# Differential protein expression and novel biomarkers related to 5-FU resistance in a 3D colorectal adenocarcinoma model

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**Abstract.** The multicellular spheroid (MCS) is an *in vitro* model which is highly representative of the avascular region of solid tumors by reflecting microenvironmental conditions *in vivo*. Hence, it is considered the most appropriate model for studying drug resistance. We compared chemosensitivity to 5-fluorouracil (5-FU) and differential protein expression between the 3D MCS model and the 2D monolayers using DLD-1 cells. We analyzed several significant signaling molecules through western blot analysis. Although various changes in the expression level were observed depending on the samples, we did not obtained remarkable findings. Notably, the level of *p*-mTOR decreased upon 5-FU exposure in the monolayers, while its level was higher in the MCSs. Nine novel proteins were identified by 2-DE and MALDI-TOF analysis for exhibiting differential expression between the MCS model and the monolayers. Among these, collapsin response mediator protein 2 (CRMP-2), DNA replication complex GINS protein PSF2 (PSF-2) and selenium-binding protein 1 (SBP-1) were notable not only for their differential expression but also for decreased expression following 5-FU exposure, indicating their possible roles as novel biomarkers for sensitivity (CRMP-2, PSF-2) as well as resistance (SBP-1) to 5-FU. Overall, the present study demonstrated greater 5-FU resistance in human colorectal cancer cells grown as MCSs compared to monolayers and identified *p*-mTOR, CRMP-2, PSF-2 and SBP-1 as novel potential biomarkers of 5-FU chemosensitivity/resistance for human colorectal cancer, findings which warrant further investigation.

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*Key words:* colorectal adenocarcinoma, multicellular spheroid, 5-FU, resistance, biomarker

## Introduction

The main cause of chemotherapeutic failure is drug resistance in solid tumors. In many cases, resistance to chemotherapy already exists before drug treatment (intrinsic resistance), which usually further develops into broad spectrum resistance (acquired resistance) following treatments. Chemotherapeutic resistance can be induced through various mechanisms, e.g., metabolic inactivation and efflux of drugs; however, recent studies have reported that the tumor microenvironment is an additional cause of drug resistance (1,2). All efforts to overcome chemoresistance have not been successful thus far, owing not only to the complex biology of cancer cells but also to the use of inappropriate models that do not exhibit the type of drug resistance encountered in patients.

For a long time, most studies have relied on two dimensional (2D)-culture models. Although 2D cultures have yielded significant insights into the study of drug resistance as well as cancer biology, they are critically limited by clinical irrelevance. Drug resistance studies using 2D cultures focus on changes at the single cell level such as mutations in genes that regulate cellular processes related to proliferation and/or apoptosis and modified accumulation and metabolism of drugs (3,4). However, substantial evidence has accumulated that the tumor microenvironment should also be considered, as it is definitely involved in the resistance of solid tumors to chemotherapy. Actually, the translation of research outcomes from *in vitro* 2D-based culture models has shown a poor success rate of <5% (5,6). *In vivo*, cancer cells grow together to form lump-like structures and are surrounded by extracellular matrix (ECM) and microenvironmental stromal cells. In three dimensional (3D) architecture, tumor cells interact with adjacent cells and ECM which can alter their behavior. The interaction between tumor cells and the microenvironment plays a significant role in anticancer drug resistance (7,8). The conventional 2D culture models may not be suitable for representing the *in vivo* microenvironment such as cell-cell interactions, cell-ECM interactions, non-uniform distribution of oxygen and nutrients as well as other physical and chemical

stresses, resulting in so-called multicellular resistance (MCR). Three-dimensional models *in vitro* such as multicellular spheroids (MCSs) and histocultures have been used successfully to elucidate tumor growth kinetics as well as the mechanisms involved in the resistance of tumor cells to anticancer drugs, cytokines and radiation. The significance of 3D models in the study of tumor biology and oncopharmacology has recently been emphasized, hence they have been exploited in new anticancer drug discovery (9,10).

In recent years, 3D culture models of several cancer types have been vigorously utilized in studies of anticancer drugs. It has been reported that the molecular features of 3D culture models of ovarian and endometrial cancers showed a higher level of similarity to *in vivo* primary tumors in terms of response and resistance to anticancer drugs than 2D monolayer cultures (11,12). Pancreatic tumor 3D spheroids also showed higher matrix-rich chemoresistance phenotypes compared to 2D monolayers (13,14) and a higher expression of the membrane protein, Cav-1, causing radio-resistance (15). The reduced sensitivity to anticancer drugs in 3D spheroid cultures of lung cancer has been attributed to a significant decrease in apoptotic signals after drug exposure was decreased (16,17).

