Establishment of a 5-fluorouracil-resistant human gastric cancer cell line and exploration of its biological characteristics

Hye Jung Chang (bacchus1220@gmail.com)
National Medical Center https://orcid.org/0000-0003-1341-8601
Min-hee Cho
Ewha Womans University, School of Medicine
Moon Young Choi
Inje University Busan Paik Hospital
Kyoung Eun Lee
Ewha Womans University, School of Medicine https://orcid.org/0000-0003-1596-8666

Research Article

Keywords: chemoresistance, gastric cancer, Notch, 5-Fluorouracil

Posted Date: February 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1302156/v1

Abstract

Background: Chemoresistance is a major problem in gastric cancer (GC) management with an unknown underlying mechanism. In this study, we aimed to establish a 5-fluorouracil (5-FU)-resistant GC cell line and explore the biological characteristics related to its chemoresistance.

Materials and Results: The chemotherapeutic agent-resistant GC cell line (AGS-R) was established by continuous exposure of AGS cells to 5-FU. These cells showed morphologic changes and increased level of thymidylate synthase (TS) mRNA compared with the parental cells. AGS-R also showed down-regulation of β-catenin activity with decreased migration and invasion. The expression of CD44 was increased in AGS-R cells and this was reversed with the inhibition of Notch1.

Conclusions: 5-FU resistant gastric cancer cell line was well established and these cells showed increased stemness which was reversed with Notch1 inhibition.

Introduction

Gastric cancer is a common malignancy worldwide, particularly in Asia. Although the incidence of gastric cancer has been decreasing, the associated mortality remains high. Unfortunately, 20–40% of patients with gastric cancer experience recurrence within 5 years of surgery, with a poor prognosis. In gastric cancer, a 5-fluorouracil (5-FU)-based adjuvant chemotherapy regimen has led to a substantial improvement in survival [1]. However, counteracting chemoresistance is the biggest hurdle in treating patients with cancer. The current therapeutic options are limited and associated with only a modest improvement in survival in the majority of patients with gastric cancer. Recently, studies have investigated the underlying mechanisms of chemoresistance to develop methods that can help patients to overcome this treatment hurdle [2, 3].

Although the molecular mechanisms underlying chemoresistance are not completely understood, studies have reported that specific cancer stem cells (CSCs) play an important role in chemoresistance [4, 5]. The CSCs are a subpopulation of tumor cells that are characterized by self-renewal, differentiation, and asymmetrical division. Moreover, they remain quiescent in some conditions (e.g., hypoxia) and resist conventional therapies, thereby leading to tumor relapse and metastasis. Therefore, the cellular pathways that drive stemness represent a rational target for cancer management [6]. Incidentally, the CSCs have aberrations in one or more signaling pathways.

Studies have been conducted to elucidate the relationship between the Wnt/β-catenin signaling pathway and the CSCs with respect to gastric cancer. The Wnt/β-catenin signaling pathway is well-documented, and its dysregulation plays an important role in the development of human cancers [7]. Particularly, more than 30% of patients with gastric cancer present activated Wnt/β-catenin signaling. Moreover, it has been demonstrated that Wnt/β-catenin signaling has a fundamental role in the self-renewal of gastric cancer stem cells (GCSCs) [8, 9]. Therefore, in this study, the parental gastric cancer cell line, AGS, was selected

for evaluating 5-FU resistance because this cell line has a constitutively activated β-catenin pathway owing to a *CTNNB1* mutation [10].

A previous study identified gastric cancer-initiating cells from a panel of human gastric cancer cell lines using the cell surface marker CD44 [11]. Moreover, another study detected CSCs in tumor tissues and peripheral blood of patients with gastric cancer using CD44 and CD54, both of which are closely related to cancer metastasis [12]. The CSCs in gastric cancer have been extensively studied using gastric cancer cell lines and primary gastric cancer tissues [13–15]. Therefore, targeting the critical pathways and molecules in GCSCs and their environment may represent a potential therapeutic strategy for patients with gastric cancer. In this study, we aimed to establish a 5-FU-resistant gastric cancer cell line, which simulates the real-world recurrent gastric cancer cells, to elucidate the characteristics of this cell line and suggest a treatment target for overcoming chemoresistance.

