oncogene 2004;23(43):7283-9.

7. Vormoor J, Lapidot T, Pflumio F, Risdon G, Patterson B, Broxmeyer HE, et al. Immature human cord blood progenitors engraft and proliferate to high levels in severe combined immunodeficient mice. Blood 1994;83(9):2489-97.

8. Dick JE. Normal and leukemic human stem cells assayed in SCID mice. Semin Immunol 1996;8(4):197-206.

9. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100(7):3983-8.

10. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003;63(18):5821-8.

11. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature 2004;432(7015):396-401.

12. Sanai N, Alvarez-Buylla A, Berger MS. Neural stem cells and the origin

of gliomas. N Engl J Med 2005;353(8):811-22.
13. Fang D, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S, et al. A tumorigenic subpopulation with stem cell properties in melanomas. Cancer Res 2005;65(20):9328-37.
14. Monzani E, Facchetti F, Galmozzi E, Corsini E, Benetti A, Cavazzin C, et al. Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. Eur J Cancer 2007;43(5):935-46.
15. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65(23):10946-51.
16. Miki J, Furusato B, Li H, Gu Y, Takahashi H, Egawa S, et al. Identification of putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized primary nonmalignant and malignant tumor-derived human prostate epithelial cell lines and in prostate cancer specimens. Cancer Res 2007;67(7):3153-61.
17. Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007;132(7):2542-56.
18. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. Cancer Res 2007;67(3):1030-7.
19. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445(7123):111-5.
20. Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, et al. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci USA 2007;104(24):10158-63.
21. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445(7123):106-10.

22. Neuzil J, Stantic M, Zobalova R, Chladova J, Wang X, Prochazka L, et al. Tumour-initiating cells vs. cancer 'stem' cells and CD133: what's in the name? Biochem Biophys Res Commun 2007;355(4):855-9.
23. Phillips TM, McBride WH, Pajonk F. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. J Natl Cancer Inst 2006;98(24):1777-85.
24. Hill RP, Perris R. "Destemming" cancer stem cells. J Natl Cancer Inst 2007;99(19):1435-40.
25. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005;5(4):275-84.
26. Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 1997;3(12):1337-45.
27. Clayton H, Titley I, Vivanco M. Growth and differentiation of progenitor/stem cells derived from the human mammary gland. Exp Cell Res 2004;297(2):444-60.
28. Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM, Goodell MA. Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol 2002;245(1):42-56.
29. Summer R, Kotton DN, Sun X, Ma B, Fitzsimmons K, Fine A. Side population cells and Bcrp1 expression in lung. Am J Physiol Lung Cell Mol Physiol 2003;285(1):L97-104.
30. Summer R, Kotton DN, Sun X, Fitzsimmons K, Fine A. Translational physiology: origin and phenotype of lung side population cells. Am J Physiol Lung Cell Mol Physiol 2004;287(3):L477-83.
31. Shimano K, Satake M, Okaya A, Kitanaka J, Kitanaka N, Takemura M, et al. Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1. Am J Pathol

2003;163(1):3-9.
32. Terunuma A, Jackson KL, Kapoor V, Telford WG, Vogel JC. Side population keratinocytes resembling bone marrow side population stem cells are distinct from label-retaining keratinocyte stem cells. J Invest Dermatol 2003;121(5):1095-103.
33. Triel C, Vestergaard ME, Bolund L, Jensen TG, Jensen UB. Side population cells in human and mouse epidermis lack stem cell characteristics. Exp Cell Res 2004;295(1):79-90.
34. Kim M, Morshead CM. Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. J Neurosci 2003;23(33):10703-9.
35. Iwatani H, Ito T, Imai E, Matsuzaki Y, Suzuki A, Yamato M, et al. Hematopoietic and nonhematopoietic potentials of Hoechst(low)/side population cells isolated from adult rat kidney. Kidney Int 2004;65(5):1604-14.
36. Bhatt RI, Brown MD, Hart CA, Gilmore P, Ramani VA, George NJ, et al. Novel method for the isolation and characterisation of the putative prostatic stem cell. Cytometry A 2003;54(2):89-99.
37. Tamaki T, Akatsuka A, Okada Y, Matsuzaki Y, Okano H, Kimura M. Growth and differentiation potential of main- and side-population cells derived from murine skeletal muscle. Exp Cell Res 2003;291(1):83-90.
38. Montanaro F, Liadaki K, Schienda J, Flint A, Gussoni E, Kunkel LM. Demystifying SP cell purification: viability, yield, and phenotype are defined by isolation parameters. Exp Cell Res 2004;298(1):144-54.
39. Nadin BM, Goodell MA, Hirschi KK. Phenotype and hematopoietic potential of side population cells throughout embryonic development. Blood 2003;102(7):2436-43.
40. Uchida N, Dykstra B, Lyons K, Leung F, Kristiansen M, Eaves C. ABC transporter activities of murine hematopoietic stem cells vary according to

