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Cell Cycle Dysregulation Is Associated With 5-Fluorouracil Resistance in Gastric Cancer Cells

DA SOM KIM, KYOUNGMI MIN and SUK KYEONG LEE

Department of Medical Life Sciences, Department of Biomedicine & Health Sciences, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea

Abstract. *Background/Aim: 5-Fluorouracil (5-FU) is an anticancer drug commonly used to treat gastric cancer; however, continuous 5-FU chemotherapy causes drug resistance. Materials and Methods: We established five sublines of 5-FU-resistant AGS gastric cancer cells to investigate changes that may have occurred in the development of 5-FU resistance. Drug resistance to other chemotherapeutic reagents, proliferation, cell-cycle changes, and wound healing ability were assessed for each subline. Results: Retarded cell growth, G_0/G_1 phase arrest, up-regulation of p57, and down-regulation of cyclin D1 were commonly observed in all five sublines. Resistance to paclitaxel and cisplatin was also observed in most of the sublines. Conclusion: Our data support the notion that G_0/G_1 arrest due to changes in p57 and cyclin D1 expression may confer drug resistance, while EMT seems non-essential to 5-FU resistance in AGS gastric carcinoma cells.*

Gastric cancer (GC) is the fifth most common cancer and causes the third-most cancer-related deaths worldwide (1). GC is associated with lifestyle factors such as *Helicobacter pylori* infection, unbalanced diet, alcohol consumption, and smoking (2). Radical surgery and chemotherapy are the primary methods of treatment for early GC. Patients with advanced GC who cannot undergo surgery are treated with neoadjuvant chemotherapy, radiotherapy, and molecular-targeted therapies. However, most patients diagnosed with advanced GC show poor overall prognosis even after treatment because of high metastatic potential and poor response to chemotherapy (3, 4).

Correspondence to: Suk Kyeong Lee, Department of Medical Life Sciences, Department of Biomedicine & Health Sciences, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul, 06591, Republic of Korea. Tel: +82 222587480, Fax: +82 5042012396, e-mail: sukklee@catholic.ac.kr

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5-Fluorouracil (5-FU) is an anticancer drug used for many solid tumor types including gastric and colon cancer (5, 6). 5-FU, an analog of uracil, is transported into cells by the same mechanism as uracil. 5-FU inhibits thymidylate synthase, incorporates into RNA and DNA, and induces cell death pathways in rapidly growing cancer cells (7-9). However, the response rate to 5-FU-based chemotherapy is lower than 32% in advanced GC (10). This low response rate is mainly due to 5-FU resistance caused by several factors including degradation of 5-FU by dihydropyrimidine dehydrogenase, increased deoxyuridine triphosphatase activity, and overexpression of thymidylate synthase, B-cell lymphoma 2 (BCL2), BCL-XL, and BCL2 family member MCL1 apoptosis regulator proteins (7, 11).

Acquisition of 5-FU resistance and subsequent chemotherapy failure is a common and problematic phenomenon in patients with cancer (12). Previous studies of 5-FU-resistant GC cells showed changes in the pathway of 5-FU metabolism, increased drug transporter protein, and resistance to apoptosis (13, 14). In addition, epithelial to mesenchymal transition (EMT) was observed, similarly to other 5-FU-resistant solid tumors (15, 16). Various drug-resistant cell lines have been established to study strategies for overcoming anticancer drug resistance. However, most studies have used only one resistant cell line rather than comparing multiple cell lines derived simultaneously.

In this study, we established five 5-FU-resistant GC cell sublines simultaneously and compared their characteristics.

Materials and Methods

Cell culture and reagents. AGS cells of a GC cell line were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco BRL).

Establishing 5-FU-resistant AGS sublines. In order to establish 5-FU-resistant AGS cells, 10 aliquots of 1×10^6 cells were seeded into six-well plates and cultured with increasing concentration of 5-FU from 5 to 100 μM over 6.5 months. Control cells were treated with dimethylsulfoxide (DMSO) for 6.5 months. Five sublines of the 5-FU-resistant cells were obtained after 6.5 months and cultured in