Materials And Methods

Cell culture

AGS was purchased from the Korean Cell Line Bank (KCLB#21739; Seoul, South Korea). AGS cells were seeded at a density of 2×10^5 cells/mL in a T25 flask and cultured routinely in RPMI 1640 medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1% antibiotics (Thermo Fisher Scientific). An AGS-derived 5-FU-resistant cell line (AGS-R) was established via long-term culture of AGS cells in gradually increasing concentrations (0 to 5 μM over a period of 1 year) of 5-FU (Sigma-Aldrich, St. Louis, MO, USA). Microscopic images of the cells were captured at 100´ magnification using an Olympus CKX31 microscope.

Cell viability assay

After the treatment of both AGS and AGS-R cells with various concentrations of 5-FU, the cell viability assay was performed using the Cell Counting Kit-8 (Enzo Life Sciences, Farmingdale, NY, USA), according to the manufacturer's protocol. First, AGS and AGS-R cells were seeded in 96-well plates at a density of 5×10^3 cells/well and cultured in a CO_2 incubator for 24 h. Subsequently, both cell lines were treated with various concentrations of 5-FU (0, 1, 3, 5, 10, and 20 μM) for 24, 48, and 72 h. Finally, the absorbance of the plates was read at 450 nm using a spectrophotometer.

To examine whether β-catenin is involved in the induction of resistance against 5-FU, the cell viability assay was repeated after an initial treatment of both cell lines with ICG-001 (Selleckchem, Houston, TX, USA), which antagonizes β-catenin/TCF-mediated transcription. The cells were treated with various concentrations of ICG-001 (0, 0.5, 1, and 2 μM) for 24 and 48 h.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from AGS and AGS-R cells treated with 5-FU for 72 h. Subsequently, DNase digestion and other clean-up procedures were performed using the RNAspin Mini RNA Isolation Kit (GE Healthcare Life Science, Pittsburgh, PA, USA), and the purity and yield of RNA were analyzed using a NanoDrop spectrophotometer (NanoDrop Tech, Wilmington, DE, USA). Thereafter, cDNA was synthesized using a cDNA synthesis kit (ELPIS Biotech, Daejeon, South Korea) with 1 μg of the extracted total RNA, according to the manufacturer's instructions. TaqMan Gene Expression Assay kits (Applied Biosystems PCR 7500; Thermo Fisher Scientific), which are based on pre-validated assays with specific primers and probes for each gene, were used for cDNA quantification of the thymidylate synthase (*TS*) gene (TaqMan Gene Expression Assay ID: [TS (TYMS)], Hs00426591_m1) and an internal reference gene (*GAPDH*; TaqMan Gene Expression Assay ID: Hs99999905_m1). TaqMan Universal PCR Master Mix was used to carry out real-time RT-PCR (Thermo Fisher Scientific). The PCR was performed under the following conditions: 10 min at 95°C, followed by 40 cycles for 15 s at 95°C and 1 min at 60°C, in the StepOne System (Thermo Fisher Scientific).

Western blotting

Whole cell lysates were prepared using radioimmunoprecipitation assay buffer (25 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1% NP-40; 1% sodium deoxycholate; and 0.1% SDS) with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Thereafter, total protein was extracted from AGS and AGS-R cells treated with 5-FU for 72 h. The total protein lysates (40 μg) were loaded into each lane, size-fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. Subsequently, the membranes were blocked in Tris-buffered saline-Tween 20 (TBST) containing 5% skim milk for 1 h, and then incubated overnight with the primary antibodies at 4°C. After washing thrice with TBST, the membranes were incubated with the secondary antibodies, namely, horseradish peroxidase-conjugated anti-rabbit IgG (developed in goat) or anti-mouse IgG (developed in horse) (#7074 and #7076, respectively, Cell Signaling Technology). Finally, the immunoblots were treated with enhanced chemiluminescence reagents and visualized using an LAS-4000 Mini camera (Fujifilm, Tokyo, Japan). All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA); the antibodies used were against β-actin (D6A8, #8457), TS (D5B3, # 9045), protein kinase B (AKT) (92G2, #2978), phosphor-AKT (p-Akt, Thr308) (D25E6, #13038), C-myc (D84C12, #5605), cyclin D1 (92G2, #5605), α-tubulin (11H10, #2125), lamin B1 (D9V6H, #13435), signal transducer and activator of transcription 3 (STAT3) (124H6, #9139), phosphor-STAT3 (Tyr705) (D3A7, #9145), Notch intracellular domain (NICD; cleaved notch1) (Val1744) (D3B8, #4147), GLI1 (C68H3, #3538), β-catenin (D10A8, #8480), and nonphospho (active) β-catenin (Ser33/37/Thr41) (D13A1, #8814).