Colorectal cancer is one of the most common tumors worldwide. 5-Fluorouracil (5-FU) is a widely used agent for colorectal cancers, particularly metastatic colorectal cancer (mCRC) (18,19). Despite the increase in the understanding of the mechanisms of 5-FU, the resistance to 5-FU remains a significant limitation to the treatment of patients (20,21). Our rationale for the present study was that intrinsic drug resistance is more clinically relevant, and the 3D structure is the most suitable model for simulating drug resistance *in vivo*. Hence, we evaluated the differential expression of proteins presumably associated with 5-FU resistance and investigated novel potential biomarkers in 3D cultures of human colorectal cancer cells.

## Materials and methods

*Chemicals and reagents*. 5-Fluorouracil (5-FU) and cell culture reagents were purchased from Sigma-Aldrich and Gibco BRL, respectively. Antibodies were purchased from Santa Cruz Biotechnology (p53, PLD, EGFR, caspase-3, ERK, *p*-Akt and HSP70), Zymed Laboratories Inc. (E-cadherin, PTEN and $p27^{kip1}$) and Cell Signaling Technology (*p*-mTOR).

*Cell culture*. All the cancer cell lines utilized were obtained from the Korea Cell Line Bank (Seoul, Korea). Monolayer cultures were maintained in RPMI-1640. All cell lines were grown under 5% $CO_2$ at 37˚C in a humidified atmosphere.

*Formation of spheroids*. Multicellular spheroids (MCSs) were cultured using a liquid overlay technique as described previously (14) with some modification. The morphology and structure of spheroids were evaluated by scanning electron microscopy (SEM, Model JSM-5410 LV, Jeol, Japan) and transmission electron microscopy (TEM, Model 1010, Jeol). Paraffin-embedded sections (5 $\mu$m) were also prepared and stained with H&E to examine 3D heterogeneity within the spheroids.

Table I. Formation of MCSs using several cancer cell lines.

| Cell lines | | Degree of aggregation |
|---|---|---|
| Origin | Name | |
| Lung | A549 | $A_{cluster}$ |
| | PC14 | $A_{loose}$ |
| Breast | MCF-7 | $S_{partly}$ |
| Vulva | A431 | $S_{full}$ |
| Head and neck | PCI-1 | $S_{full}$ |
| | PCI-13 | $S_{partly}$ |
| | PCI-50 | $S_{partly}$ |
| Colon | DLD-1 | $S_{full}$ |
| Gastric | SNU-484 | $S_{full}$ |
| | SNU-216 | $A_{loose}$ |
| | SNU-601 | $A_{loose}$ |
| | AGS | $A_{loose}$ |

$A_{cluster}$, aggregate containing a fraction of clustered cells; $A_{loose}$, aggregate of cells loosely attached to each other; $S_{partly}$, partly compact multicellular spheroids; $S_{full}$, fully compact multicellular spheroids.

*Cytotoxicity assay*. Cell viability was assessed by MTS. For monolayers, cells were plated into 96-well plates at 1,500 cells/well followed by drug exposure for 96 h. For spheroids, 7 day-grown spheroids were transferred to non-coated 96-well plates and then 100 $\mu$l of fresh media was added to each well. Spheroids were treated with various concentrations of 5-FU for 96 h. The $IC_{50}$ value was determined as the drug concentration required to reduce the absorbance value to 50% as compared to the control by fitting the data to a classic sigmoid $E_{max}$ model using SigmaPlot.

*Cell cycle analysis and apoptosis*. Cells were stained using PI and subjected to FACS analysis (FACSVantage™, Becton-Dickinson Immunocytometry Systems, San Jose, CA). For simultaneous determination of cell cycle phase and percentage of apoptosis, experiments were carried out according to the User's Manual included in the Apo-Direct™ kit (BD Pharmingen). For each sample, 10,000 events were recorded.

*Western blot analysis and 2-DE & MALDI-TOF analysis*. Proteins (50 $\mu$g) were electrophoresed on 8-14% SDS gels for western blot analysis. The blots were obtained and visualized using specific antibodies (as listed in Chemicals and reagents) for each protein of interest and HRP-conjugated secondary antibodies followed by enhanced chemiluminescence detection (Amersham Pharmacia Biotech, UK). For 2-DE, 200 $\mu$g of proteins was electrophoresed. Quantitative analysis of digitized images was carried out using PDQuest software (version 7.0, Bio-Rad). Protein spots were selected for significant expression variation when they deviated >2-fold in their expression level compared with the control or normal sample. Protein analysis was performed using an Ettan MALDI-ToF (Amersham Biosciences). ProFound, developed by The Rockefeller University, was used for protein identification.