media containing 100 μM 5-FU to maintain 5-FU resistance. The five AGS sublines that acquired resistance to 5-FU were named AGS/FR1 to -5 and AGS cells that were maintained with DMSO were named AGS/D. 5-FU was purchased from Sigma-Aldrich (St Louis, MO, USA). A stock solution of 5-FU was prepared in DMSO, aliquoted, and stored at 4˚C. 5-FU is used in the range of 300-600 mg/m^2 to treat gastric, breast, and colorectal cancer (17, 18). The plasma peak level of 5-FU was 100-1000 μM when a single treatment of 300-600 mg/m^2 5-FU was administered (19). The half-maximal inhibitory concentration (IC_{50}) of 5-FU was 70-400 μM in previous reports of 5-FU-resistant cancer cells (20-22). Thus, we used up to 100 μM of 5-FU to establish 5-FU-resistant GC cells in this experiment.

Drug-sensitivity assay. A drug-sensitivity assay was performed to characterize cells. AGS/FR and AGS/D cells (1-2×10^3 cells/well) were seeded in 96-well plates, cultured overnight in humidified air with 5% CO_2 at 37˚C, and then treated with serial dilutions of 5-FU, paclitaxel (Sigma-Aldrich), or cisplatin (Sigma-Aldrich). After 72 h, 10 μl of CCK-8 solution (Dojindo Molecular Technologies, Tokyo, Japan) was added to each well. The absorbance at a wavelength of 450 nm was measured after 2 h using a SoftMax apparatus (Molecular Devices, Sunnyvale, CA, USA). The IC_{50} was calculated from the survival curves.

Observation of morphological changes. The morphological characteristics of the cells were observed using an Axiovert 200 (Carl Zeiss, Thornwood, NY, USA) microscope. The magnification was ×100.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Cells were harvested and total RNA was extracted using the RNAiso Plus (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized using 2 μg total RNA, oligo (dT) primers (Macrogen, Seoul, Republic of Korea), and M-MLV reverse transcriptase (Invitrogen). qRT-PCR was carried out using a TOPreal™ qPCR 2x Pre MIX SYBR-Green kit (Enzynomics, Daejeon, Republic of Korea) with a CFX96 Real –Time PCR System (Bio-Rad, Hercules, CA, USA). The following primers were used for PCR: E-Cadherin: forward: 5'-TTCTGCTGCTCTTGCTGTTT-3', reverse: 5'-TGG CTCAAGTCAAAGTCCTG-3'; N-cadherin: forward: 5'-ATTG GACCATCACTCGGCTTA-3', reverse: 5'-CACACTGGCAAACCT TCACG-3'; vimentin: forward: 5'-TGTCCAAATCGATGTGGATG TTTC-3', reverse: 5'-TTGTACCATTCTTCTGCCTCCTG-3'; glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*): forward: 5'-ATGGGGAAGGTGAAGGTCG-3', reverse: 5'-CCATGTAGTTGAG GTCAATGAAG-3'). PCR conditions were 95˚C for 10 min, followed by 35 cycles at 95˚C for 10 s, 60˚C for 30 s, and 72˚C for 30 s. Dissociation curves were checked routinely to confirm specific amplification of PCR products. For this process, reaction mixtures were incubated at 95˚C for 60 s and then ramped from 60 to 95˚C at a heating rate of 0.1˚C/s, with fluorescence measured continuously. Relative gene expression was calculated according to the comparative C_t method using *GAPDH*.

Wound-healing assay. To study differences in migration between AGS/FR and AGS/D cells, cells (3×10^4) were seeded into 12-well plates and cultured to 90-95% confluence. The cell layer was scratched with a sterile 200 μl pipette tip through the confluent monolayer and washed with PBS to remove cell debris. The cells were then cultured in RPMI-1640 medium without FBS at 37˚C in a humidified chamber with 5% CO_2. The scratched wounds were observed using an Axiovert 200 (Carl Zeiss) microscope just after scratching and 24 h after scratching. Photographs were captured to evaluate the level of migration in each group of cells, and wound areas were assessed using Image J software (National Institutes of Health, Bethesda, MD, USA).