To investigate the expression of β-catenin/active β-catenin in the nucleus and cytoplasm, the nuclear and cytoplasmic extracts were purified using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific).

Luciferase assay

For electroporation, AGS and AGS-R cells were seeded at a density of 1×10^5 cells/0.5 mL in 24-well plates with serum-free RPMI. The cells were serum-starved for 16 h, and then co-transfected with pGL4.49 (Promega, Madison, WI, USA) and pRL-TK constructs (Promega) using jetPRIME transfection reagent (Polyplus transfection, NY, USA). The pGL4.49 vector contains eight copies of a T-cell factor/lymphoid enhancer factor response element that drives the transcription of the firefly luciferase reporter gene *luc2p*. The pRL-TK vector provides the constitutive expression of *Renilla* luciferase, and serves as a control reporter vector. After incubation for 24 h, the luciferase assay was performed with both cell lysates using the DUAL-GLO Luciferase Assay System (Promega), according to the manufacturer's instructions. Luminescence was measured using GLOMAX (Promega). The ratio of firefly to *Renilla* luciferase activities represented the transcriptional activity of β-catenin.

Flow cytometry

AGS and AGS-R cells (1×10^6/mL) were stained with FITC-conjugated anti-human CD44 antibody (Mouse IgG2b; BD Biosciences, San Jose, CA, USA) or the isotype control antibody (Mouse IgG2b; BioLegend, San Diego, CA, USA) on ice for 40 min. Thereafter, the cells were washed thrice with phosphate-buffered saline and analyzed on the NovoCyte Flow Cytometer (ACEA Biosciences, San Diego, CA, USA); the data were assessed using NoveExpress software.

Cell invasion and scratch assay

The cell invasion assay was performed using the Chemicon Cell Invasion Assay Kit (Merck, Temecula, CA, USA). This kit consists of 12 inserts, each of which contains an 8-μm pore-size polycarbonate membrane coated with a thin layer of ECMatrix. The invasive cells migrated through the extracellular matrix layer and were attached to the bottom of the membrane; these cells were fixed, stained with 0.1% crystal violet, and photographed under a microscope (Olympus CKX31).

For the cell scratch assay, AGS and AGS-R cells (3×10^5 cells/2 mL/well) were incubated in 6-well plates with small straight-line wounds that were made in confluent monolayers using an SPLScar scratcher (width 0.5 mm; SPL Life Sciences, Pocheon-si, South Korea). Thereafter, the cells were fixed, stained with Cell Stain solution (Merck, Temecula, CA, USA), and photographed under a microscope (Olympus CKX31).

siRNA transfection

Notch1 specific siRNA (siNotch1) and non-targeting siRNA (siNC) were purchased from Bioneer (Daejeon, South Korea). siRNA transfection was performed using the jetPRIME transfection reagent (Polyplus transfection), according to the manufacturer's instructions. The control cells were transfected with non-targeting scrambled siRNA using the jetPRIME transfection reagent (mock). Further experiments were performed 72 h after transfection.

Statistical analyses

Statistical analyses were performed using SPSS version 18 (IBM Corp., Armonk, NY, USA). An independent *t*-test was used to compare the two cell lines. Statistical significance was set at $P \leq 0.05$.

Results

Establishment of 5-FU-resistant gastric cancer cells

The chemoresistant gastric cancer cell line AGS-R was established through continuous exposure of AGS cells to 5-FU for 1 year. The morphological differences between AGS and AGS-R cells were observed by microscopy (Fig. 1a); although the majority of AGS cells were initially cuboidal in shape, there was a gradual transformation into spindle-shaped cells with pseudopodia formation. Both AGS and AGS-R cells displayed a decrease in viability upon 5-FU treatment in a time- and concentration-dependent manner; however, AGS-R cells were more resistant to 5-FU than their parental cell line (Fig. 1b). The viability of AGS cells was below 60% when treated with >3 μM 5-FU, regardless of the treatment duration. In contrast, the viability of AGS-R cells was below 60% only when treated with >10 μM 5-FU for 72 h.