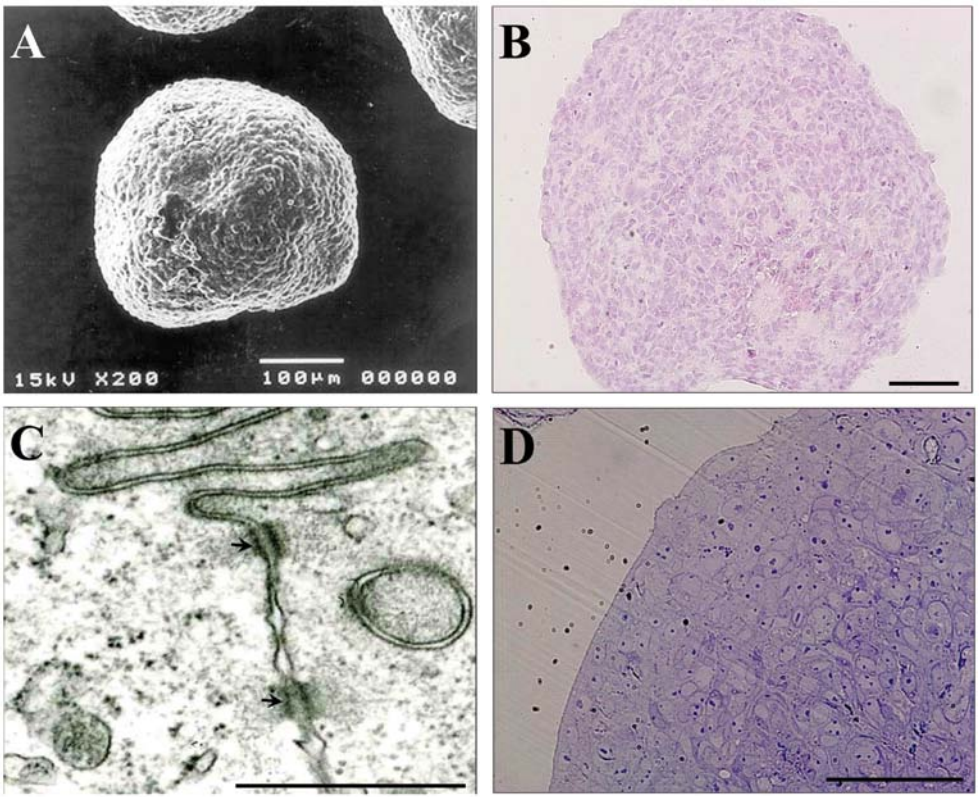


Figure 1. Morphological characteristics of the DLD-1 MCSs. (A) Scanning electron micrographs. (B) H&E-stained paraffin-embedded sections. (C) Transmission electron micrographs (arrows indicate desmosomes). (D) Semi-thin section of an MCS. Scale bars: 100 $\mu$m (A, B and D) and 200 nm (C). MCS, multicellular spheroid.

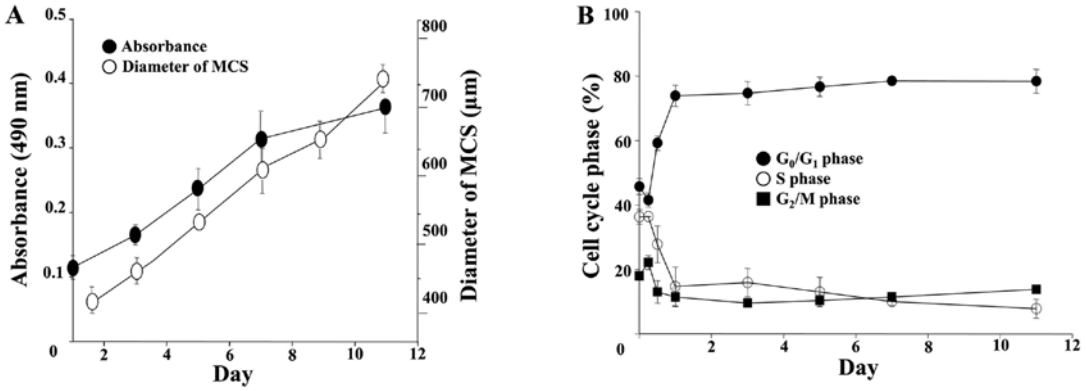


Figure 2. Growth characteristics of the DLD-1 MCSs. (A) The growth of the DLD-1 MCSs was monitored using an MTS assay (●) and the diameter (○) over 11 days after plating. (B) Changes in cell cycle distribution from day 0 to day 11 were determined by flow cytometric analysis (●, $G_0/G_1$ phase; ○, S phase, ■, $G_2$/M phase). MCS, multicellular spheroid.