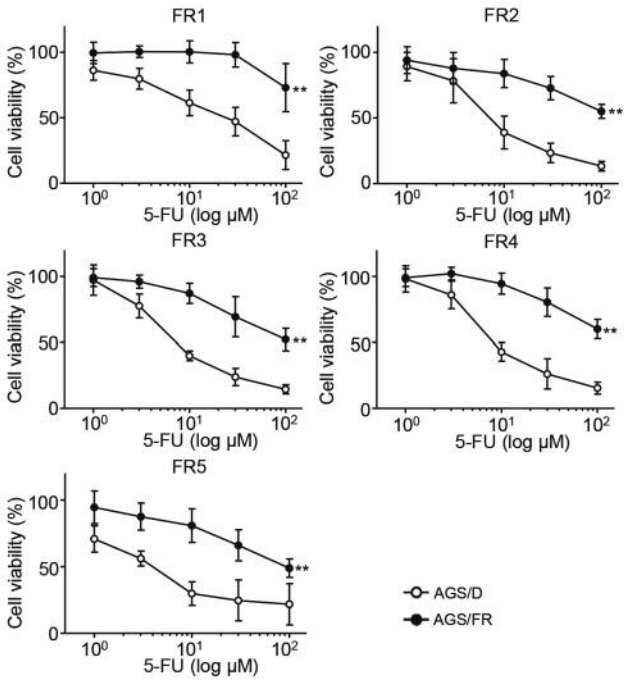

Figure 1. *5-Fluorouracil (5-FU) sensitivity assay of AGS gastric cancer cells (AGS/D; open symbols) and five 5-FU-resistant sublines (AGS/FR1-5; closed symbols). Cells were plated in a 96-well plate and treated with 5-FU at concentrations ranging from 0 to 100 μM. After 72 h, CCK-8 solution was added to each well and absorbance at 450 nm was measured to assess cell growth. The half-maximal inhibitory concentration (IC_{50}) for 5-FU was calculated. Data are expressed as the mean±SD of three independent experiments. **Significantly different at p<0.01.*

Cell growth assay. Cell growth was determined by seeding 12-well plates with 5×10^3 cells/well in triplicate and allowing them to grow for 120 h. Every 24 h, the cells were trypsinized and counted using a hemocytometer. The doubling time (T_d) of each cell line was calculated according to the formula: $T_d=\Delta t\times lg2/(lgN_t- lgN_0)$; where N_0 was the cell number at the beginning of the experiment, Nt the cell number at the end, and Δt was the time from N_0 to N_t.

Flow cytometric analysis of the cell cycle. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum for 48 h. The cells were harvested, washed with ice-cold phosphate-buffered saline, and fixed by dropwise addition of 70% ethanol. The fixed cells were stained with 50 μg/ml propidium iodide (Sigma-Aldrich) solution containing 10 μg/ml RNase A (Invitrogen, Carlsbad, CA, USA). The cell-cycle profile was assessed for 10,000 cells by flow cytometry using a FACS Canto II (Becton-Dickinson, San Jose, CA, USA).