Thymidylate synthase is the rate-limiting enzyme in *de novo* pyrimidine biosynthesis, and its inhibition leads to the inhibition of DNA synthesis; the 5-FU treatment is based on this mechanism of TS-inhibition. Therefore, resistance to 5-FU is associated with the upregulation of TS expression. Our study confirmed that the TS level was higher in AGS-R cells than in AGS cells (Fig. 1c and d).

Decreased ß-catenin activity in and invasive capacity of AGS-R cells

The transcriptional activity of β-catenin was lower in AGS-R cells than in AGS cells (Fig. 2a). Additionally, cytoplasmic and nuclear β-catenin, cyclin D1, and c-myc levels were higher in AGS-R cells than in AGS cells (Fig. 2b). Incidentally, the ratio of total β-catenin in the nucleus and cytoplasm was similar in AGS and AGS-R cells.

Although the β-catenin pathway is upregulated in AGS-R cells, the inhibition of β-catenin with various concentrations of ICG-001 did not inhibit the proliferation of AGS-R cells compared with that in AGS cells. AGS-R cells showed a viability of more than 90%, regardless of the ICG-001 concentration and treatment duration (Fig. 2c).

The invasiveness and migratory capacity of AGS-R cells were lower than those of AGS cells (Fig. 3).

Aggravated stemness in AGS-R cells

Cancer stemness is induced by the continuous exposure of cancer cells to chemotherapeutic drugs, and CD44 expression is considered an important characteristic feature of cancer stem cells. Here, CD44

expression on the surface of AGS and AGS-R cells was assessed by flow cytometry. The percentage of CD44+ cells in AGS was 5.1%, but it was upregulated by approximately 20% in AGS-R (Fig. 4a).

Moreover, CD44 expression was upregulated in AGS-R cells despite the inactivation of the Wnt/β-catenin signaling pathway. Therefore, we investigated the exact signaling pathway in AGS-R cells that led to an increase in CD44 expression in gastric cancer cells. Furthermore, the protein levels of glioma-associated oncogene homolog 1 (GLI1, which is a nuclear mediator of the Hedgehog pathway), p-STAT3, and NICD were compared between the cell lines (Fig. 4b). NICD expression was five times (5.1 ± 1.6) higher in AGS-R cells than in AGS cells. These findings imply that the Notch signaling pathway has a role in the development of chemoresistance in AGS-R cells and could be the target pathway for the restoration of chemosensitivity in this 5-FU-resistant gastric cancer cell line.

To confirm whether the Notch signaling pathway regulates the stem cell-like characteristics of AGS-R cells, especially the expression of CD44, the expression of NICD was silenced via *Notch1*-specific siRNA transfection (siNotch1) (Fig. 4c). Although not significant, there was a decrease in CD44 expression in AGS-R cells after *Notch1* silencing compared with that in the cells transfected with non-targeting siRNA (Fig. 4d). These results suggest that the activation of the Notch signaling pathway plays a role in generating 5-FU-resistant gastric cancer cells by increasing CD44 expression.

Discussion

In the past 60 years, 5-FU has been used in the treatment of various cancers such as gastric cancer, colon cancer, and head and neck cancer. In gastric cancer, 5-FU is the mainstay treatment drug in adjuvant and palliative settings; hence, the development of 5-FU resistance limits the treatment options for patients with gastric cancer in the real-world setting.

Therefore, several studies have been conducted to determine the possible means of overcoming chemoresistance [2, 3]. In the present study, a 5-FU-resistant gastric cancer cell line, AGS-R, was successfully established by exposing AGS cells to gradually increasing concentrations of 5-FU for 1 year.

With respect to cell morphology, AGS-R cells were spindle-shaped with pseudopodia, as opposed to the cuboidal shape of AGS cells. Although AGS-R cells exhibited EMT-like cell morphology, their invasiveness and migration abilities were decreased compared with those of AGS cells. Previously, it has been demonstrated that the migration capability of cells does not always affect only migration, as migration and invasion can be uncoupled [16]. Another study reported that EMT confers increased invasion and migration abilities to the cells, and that it is associated with drug resistance [17]. The EMT-induced stemness of cancer cells may characterize one of the important mechanisms underlying EMT-mediated chemoresistance; furthermore, tumorigenesis may regulate various EMT signaling pathways, such as the Wnt, Notch, and Hedgehog pathways, thereby triggering the renewal and maintenance of CSCs [18–20].