## Results

*Characteristics of the DLD-1 MCS model*. Firstly, we evaluated a three dimensional multicellular spheroid (3D MCS) culture of various human cancer cell lines including DLD-1, and their characteristics in regards to aggregation and compact conditions are summarized in Table I. DLD-1 cells along with SNU-484, PCI-1 and A432 formed MCSs with a fully compact structure. Among the fully compact MCSs, DLD-1, a human colon carcinoma cell line, was selected for further study. Representative images of morphologic and microstructure characteristics of the DLD-1 spheroids are shown in Fig. 1. DLD-1 MCSs showed a compact structure as shown by the smooth surface of the MCSs resulting from tight interactions between the cells. After 7 days of culture, DLD-1 MCSs reached ~580 $\mu$m in diameter, which was quite large in size, but neither necrotic nor apoptotic cells were found within the spheroids. Adhesion structures such as desmosomes were observed in the TEM images (Fig. 1D). The growth of the DLD-1 MCSs was examined by measuring size and by MTS assay (Fig. 2A). The spheroids showed a steady growth profile for up to 11 days, reaching 700 $\mu$m in diameter. An increase

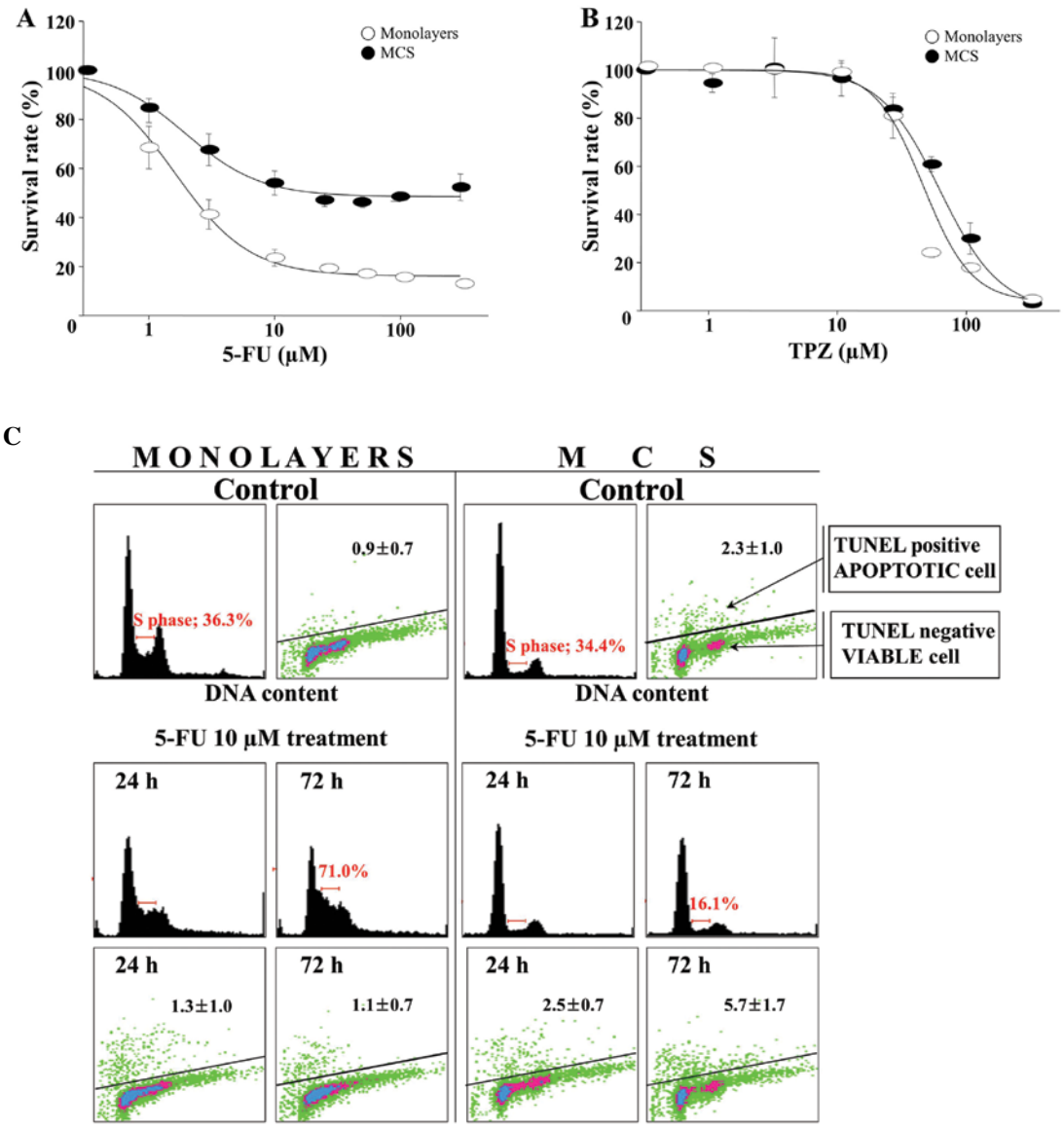


Figure 3. Effect of 5-FU on DLD-1 cells grown as monolayers or MCSs. Dose-response curves for 5-FU (A) and TPZ (B) in DLD-1 cells grown as monolayers (○) or MCSs (●). Cells were exposed to 5-FU for 96 h, and viability was determined using an MTS assay. Error bars represent the SE of at least three replicates. (C) Representative DNA histograms and apoptosis of DLD-1 cells following 5-FU exposure. DLD-1 cells were grown as monolayers and MCSs with or without 10 $\mu$M 5-FU. TUNEL data were obtained by multi-color density plots using FITC-dUTP (TUNEL labeling) and PI (DNA content) double-stained samples. MCS, multicellular spheroid; 5-FU, 5-fluorouracil.

in OD for the MTS with time was parallel to that of the size increase, indicating that the viability of cells within the MCSs was sustained, although a subtle decrease was noticeable after 7 days. Cell cycle changes along with spheroid formation were analyzed as shown in Fig. 2B. A significant increase in the percentage of cell in the $G_0/G_1$ phase and a concomitant decrease in the percentage of cells in the S and $G_2$/M phase were observed in cells grown as MCSs. It is noted that the change in cell cycle distribution was induced as early as 24 h and no further changes were noted afterwards.