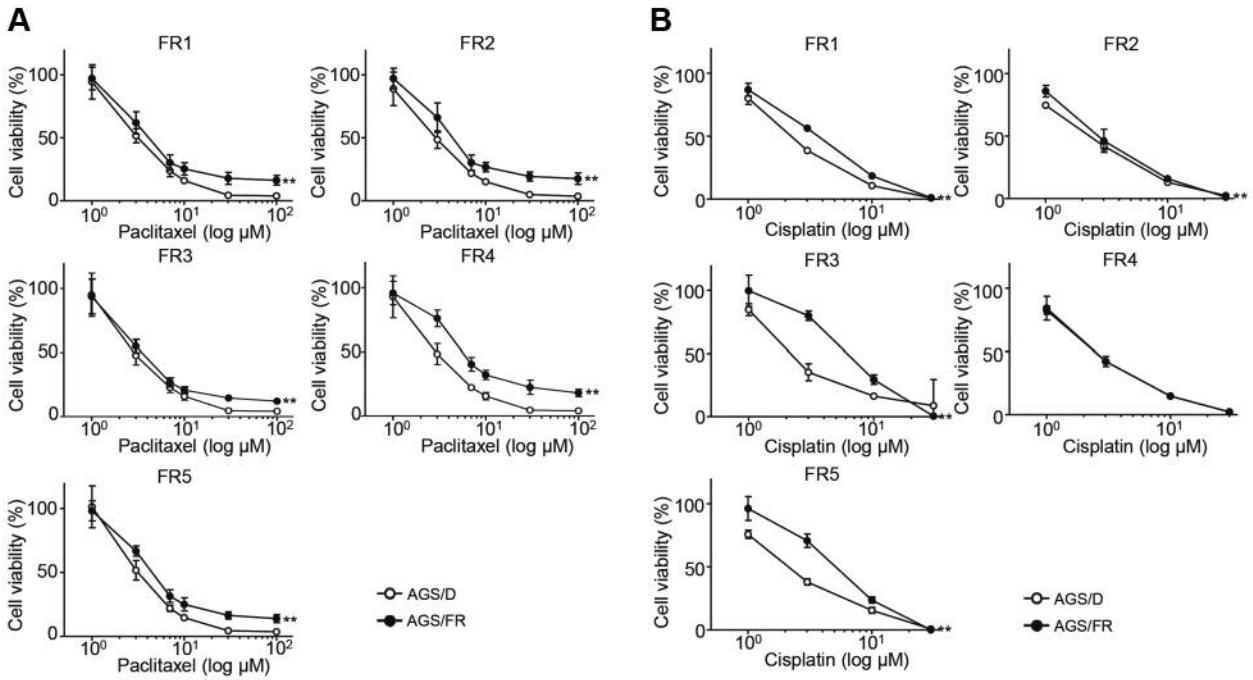

Figure 2. *Drug-sensitivity assay of AGS gastric cancer cells (AGS/D; open symbols) and five 5-FU-resistant sublines (AGS/FR1-5; closed symbols) to paclitaxel (A) and cisplatin (B). Cells were plated in a 96-well plate and treated with anticancer agents at increasing concentrations. After 72 h, CCK-8 solution was added to each well and absorbance at 450 nm was measured to assess cell growth. The half-maximal inhibitory concentrations (IC_{50}) of paclitaxel and cisplatin were calculated. Data are expressed as mean±SD of three independent experiments. **Significantly different at p<0.01.*

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer containing 0.1 mM NaF, 0.02 mM phenylmethylsulphonyl fluoride, 0.01 mM Na_3VO_4, 0.1 μg/ml pepstatin, and 0.1 μg/ml leupeptin. The cell lysate was mixed with 5× loading buffer and heated at 95˚C for 5 min. Samples were separated by electrophoresis on 12% sodium dodecyl sulfate polyacrylamide gels and the separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Membranes were incubated overnight at 4˚C with rabbit anti-p21 (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-p57 (1:500; Santa Cruz Biotechnology), mouse anti-cyclin D1 (1:500; Santa Cruz Biotechnology), rabbit anti-vimentin (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-E-cadherin (1:500 Cell Signaling Technology) or rabbit anti-N-cadherin (1:500 Cell Signaling Technology). Following incubation with horseradish peroxidase-conjugated anti-rabbit (1:3000; Cell Signaling Technology) or anti-mouse (1:3000; GeneTex, Irvine, CA, USA) secondary antibodies, protein bands were visualized using an ECL detection system (Amersham, Uppsala, Sweden) followed by membrane exposure to X-ray film (Agfa, Mortsel, Belgium). Antibody specific to β-actin (1:2000; Cell Signaling Technology) was used to confirm comparable loading between gel lanes. The density of each protein band was quantified using image J software (National Institutes of Health).

Statistical analysis. Data were analyzed using Student's *t*-test. Curve fit and analysis were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). *p*-Values <0.05 were considered statistically significant. All results are expressed as the mean±standard deviation (SD).

Results

Establishment of 5-FU-resistant cell sublines. Ten aliquots of AGS cells were each treated with increasing concentrations of 5-FU, from 5 μM to 100 μM, to develop 5-FU-resistant GC cell lines. Five cell sublines survived 6.5 months after treatment with 5-FU. The sensitivities to 5-FU of the five resistant cell sublines (FR1-5) and that of the control AGS/D cells were measured using a CCK-8 assay. The IC_{50} for 5-FU of the five AGS/FR cells was significantly higher (>100 μM) than that of the control cells (22.1±7.7 μM) (Figure 1).

Resistance to other chemotherapeutic reagents. Experiments were performed to determine whether the 5-FU-resistant cells acquired resistance to other anticancer drugs. The sensitivity to paclitaxel and cisplatin was measured with a CCK-8 assay. The IC_{50} value for paclitaxel was 5.6±0.2 μM in AGS/D, while that for paclitaxel increased in all 5-FU-resistant sublines (Figure 2A). The IC_{50} values for cisplatin were higher for all AGS/FR cells except AGS/FR4 compared to that for AGS/D (3.7±0.6 μM) (Figure 2B).