Activation of the Wnt/β-catenin signaling pathway can confer chemoresistance to 5-FU in various carcinomas [21], and it plays an important role in the self-renewal of GCSCs [9]. In this study, AGS-R cells

were generated from AGS cells that harbor a heterozygous mutation in the β-catenin gene (*CTNNB1*) at codon 34 [10], thereby exhibiting elevated β-catenin/TCF transcriptional activity, including *CDH1, KRAS*, and *PIK3CA* mutations. Our study showed that the acquisition of 5-FU resistance by AGS cells downregulated the intrinsic activation of β-catenin. We analyzed the probable genetic abnormalities in AGS-R cells using the NGS panel technique (data not shown; Oncomine Comprehensive Panel; Thermo Fisher Scientific) but did not observe any significant genetic changes. Although AGS-R cells harbor the *CTNNB1* gain-of-function mutation, they exhibited a decrease in β-catenin activity. It has been demonstrated that Wnt/β-catenin signaling is one of the major pathways involved in the EMT of cancer cells; therefore, it must have a critical role in the regulation of metastasis [22]. Indeed, it has been established that the inhibition of the Wnt/β-catenin pathway can increase the anti-metastatic activity of gastric cancer cells [23, 24]. The results of our present study are consistent with those of previous studies with respect to the decrease in tumor aggressiveness, invasiveness, and migratory activity in conjunction with the decreased activity of β-catenin.

Several signaling pathways, such as the Notch, Wnt/β-catenin, and Sonic hedgehog (Shh) pathways, are associated with the stemness of gastric cancer cells [13, 25]. Previous studies have established that the Notch signaling pathway can manipulate physiological and pathological processes, including stem cell renewal and differentiation. Moreover, it has a substantial role in the development of malignant tumors, such as breast, lung, pancreas, and basal cell carcinomas, in humans [26, 27]. Additionally, some recent evidence implies that Notch signaling regulates the self-renewal and survival of CSCs, which, in turn, induce tumor initiation and repopulation in patients with cancer, following chemotherapy. Furthermore, this pathway increases the CSC population in various cancers, including the GCSCs, thereby contributing to therapy resistance [28]. We observed that the Notch signaling pathway was considerably upregulated in AGS-R cells, and the expression of CD44 was notably decreased after *Notch1* knockdown in AGS-R cells. This observation suggests that Notch signaling mediates the increase in stemness and induces 5-FU resistance in AGS-R cells. Therefore, inhibition of this pathway may form a potential therapeutic strategy of curing gastric cancer by eradicating CSCs and overcoming chemoresistance.

In conclusion, in this study, we successfully established a 5-FU-resistant gastric cancer cell line, AGS-R, which simulated the real-world chemoresistant gastric cancer cells. The AGS-R cell line showed altered characteristics of β-catenin activity, invasion/migration, and stemness patterns compared with the AGS cell line. Incidentally, these changes might be specific to the gastric cancer cell line; the administration of precision medicine based on the results of NGS could not alter the changes in the drug sensitivity of the resistant cancer cells. However, based on our observations of the Notch signaling pathway in this 5-FU-resistant gastric cancer cell line, it is expected that targeted inhibition of the Notch signaling pathway might have therapeutic potential against 5-FU-resistant gastric cancer.

Further research is required to determine the comprehensive practical target of treating drug-resistant cancer cells, depending on relapsed and chemotherapy-refractory patients.

Declarations

Acknowledgements

We would like to thank Editage (www.editage.co.kr) for editing and reviewing this manuscript for English language editing. This work was supported by the National Research Foundation of Korea (NRF), Ministry of Science and ICT, Government of Korea (grant number NRF-2017R1D1A1B04035892).

Funding

This work was supported by the National Research Foundation of Korea (NRF), Ministry of Science and ICT, Government of Korea (Grant number NRF-2017R1D1A1B04035892).