*Effect of 5-FU on DLD-1 cells grown as monolayers and MCSs.* The growth inhibitory effects of 5-FU were evaluated against various human colon cancer cell lines, DLD-1, HT-29, SW480, HCT-15, KM1214 and KM12C. The $IC_{50}$ value after 96 h of continuous exposure to 5-FU showed a range of 2.6-9.3 $\mu$M (data not shown), where DLD-1 showed the greatest sensitivity as indicated by an $IC_{50/96\ h}$ of 2.1 $\mu$M (Fig. 3A). As expected, MCSs showed a reduced sensitivity toward 5-FU and the $IC_{50}$ increased by 10-fold when compared to the monolayers, i.e. an $IC_{50/96\ h/MCS}$ of 21.6 $\mu$M vs. $IC_{50/96\ h/monolayers}$ of 2.1 $\mu$M (Fig. 3A). In contrast, the growth inhibition induced by tirapazamine (TPZ, a hypotoxin) appeared similar between the monolayers and the MCSs. TPZ is a cytotoxic anticancer drug that is activated to a toxic radical only at very low levels of oxygen (hypoxia) (22). TPZ was used as a reference drug to indirectly assess the microenvironmental oxygen conditions, which can offset 3D drug resistance in MCSs, i.e., for TPZ the $IC_{50/96\ h/MCS}$ was 62.6 vs. 48.0 $\mu$M for $IC_{50/96\ h/monolayers}$ (Fig. 3B). The DLD-1 cells were grown in monolayers or MCSs and exposed to 10 $\mu$M of 5-FU for 24 and 72 h. When the monolayer cells were treated, the percentage of cells in the S phase was markedly

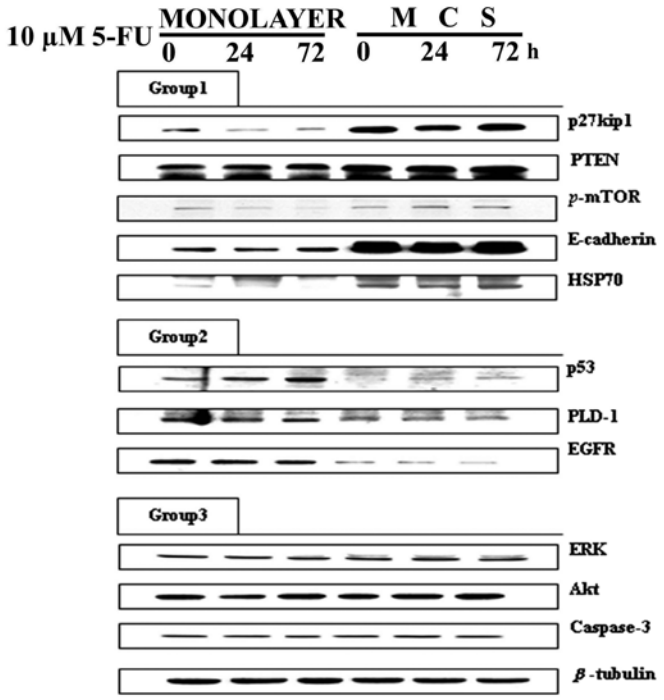


Figure 4. Western blot analysis of DLD-1 cells grown as monolayers and MCSs. DLD-1 cells grown as monolayers and MCSs were exposed to 10 $\mu$M of 5-FU and 11 proteins were analyzed. The proteins were divided into three groups: Groups 1 and 2 for higher and lower basal expression in MCSs than in monolayers, respectively, and Group 3 for similar basal expression in both cultures. MCS, multicellular spheroid; 5-FU, 5-fluorouracil.

increased. In MCSs, the percentage of cells in the S phase was slightly reduced and the cell cycle profile was similarly maintained for 72 h post-treatment (Fig. 3C). In contrast, there was no evidence of a hypodiploid DNA peak in both cultures exposed to 10 $\mu$M of 5-FU for 72 h. Similar results were also obtained with the TUNEL assay. Even though a slight increase in TUNEL-positive apoptotic cells was shown in the MCSs at 72 h (5.7%), only a minimal induction of apoptosis was noted in both cultures.