EMT phenotype of 5-FU-resistant cell lines. AGS/FR1 and AGS/FR2 cells were spindle shaped, which is characteristic of mesenchymal cells, while the other sublines were round,

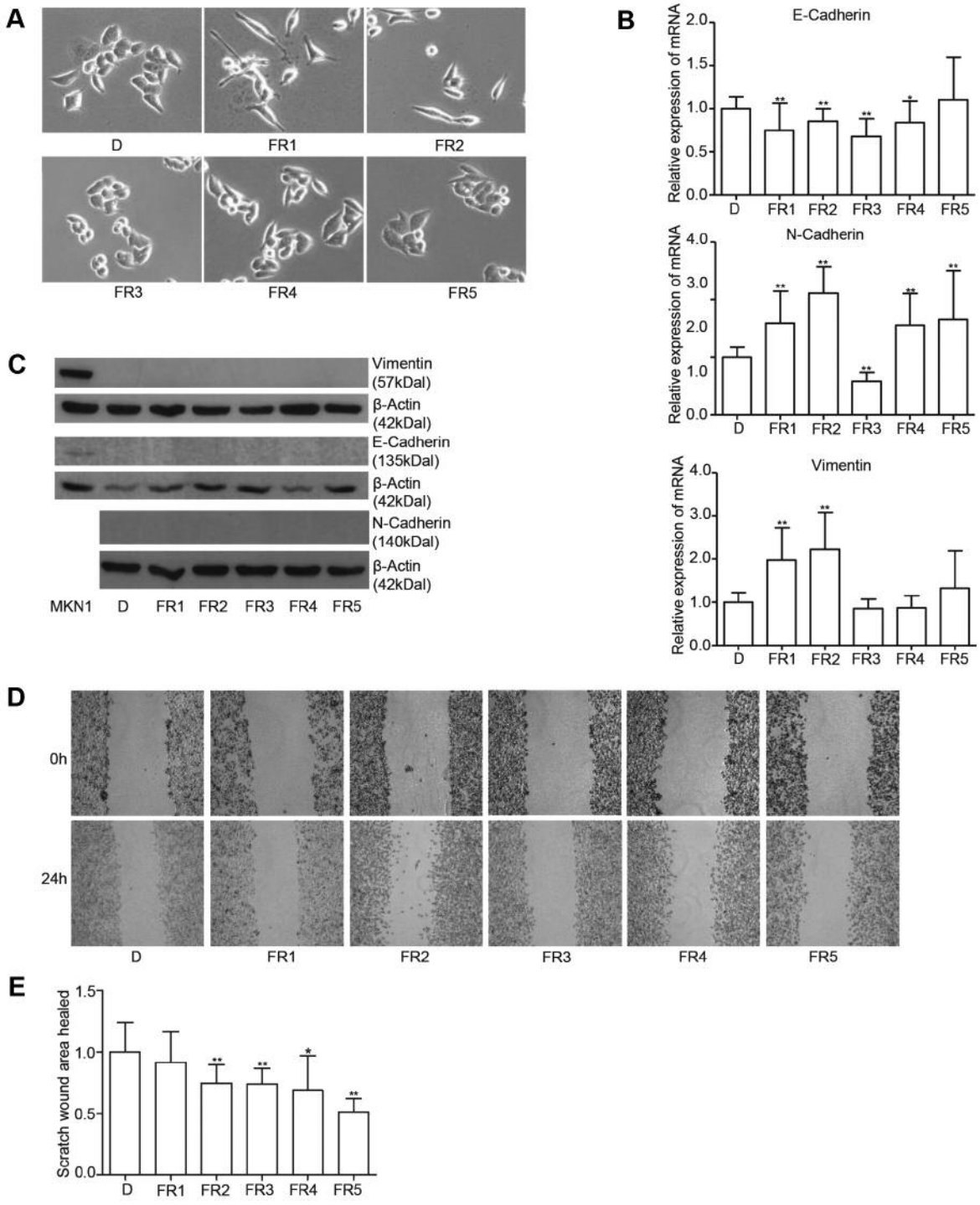

Figure 3. *Epithelial–mesenchymal transition phenotype of AGS gastric cancer cells (AGS/D) and five 5-FU-resistant sublines (AGS/FR1-5). A: AGS/D and AGS/FR1-5 were each seeded in a 60 mm dish. Cell morphology was observed using a Leica DM at an original magnification of ×100. B: mRNA expression of E-cadherin, N-cadherin, and vimentin was measured by quantitative reverse transcription polymerase chain reaction using a SYBR green qPCR kit. Relative gene expression was calculated according to the comparative Ct method, using glyceraldehyde 3-phosphate dehydrogenase as an internal control. C Level of vimentin, E-cadherin, and N-cadherin protein was determined by western blot analysis using antibody to vimentin (1:500), anti-E-cadherin (1:500), and anti-N-cadherin (1:500). Anti-β-actin (1:2,000) was used to confirm equivalent loading. MKN1 cells were used as a positive control. D: A wound-healing assay was performed to compare the cell migration ability of AGS/D and AGS/FR cells. Representative images captured 0 and 24 h after wounding (magnification, ×50) are shown. E: Quantified wound-healing percentage of AGS/D and AGS/FR cells 24 h after being wounded. Data are expressed as the mean±SD of three independent experiments. Significantly different at: *p<0.05, and **p<0.01.*

Table I. *Doubling time of AGS gastric cancer cells (AGS/D) and five 5-FU-resistant sublines (AGS/FR1-5). Data are expressed as mean±SD of three independent experiments.*

Cell line	Doubling time (h)
AGS/D	18.8±0.3
AGS/FR1	25.8±0.4*
AGS/FR2	21.1±0.5*
AGS/FR3	20.9±0.2*
AGS/FR4	20.4±0.5*
AGS/FR5	20.0±0.3*

*Significantly different at $p<0.05$.