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics statement, consent to participate, consent to publish and availability of data and material

The authors did not research involving human participants and/or animals.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hye Jung Chang, Min-hee Cho, Moon Young Choi and Kyoung Eun Lee. The first draft of the manuscript was written by Hye Jung Chang and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Details are following,

Conceptualization : Hye Jung Chang and Kyoung Eun Lee

Data curation : Hye Jung Chang, Min-hee Cho, Moon Young Choi and Kyoung Eun Lee

Formal analysis : Hye Jung Chang and Min-hee Cho

Funding acquisition : Kyoung Eun Lee

Investigation : Hye Jung Chang, Min-hee Cho, Moon Young Choi and Kyoung Eun Lee

Methodology: Hye Jung Chang, Min-hee Cho, Moon Young Choi and Kyoung Eun Lee

Project administration: Kyoung Eun Lee

Resources : Kyoung Eun Lee

Software : Hye Jung Chang, Min-hee Cho and Moon Young Choi

Supervision : Kyoung Eun Lee

Validation : Kyoung Eun Lee

Visualization: Hye Jung Chang, Min-hee Cho, Moon Young Choi and Kyoung Eun Lee

Writing original draft: Hye Jung Chang

Writing review & editing: Min-hee Cho, Moon Young Choi and Kyoung Eun Lee

References

1. Kilic L, Ordu C, Yildiz I, Sen F, Keskin S, Ciftci R, Pilanci KN (2016) Current adjuvant treatment modalities for gastric cancer: from history to the future. World J Gastrointest Oncol 8:439–449. https://doi.org/10.4251/wjgo.v8.i5.439
2. Shi WJ, Gao JB (2016) Molecular mechanisms of chemoresistance in gastric cancer. World J Gastrointest Oncol 8:673–681. https://doi.org/10.4251/wigo.v8.i9.673
3. Moon KJ, Park JJ, Chun HJ (2016) Recent updateds of precision therapy for gastric cancer: Towards optimal tailored management. World J Gastrointerol 22:4638–4650. https://doi.org/10.3748/wig.v22.i19.4638
4. Dean M, Fojo T, Bates S (2005) Tumour stem cells and drug resistance. Nat Rev Cancer 5:275–284. https://doi.org/10.1038/nrc1590
5. Xu ZY, Tang JN, Xie HX, Du YA, Huang L, Yu PF, Cheng XD (2015) 5-Fluorouracil chemotherapy of gastric cancer generates residual cells with properties of cancer stem cells. Int J Biol Sci 11:284–294. https://doi.org/10.7150/ijbs.10248
6. Nassar D, Blanpain C (2016) Cancer stem cells: basic concepts and therapeutic implications. Annu Rev Pathol 11:47–76. https://doi.org/10.1146/annurev-pathol-012615-044438
7. Koushyar S, Powell AG, Vincan E, Phesse TJ (2020) Targeting Wnt signaling for the treatment of gastric cancer. Int J Mol Sci 21:3927. https://doi.org/10.3390/ijms21113927
8. Ishimoto T, Oshima H, Oshima M, Kai K, Torii R, Masuko T, Baba H, Saya H, Nagano O (2010) CD44+ slow-cycling tumor cell expansion is triggered by cooperative actions of Wnt and prostaglandin E2 in gastric tumorigenesis. Cancer Sci 101:673–678. https://doi.org/10.1111/j.1349-7006.2009.01430.x
9. Cai C, Zhu X (2012) The Wnt/β-catenin pathway regulates self-renewal of cancer stem-like cells in human gastric cancer. Mol Med Rep 5:1191–1196. https://doi.org/10.3892/mmr.2012.802
10. Caca K, Kolligs FT, Ji X, Hayes M, Qian J, Yahanda A, Rimm DL, Costa J, Fearon ER (1999) Beta- and gamma-catenin mutations, but not E-cadherin inactivation, underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. Cell Growth Differ 10:369–376
11. Takaishi S, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, Gordon SA, Shimada Y, Wang TC (2009) Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells 27:1006–1020. https://doi.org/10.1002/stem.30