*Comparison of protein expression following 5-FU exposure between monolayers and the MCSs*. Western blot analysis was performed to determine whether the 3D conditions in the MCSs induced any changes in the expression of proteins associated with cell cycle check points and intracellular signaling for proliferation. The analyzed proteins were divided into three groups by a change in expression pattern: one where the basal expression was higher in MCSs, another when it was higher in monolayers and the other when expression was similar in both (Fig. 4). $p27^{kip1}$, PTEN, *p*-mTOR, E-cadherin and HSP70 showed a significantly higher basal expression level in MCSs than in the monolayers (Group 1 in Fig. 4). Among these, $p27^{kip1}$ and *p*-mTOR showed decreased expression upon 5-FU exposure in the monolayers whereas no change ($p27^{kip1}$) or even an increase (*p*-mTOR) was observed in the MCSs. The other three proteins in Group 1, PTEN, E-cadherin and HSP70, showed no changes upon drug exposure. Based on these data, it can be suggested that 5-FU resistance in MCSs can be attributed to increased expression of $p27^{kip1}$ and *p*-mTOR, which resulted from growth under 3D conditions and is related with the increased expression of PTEN, E-cadherin and HSP70. On the other hand, the basal expression levels of p53, PLD-1 and EGFR were higher in the monolayers than levels in the MCSs, which were assigned in Group 2 (Group 2 in Fig. 4). The expression level of p53 showed a time-dependent increase in both the monolayers and MCSs upon 5-FU treatment. The other two proteins, PLD-1 and EGFR, did not show any change. Regardless of the unchanged level of expression upon 5-FU treatment, the significantly lower level of p53, PLD-1 and EGFR in MCSs suggested their association with 3D drug resistance. The remaining proteins, including ERK, Akt and caspase-3, showed a similar level in the monolayers and MCSs and no changes upon 5-FU treatment (Group 3 in Fig. 4).

*2D gel mapping and mass spectrometry for novel biomarkers of 5-FU resistance*. In order to identify novel protein markers for 5-FU resistance, we carried out (2-DE) MALDI-TOF. Nine protein spots (a through i) were selected based on differential expression profiles between the monolayers and MCSs (Fig. 5A), and are listed in Table II. Collapsin response mediator protein 2 (CRMP-2) (spot a in Fig. 5 and Table II) and DNA replication complex GINS protein PSF2 (spot h) were grouped into the first group, which showed a higher expression in the monolayers than the MCSs and a decreased level upon 5-FU (Group I). The other proteins (Group II) showed higher expression in the MCSs than in the monolayers, with accompanying changes upon 5-FU treatment and included selenium binding protein-1 (SBP-1) (spot b), keratin 20 (spot c), β-succinyl CoA synthetase (spot d), sialic acid synthase (spot e), Annexin A4 (spot f) and dimethylarginine dimethylaminohydrolase 1 (DDAH1) (spot g). Among Group II, SBP-1 appeared to be downregulated by 5-FU exposure in both cultures. On the other hand, keratin 20, β-succinyl CoA synthetase, DDAH1 and Annexin A4 were upregulated after 5-FU treatment in the monolayers only.

## Discussion

The detailed morphological structure of DLD-1 spheroids, observed using SEM and semi-thin sections after 7 days of culture, clearly showed a tight compaction and a close interaction among tumor cells. Neither necrotic areas nor signs of nuclear damage throughout spheroids were observed (Fig. 1), indicating that for DLD-1 cells, the MCS model was an appropriate 3D cell culture model to study drug resistance. The changes in cell cycle distribution of the growing DLD-1 MCSs were dramatic within 12 h after seeding, showing G1 cell cycle arrest with a significant decrease in the percentage of S and $G_2$/M phase cells. Despite the slow growth expected from this cell cycle arrest, the diameters of spheroids gradually increased from 350 $\mu$m at 24 h up to ~700 $\mu$m at 11 days (Fig. 2). It is well known that cell cycle arrest in the inner regions of 3D spheroids is induced in response to microenvironmental stresses and is closely related to the p53 signaling network. Since the upregulation of p53 was not observed in the DLD-1 MCSs, these quiescent (Q) cells could possibly be due to marked cell contact-dependent upregulation of cyclin-dependent kinase inhibitors such as $p27^{kip1}$ (Fig. 4). These Q cells are expected to be viable yet insensitive to chemotherapeutic drugs (23,24) as demonstrated in our study (Fig. 3C).