similar to the control cells (Figure 3A). Because a spindle shape is typical of EMT, we compared the level of expression of EMT markers in the 5-FU-resistant cells. The level of vimentin mRNA was elevated in AGS/FR1 and AGS/FR2 but not in other cells compared to the control cells. qRT- PCR showed that E-cadherin expression was lower in all 5-FU-resistant cells except AGS/FR5 compared to the control cells. In addition, N-cadherin mRNA level was elevated in all but AGS/FR3 compared to the control cells. In AGS/FR3, N-cadherin expression was lower than that in AGS/D (Figure 3B). Vimentin protein was not detectable by western blot in any of the AGS/FR or AGS/D cells, even though vimentin expression was clearly detected in another gastric cell line, MKN1 (Figure 3C). Similarly, E-cadherin and N-cadherin protein expression was not detectable by western blot in any of the AGS/FR cells (Figure 3C).

A wound-healing assay was performed to examine cell migration, which can increase in some cases of EMT. AGS/FR1 showed similar migration ability to AGS/D, while AGS/FR2-AGS/FR5 showed reduced migration compared to AGS/D (Figure 3D and E). These results suggest that EMT is not essential in the acquisition of 5-FU resistance by AGS/FR cells.

Changes in proliferation of 5-FU-resistant cells. We performed cell proliferation assays and found that all resistant cell lines had a longer doubling time than the control cells (Table I). Cell cycle analysis was performed by propidium iodide staining to identify the cause of the slow proliferation of 5-FU-resistant cells. The ratios of AGS/FR1-5 in G_0/G_1 phase were 58.3%, 49.3%, 46.6%, 43.8%, and 46.4%, respectively, significantly higher than that of the control cells (38.7%). In contrast, the ratios in G_2/M phase were lower in all 5-FU-resistant cells compared to D (Figure 4A and B). Therefore, 5-FU-resistant cells appear to be arrested in G0/G1 phase resulting in delayed cell proliferation.

Expression of cell-cycle regulatory proteins in 5-FU-resistant cells. To determine why 5-FU-resistant cells show G_0/G_1 arrest, the expression of p21, p57, and cyclin D1, which regulate the G_1/S phase transition, was examined by western blot. The expression of p21 protein tended to be higher and the expression of p57 in all AGS/FR cell lines was 1.5 to 2 times higher compared to AGS/D cells. In contrast, expression of cyclin D1 in all AGS/FR cell lines was 29-50% of that in AGS/D cells (Figure 4C and D).