their developmental and activation status. Blood 2004;103(12):4487-95.
41. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001;7(9):1028-34.
42. Doyle LA, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene 2003;22(47):7340-58.
43. Watanabe K, Nishida K, Yamato M, Umemoto T, Sumide T, Yamamoto K, et al. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. FEBS Lett 2004;565(1-3):6-10.
44. Lechner A, Leech CA, Abraham EJ, Nolan AL, Habener JF. Nestin-positive progenitor cells derived from adult human pancreatic islets of Langerhans contain side population (SP) cells defined by expression of the ABCG2 (BCRP1) ATP-binding cassette transporter. Biochem Biophys Res Commun 2002;293(2):670-4.
45. Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, et al. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci USA 2004;101(39):14228-33.
46. Benchaouir R, Rameau P, Decraene C, Dreyfus P, Israeli D, Pietu G, et al. Evidence for a resident subset of cells with SP phenotype in the C2C12 myogenic line: a tool to explore muscle stem cell biology. Exp Cell Res 2004;294(1):254-68.
47. Patrawala L, Calhoun T, Schneider-Broussard R, Zhou J, Claypool K, Tang DG. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 2005;65(14):6207-19.
48. Bapat SA, Mali AM, Koppikar CB, Kurrey NK. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 2005;65(8):3025-9.

49. Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, Dinulescu DM, Connolly D, Foster R, et al. Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. Proc Natl Acad Sci USA 2006;103(30):11154-9.
50. Moserle L, Indraccolo S, Ghisi M, Frasson C, Fortunato E, Canevari S, et al. The side population of ovarian cancer cells is a primary target of IFN-alpha antitumor effects. Cancer Res 2008;68(14):5658-68.
51. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996;183(4):1797-806.
52. Barrett MT, Scheffer A, Ben-Dor A, Sampas N, Lipson D, Kincaid R, et al. Comparative genomic hybridization using oligonucleotide microarrays and total genomic DNA. Proc Natl Acad Sci USA 2004;101(51):17765-70.
53. Johnson NA, Hamoudi RA, Ichimura K, Liu L, Pearson DM, Collins VP, et al. Application of array CGH on archival formalin-fixed paraffin-embedded tissues including small numbers of microdissected cells. Lab Invest 2006;86(9):968-78.
54. Little SE, Vuononvirta R, Reis-Filho JS, Natrajan R, Iravani M, Fenwick K, et al. Array CGH using whole genome amplification of fresh-frozen and formalin-fixed, paraffin-embedded tumor DNA. Genomics 2006;87(2):298-306.
55. Wu H, Kim KJ, Mehta K, Paxia S, Sundstrom A, Anantharaman T, et al. Copy number variant analysis of human embryonic stem cells. Stem cells 2008;26(6):1484-9.
56. Ishigami T, Uzawa K, Higo M, Nomura H, Saito K, Kato Y, et al. Genes and molecular pathways related to radioresistance of oral squamous cell carcinoma cells. Int J Cancer 2007;120(10):2262-70.
57. Dahiya N, Sherman-Baust CA, Wang TL, Davidson B, Shih Ie M, Zhang Y, et al. MicroRNA expression and identification of putative miRNA targets in

ovarian cancer. PLoS ONE 2008;3(6):e2436.
58. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci USA 2004;101(3):781-6.
59. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ. Opinion: the origin of the cancer stem cell: current controversies and new insights. Nat Rev Cancer 2005;5(11):899-904.
60. Nguyen HG, Ravid K. Tetraploidy/aneuploidy and stem cells in cancer promotion: The role of chromosome passenger proteins. J Cell Physiol 2006;208(1):12-22.
61. Lagasse E. Cancer stem cells with genetic instability: the best vehicle with the best engine for cancer. Gene Ther 2008;15(2):136-42.
62. Hu J, Khanna V, Jones MW, Surti U. Comparative study of primary and recurrent ovarian serous carcinomas: comparative genomic hybridization analysis with a potential application for prognosis. Gynecol Oncol 2003;89(3):369-75.
63. Kiechle M, Jacobsen A, Schwarz-Boeger U, Hedderich J, Pfisterer J, Arnold N. Comparative genomic hybridization detects genetic imbalances in primary ovarian carcinomas as correlated with grade of differentiation. Cancer 2001;91(3):534-40.
64. Makhija S, Sit A, Edwards R, Aufman K, Weiss H, Kanbour-Shakir A, et al. Identification of genetic alterations related to chemoresistance in epithelial ovarian cancer. Gynecol Oncol 2003;90(1):3-9.
65. Bruchim I, Israeli O, Mahmud SM, Aviram-Goldring A, Rienstein S, Friedman E, et al. Genetic alterations detected by comparative genomic hybridization and recurrence rate in epithelial ovarian carcinoma. Cancer Genet Cytogenet 2009;190(2):66-70.
66. Iwabuchi H, Sakamoto M, Sakunaga H, Ma YY, Carcangiu ML, Pinkel D, et al. Genetic analysis of benign, low-grade, and high-grade ovarian tumors.