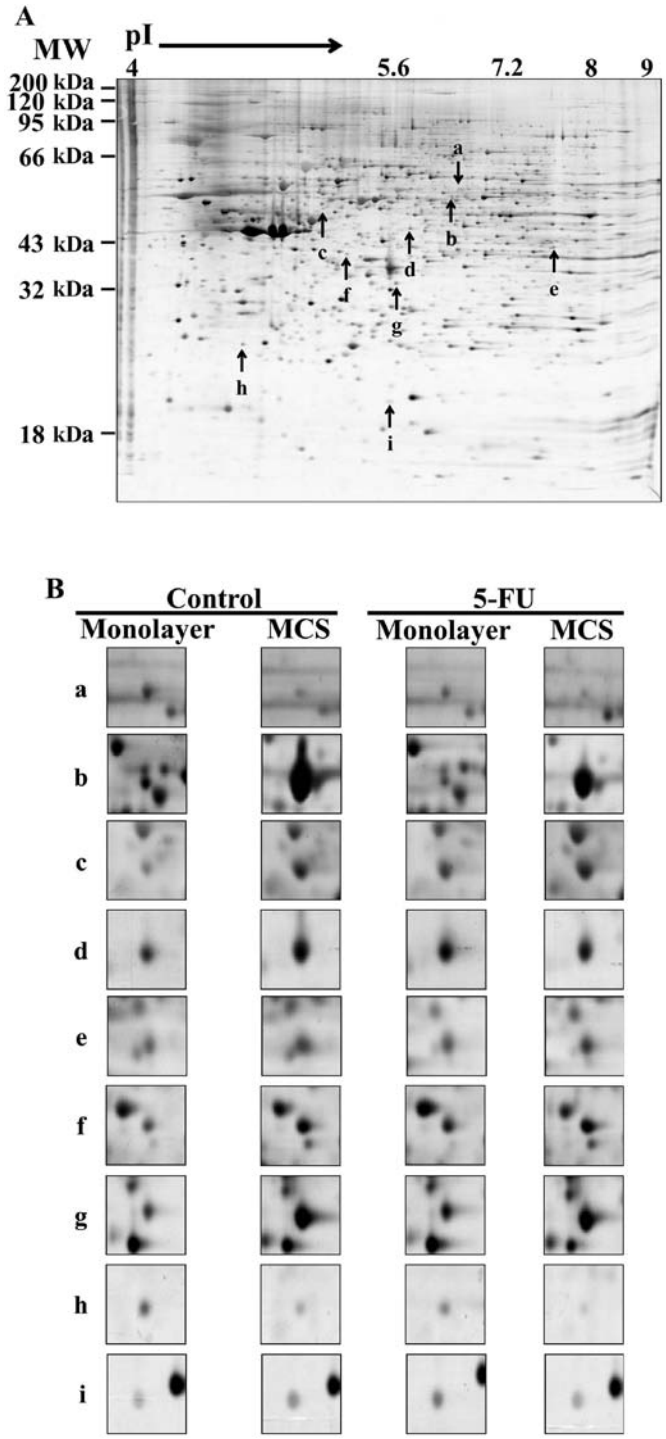


Figure 5. 2-DE gel analysis of novel biomarkers for 5-FU resistance in DLD-1 cells. Proteins extracted from DLD-1 cells grown as monolayers and MCSs with or without 5-FU exposure for 72 h were analyzed, and 9 protein spots (a-i) (A) showing significant differences (B) between the two cultures were subjected to further analysis by MALDI-TOF for identification. A list of the 9 proteins is shown in Table II. MCS, multicellular spheroid; 5-FU, 5-fluorouracil.

Table II. The 9 proteins identified by MALDI-TOF analysis following 2-DE.

| | | Parameters | MALDI-TOF |
|---|---|---|---|
| ID | Protein | MW (kDa) | Coverage (%) |
| Group I | | | |
| a | Collapsin response mediator protein 2 | 62.73 | 23 |
| h | DNA replication complex GINS protein PSF2 | 21.47 | 20 |
| Group II | | | |
| b | Selenium-binding protein 1 | 52.94 | 38 |
| c | Keratin 20 | 48.53 | 35 |
| d | β-succynyl CoA synthetase | 43.93 | 34 |
| e | Sialic acid synthase | 40.75 | 23 |
| f | Annexin A4 | 36.09 | 39 |
| g | Dimethylarginine dimethylaminohydrolase 1 | 31.45 | 29 |
| i | Immunoglobulin heavy chain VHDJ region | 13.03 | 34 |

The proteins were divided into two groups and the spot (ID), protein description, molecular mass and sequence coverage are listed. Group I is comprised of proteins with lower expression in the MCSs than that in the monolayers, with decreased expression upon 5-FU exposure. Group II consists of proteins with higher expression in the MCSs than that in the monolayers with expression level changes upon 5-FU exposure. MCS, multicellular spheroid; 5-FU, 5-fluorouracil.

Our results indicated that DLD-1 was much more resistant to 5-FU in MCSs compared to the monolayers; the differential sensitivity to 5-FU was shown by a significantly (10-fold) higher $IC_{50/96\ h}$ (21.6 vs. 2.1 $\mu$M in MCSs vs. monolayers, respectively Fig. 3A) and the absence of S phase arrest after 5-FU treatment in the MCSs (Fig. 3C). Hence, it may be caused by certain specific changes induced in a cellular signaling pathway related to drug response. This prompted us to investigate changes in major proteins involved in drug-induced cytotoxicity as well as the differential expression between the two culture models for novel biomarkers for 5-FU resistance in the MCS model.