Discussion

We established and characterized five 5-FU-resistant GC cell sublines. The 5-FU-resistant cells commonly showed G_0/G_1 arrest, up-regulation of p57 and p21, and downregulation of cyclin D1. Resistance to paclitaxel and cisplatin was also observed in most of the AGS/FR cell lines. However, cell migration was suppressed rather than increased in the 5-FU-resistant cells compared to the control cells.

Paclitaxel is a drug that targets a cytoskeletal protein, tubulin (23). Abnormal stabilization of the microtubule polymer by paclitaxel causes mitotic arrest and apoptotic cell death, resulting in an anticancer effect (24). Cisplatin induces apoptosis in cells by producing unrepairable platinum–DNA adducts on purine bases (25). Despite the different mechanism of action for each anticancer agent, resistance to 5-FU, paclitaxel, and cisplatin is observed in GC cell lines (12). Many factors affect multidrug resistance. Arrest of tumor cells in the G_0/G_1 phase, as observed in our study, provides prolonged DNA damage-repair time and induces drug resistance following anticancer drug treatment (26).

All the AGS/FR cells we established showed G_0/G_1 phase arrest and reduced proliferation rate. Similarly, an increased ratio of cells in the G_0/G_1 phase was reported in 5-FU- as well as 5-FU- and paclitaxel-resistant GC (27, 28). Together, these results suggest that cell-cycle arrest causes multidrug resistance to anticancer agents that act on rapidly dividing cancer cells.

In our study, p57 protein expression was up-regulated in all five AGS/FR cell lines. p57 is a cyclin-dependent kinase inhibitor that regulates the cell cycle (29). p57 overexpression induced cell growth inhibition and G_0/G_1 arrest in GC (30). In addition, up-regulation of p57 was also observed in pancreatic cancer stem cells resistant to gemcitabine and abraxane (31). Thus, up-regulation of p57 causing G_0/G_1 arrest may have contributed to the increased doubling time and chemoresistance of AGS/FR cells in our study.

Cyclin D1 is one allosteric activator of cyclin-dependent kinase that promotes transition to the G_1/S phase through retinoblastoma protein phosphorylation (32). Cyclin D1 is overexpressed and plays an oncogenic role in many cancer types, including colonic and breast cancer (33, 34). However, overexpression of cyclin D1 was associated with good prognosis in one colonic cancer study (35), suggesting that the role of cyclin D1 is dependent on tumor type. Our data showed an inverse relationship between cyclin D1 expression