12. Chen T, Yang K, Yu J, Meng W, Yuan D, Bi F, Liu F, Liu J, Dai B, Chen X, Wang F, Zeng F, Xu H, Hu J, Mo X (2012) Identification and expansion of cancer stem cells in tumor tissues and peripheral blood derived from gastric adenocarcinoma patients. Cell Res 22:248–258. https://doi.org/10.1038/cr.2011.109
13. Bekaii-Saab T, El-Rayes B (2017) Identifying and targeting cancer stem cells in the treatment of gastric cancer. Cancer 123:1303–1312. https://doi.org/10.1002/cncr.30538
14. Lee JW, Sung JS, Park YS, Chung S, Kim YH (2018) Isolation of spheroid-forming single cells from gastric cancer cell lines: enrichment of cancer stem-like cells. Biotechniques 65:197–203. https://doi.org/10.2144/btn-2018-0046
15. Liu HQ, Shu X, Ma Q, Wang R, Huang MY, Gao X, Liu YN (2020) Identifying specific miRNAs and associated mRNAs in CD44 and CD90 cancer stem cell subtypes in gastric cancer cell line SNU-5. Int J Clin Exp Pathol 13:1313–1323
16. Schaeffer D, Somarelli JA, Hanna G, Palmer GM, Garcia-Blanco MA (2014) Cellular migration and invasion uncoupled: increased migration is not an inexorable consequence of epithelial-to-mesenchymal transition. Mol Cell Biol 34:3486–3499. https://doi.org/10.1128/MCB.00694-14
17. Shibue T, Weinberg RA (2017) EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nat Rev Clin Oncol 14:611–629. https://doi.org/10.1038/nrclinonc.2017.44
18. Malanchi I, Peinado H, Kassen D, Hussenet T, Metzger D, Chambon P, Huber M, Hohl D, Cano A, Birchmeier W, Huelsken J (2008) Cutaneous cancer stem cell maintenance is dependent on beta-catenin signalling. Nature 452:650–653. https://doi.org/10.1038/nature06835
19. Singh A, Settleman J (2010) EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 29:4741–4751
20. Najafi M, Farhood B, Mortezaee K (2019) Cancer stem cells (CSCs) in cancer progression and therapy. J Cell Physiol 234:8381–8395. https://doi.org/10.1002/jcp.27740
21. Liu Y, Chen H, Zheng P, Zheng Y, Luo Q, Xie G, Ma Y, Shen L (2017) ICG-001 suppresses growth of gastric cancer cells and reduces chemoresistance of cancer stem cell-like population. J Exp Clin Cancer Res 36:125. https://doi.org/10.1186/s13046-017-0595-0
22. Talbot LJ, Bhattacharya SD, Kuo PC (2012) Epithelial-mesenchymal transition, the tumor microenvironment, and metastatic behavior of epithelial malignancies. Int J Biochem Mol Biol 3:117–136
23. Hanaki H, Yamamoto H, Sakane H, Matsumoto S, Ohdan H, Sato A, Kikuchi A (2012) An anti-Wnt5a antibody suppresses metastasis of gastric cancer cells in vivo by inhibiting receptor-mediated endocytosis. Mol Cancer Ther 11:298–307. https://doi.org/10.1158/1535-7163.MCT-11-0682
24. Wang B, Guan G, Zhao D (2020) Silence of FAM83H-AS1 promotes chemosensitivity of gastric cancer through Wnt/β-catenin signaling pathway. Biomed Pharmacother 125:109961. https://doi.org/10.1016/j.biopha.2020.109961
25. Stojnev S, Krstic M, Ristic-Petrovic A, Stefanovic V, Hattori T (2014) Gastric cancer stem cells: therapeutic targets. Gastric Cancer 17:13–25. https://doi.org/10.1007/s10120-013-0254-x

26. Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, Artavanis-Tsakonas S (2005) Notch signals control the fate of immature progenitor cells in the intestine. Nature 435:964–968. https://doi.org/10.1007/s10120-013-0254-x
27. Yuan X, Wu H, Xu H, Xiong H, Chu Q, Yu S, Wu GS, Wu K (2015) Notch signaling: an emerging therapeutic target for cancer treatment. Cancer Lett 369:20–27. https://doi.org/10.1016/j.canlet.2015.07.048
28. Li LQ, Pan D, Zhang SW, Y-Xie D, Zheng XL, Chen H (2018) Autophagy regulates chemoresistance of gastric cancer stem cells via the Notch signaling pathway. Eur Rev Med Pharmacol Sci 22:3402–3407. https://doi.org/10.26355/eurrev_201806_15162