The analyzed 11 proteins by western blot analyses were divided into three groups (Fig. 4). Proteins in Group 1 ($p27^{kip1}$, PTEN, *p*-mTOR, E-cadherin, HSP-70) showed a higher level of expression in the MCSs, yet no changes were detected upon 5-FU exposure except a weak increase in *p*-mTOR expression in the MCSs (Fig. 4). Reports have discussed the association of E-cadherin and $p27^{kip1}$ with 3D chemoresistance to cisplatin, fluorouracil and adriamycin in breast cancer cell lines, and an anti-E-cadherin neutralizing antibody was shown to successfully decrease $p27^{kip1}$ expression resulting in restoration of chemosensitivity in 3D (25). Although upregulation of $p27^{kip1}$ is often induced by p53, our results showed suppressed expression of p53 in the DLD-1 3D cultures, suggesting that the increased $p27^{kip1}$ in MCSs may be associated with a p73-related pathway rather than a p53-related pathway (26). Our data also suggest that resistance to 5-FU in MCSs can be attributed to the increased expression of PTEN and *p*-mTOR (Fig. 4). PTEN is known to be involved in the regulation of Akt activity, leading to broad-spectrum chemoresistance (27,28). The level of *p*-mTOR decreased after 5-FU exposure in the monolayers,

probably in response to reduced proliferative activity of the cells. In contrast, its level increased in the MCSs. Although this response was weak, it still suggests that *p*-mTOR may be a novel biomarker for 5-FU resistance in 3D conditions (29).

Proteins in Group 2 (p53, PLD-1 and EGFR) showed a higher level of expression in the 2D cultures than the 3D cultures. In this group, only p53 showed a weak increase upon 5-FU exposure (Fig. 4). The role of p53 is to induce damage repair or apoptosis upon exposure to genotoxic agents such as 5-FU (30); hence, the decreased sensitivity (avoidance of drug-induced apoptosis) may be associated with the lower level of p53 in the DLD-1 MCS model observed in this study (Fig. 5). Reports have recently discussed the roles of EGFR and its downstream effector, PLD1, in tumor survival, both of which showed decreased levels of expression in MCSs (Fig. 4). With this suppressed signaling via EGFR and PLD-1, cells may not have sufficient levels of proliferative activity, by which cells may show resistance to cell cycle-specific drugs such as 5-FU (31,32).

Using (2-DE) MALDI-TOF, we identified 9 important proteins: collapsin response mediator protein 2 (CRMP-2), DNA replication complex GINS protein PSF2 (PSF2) and selenium binding protein 1 (SBP1). In our study, CRMP-2 and PSF2 showed decreased expression after 5-FU treatment in both cultures, and the relative level of expression was lower in the MCSs than in the monolayers. These two proteins have recently been reported to have oncogenic properties. CRMP-2 is known to mediate microtubule polymerization. Recently, phosphorylated CRMP-2 has been suggested as a candidate therapeutic target for NSCLC based on a correlation between a high level of nuclear phosphorylated CRMP-2 and poor prognosis (33). PSF2 is a member of the GINS complex. In cancer cells, PSF2 is frequently upregulated, but the elimination of PSF2 provokes chromosome missegregation (34,35). Although it has not been determined whether these two proteins are associated with the mechanism of action of 5-FU, decreased expression after 5-FU exposure in both cultures may suggest their involvement in the antiproliferative activity of 5-FU in cancer cells. In addition, the relatively low expression in MCSs warrants further study regarding its role as a 5-FU resistance biomarker.

SBP-1 showed a significant decrease after 5-FU treatment (Fig. 5). SBP-1 is a cytoplasmic selenium binding protein that is abundantly expressed in most normal tissues. Several recent studies have reported that the expression level of SBP-1 is markedly reduced in many cancer tissues, including colorectal, breast and gastric, when compared to their normal counterparts, suggesting its role as a tumor suppressor (36,37). Its low expression has even been suggested as a survival predictor in stage III colorectal cancer patients (38). Although it is not clear whether SBP-1 is associated with resistance to 5-FU, its decreased expression after 5-FU treatment in both cultures and its significantly higher level of expression in MCSs may support its potential role as a 5-FU resistance biomarker and is thus worthy of further study.

In the present study, we demonstrated greater 5-FU resistance in human colorectal DLD-1 cells grown as MCSs compared to monolayers, and showed that cell cycle deregulation accompanied by the altered expression of several signaling molecules may be related to 5-FU resistance in a 3D MCS model of colorectal cancer. In addition, our data suggest *p*-mTOR as a candidate factor and CRMP-2, PSF2 and SBP-1 as potential biomarkers of 5-FU chemosensitivity/resistance in a 3D model of colorectal cancer, which warrants further study.

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