Cancer Res 1995;55(24):6172-80.
67. Suzuki S, Moore DH, 2nd, Ginzinger DG, Godfrey TE, Barclay J, Powell B, et al. An approach to analysis of large-scale correlations between genome changes and clinical endpoints in ovarian cancer. Cancer Res 2000;60(19):5382-5.
68. Fishman A, Shalom-Paz E, Fejgin M, Gaber E, Altaras M, Amiel A. Comparing the genetic changes detected in the primary and secondary tumor sites of ovarian cancer using comparative genomic hybridization. Int J Gynecol Cancer 2005;15(2):261-6.
69. Helou K, Padilla-Nash H, Wangsa D, Karlsson E, Osterberg L, Karlsson P, et al. Comparative genome hybridization reveals specific genomic imbalances during the genesis from benign through borderline to malignant ovarian tumors. Cancer Genet Cytogenet 2006;170(1):1-8.

< ABSTRACT(IN KOREAN)>

상피 난소암에서 side population의 비교유전체 분석

<지도교수 조남훈>

연세대학교 대학원 의학과

심 효 섭

난소암은 부인과 암중에서 가장 예후가 불량하다. 진행된 병기의 경우 초기 치료에 반응을 하더라도 대부분의 경우 재발하며 항암제에 내성을 보이는 경우가 많다. 최근 많은 연구에서 종양내에 암줄기세포가 존재하고, 이러한 암줄기세포가 암의 예후와 항암제 내성에 관여할 것으로 보고되고 있다. Side population (SP) 기법은 암줄기세포를 동정하는 방법 중 하나로 Hoechst 33342 dye에 염색되지 않는 세포 집단을 동정하는 것이다. SP 세포에는 약물 내성과 관련된 ATP-binding cassette 수송체가 발현되는 것으로 알려져 있고, 이 집단에 암줄기세포가 많이 존재한다고 알려져 있다. 따라서 본 연구에서는 상피 난소암의 세포 배양을 통하여 SP를 동정하고 SP의 특징을 살펴봄으로써 난소암 줄기세포의 특징을 알아보고자 하였다. 상피 난소암으로 진단된 조직에서 일차 세포배양을 시행하였다. 같은 조직에서 총 20개의 세포주가 확립되었고, 그 중 28 계대 배양까지 생존한 3개 (C2, C4, C7.2)의 세포주를 대상으로 연구를 진행하였다. 먼저 세포주의 기본 특성을 살펴보기 위하여 시행한 세포 표지자에 대한 RT-PCR, 증식속도 검사, 세포주기 분포도 검사 결과 각 세포주 별로 상이한 결과를 보였다. SP 분석 결과 SP 비율이 세개의 세포주에서 약 0.5-1% 정도 존재하는 것을 확인하였다. SP가 가장 많은 C7.2를 대상으로 비교유전체 부합화 검사를 시행하였고, non-side population (NSP)과 그 결과를 비교하였다. SP와 NSP에서 공통으로 증가하거나 감소한 것, NSP에서만 증가하거나 감소한 것을 제거한 후 SP에서만 특이적으로 변화를 보인 유전자는

총 2383개였다. 이 중에서 2배 이상 차이를 보인 198개의 유전자들은 서로 연결된 10개의 분자망에 관여하는 분자들이었고, 그들의 주요 기능은 세포 주기 혹은 세포나 배아 발달이었다. Ingenuity Pathway Analysis를 통하여 좀더 의미있는 유전자를 64개로 좁힐 수 있었으며, 이러한 분자들은 Notch signaling, Wnt/β-catenin signaling, PTEN signaling, G1/S and G2/M checkpoint regulation, PI3K/AKT signaling, p53 signaling 등에서 중요한 역할을 담당하는 것을 확인하였다. 난소암 조직에서 유전체의 변화를 분석한 기존의 여러 연구들과 비교한 결과, 암의 재발과 연관된 유전체 변화 중에서 19p13.1의 증가는 본 연구의 유전체 변화 결과와 일치하였다. 본 연구는 일차세포배양을 통하여 SP의 유전체 변화를 분석했다는 점에서 기존의 연구결과와 직접적으로 비교한다는 것은 무리가 있으나, 암줄기세포가 암의 재발과 예후에 관여한다는 점을 고려했을 때 이러한 유전자에 대한 추가 연구가 필요할 것으로 생각한다. 본 연구에서는 암줄기세포가 많을 것으로 예상되는 SP에서 직접 유전체의 변화가 있음을 증명하였고 이는 암줄기세포의 발생에 유전체의 변화가 관여할 것임을 시사한다. 또한 SP에서 변화를 보인 유전자들이 세포주기와 배아 발생에 관여한다는 점을 볼 때 줄기세포로써의 특징이 반영되어 있음을 시사한다. 본 연구는 난소암 줄기세포와 비 줄기세포의 특징에 대한 이해를 제공하고, 장기적으로 암줄기세포에만 작용하는 표적 치료제를 개발하는데 기초적인 정보를 제공하리라 기대한다.

핵심되는 말 : epithelial ovarian cancer, cancer stem cells, side population, comparative genomic analysis