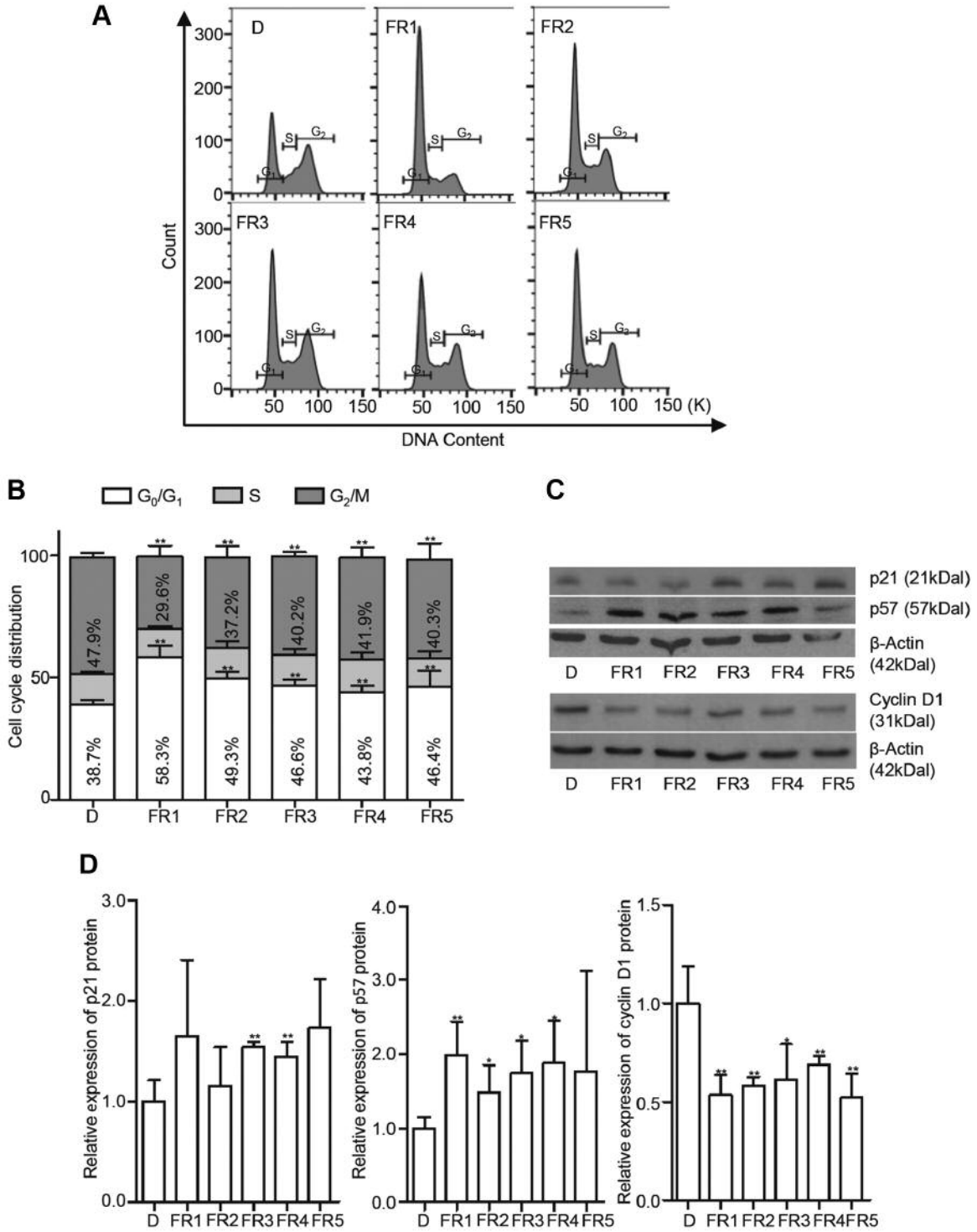

Figure 4. *Increased accumulation of 5-FU-resistant cells in the G_0/G_1 phase. A: The cell-cycle distribution of AGS gastric cancer cells (AGS/D) and five 5-FU-resistant sublines (AGS/FR1-5) was examined by flow cytometry following propidium iodide staining. Representative images of three independent experiments are shown. B: Flow cytometric data are displayed in a histogram. Data are expressed as the mean±SD of three independent experiments. C: Protein levels of p21, p57, and cyclin D1 were determined by western blot analysis using anti-p21 (1:500), anti-p57 (1:500), and anti-cyclin D1, respectively. Anti-β-actin (1:2,000) was used to confirm equivalent loading. D: The band intensities of p21, p57, and cyclin D1 protein were quantified by image J software and normalized to the band intensity of β-actin. Data are expressed as mean±SD of three independent experiments. Significantly different at: *p<0.05 and **p<0.01.*

and the proportion of AGS/FR cells in the G_0/G_1 phase, supporting the notion that cyclin D1 functions to reduce chemoresistance in AGS/FR cells.

Drug-resistant cells often exhibit the EMT phenotype and enhanced cell migration (36, 37). Some of the AGS/FR cells we established showed spindle shaped morphology unlike their round parental cells. However, the ability of the five AGS/FR cell lines to migrate was either unchanged or reduced. The reduced migration of AGS/FR cells may partly be due to the slow proliferation rates of these cells compared to AGS/D. Our results indicate that EMT is not essential for 5-FU resistance, which is consistent with the results of other studies (38, 39).

In this study, we compared the characteristics of five 5-FU-resistant GC cell sublines. Increased p57 expression, reduced cyclin D1 expression, and reduced cell growth were observed in all 5-FU-resistant cell Iines. Most of these also exhibited cisplatin and paclitaxel resistance. This is similar to clinical experience as patients tend to develop multidrug resistance even when they are treated with only one specific anticancer drug, resulting in chemotherapy failure. Our results suggest that new therapeutic strategies should aim to modulate p57 and cyclin D1 expression in patients who suffer from 5-FU resistant disease.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

D.S. Kim carried out most of the experiments and discussed the results with S.K. Lee. K. Min carried out some of the experiments and edited the initial draft of the article. S.K. Lee designed and supervised the work and edited the article.

Acknowledgements

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