Figures

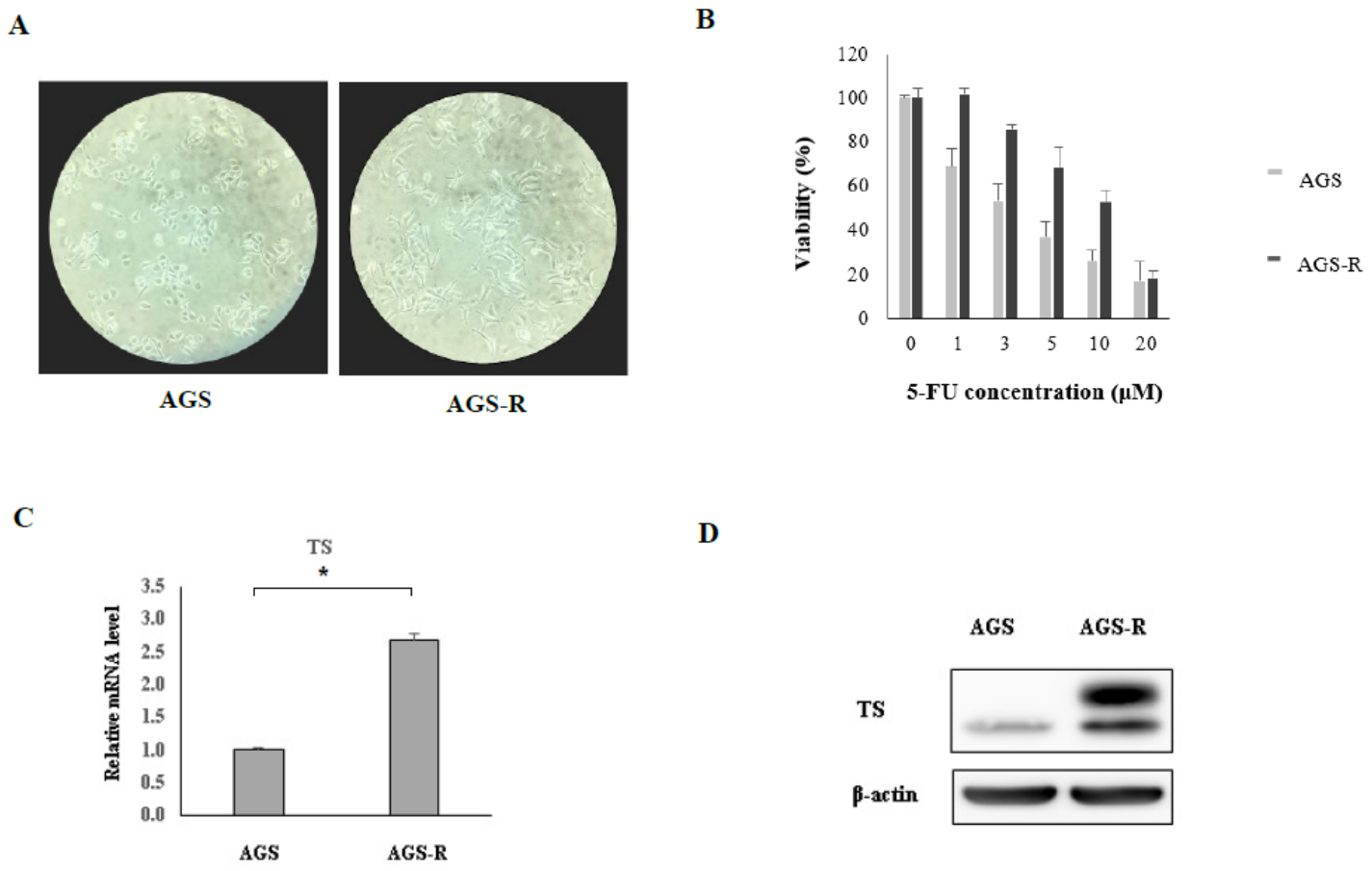

Figure 1

Establishment of 5-fluorouracil (5-FU)-resistant gastric cell line. (a) Morphological differences between parental gastric cancer cells (AGS) and 5-FU-resistant gastric cancer cells (AGS-R). AGS-R cell line was established by continuous exposure of AGS cells to 5-FU, as described in the Materials and Methods. Morphological changes in AGS-R cells were compared with those of its parental cell line AGS on day 3 of culture under an inverted microscope (X100). (b) *In vitro* sensitivity of AGS and AGS-R cells to 5-FU. AGS

and AGS-R cells were treated with 0, 1, 3, 5, 10, and 20 μM 5-FU for 24, 48, and 72 h. Data are representative of more than three independent experiments. (c) The level of thymidylate synthase (TS) mRNA expression in AGS and AGS-R cells. Single asterisk indicates a significant difference (*P < 0.05*). (d) Changes in the TS level in AGS and AGS-R cells, as analyzed by western blotting

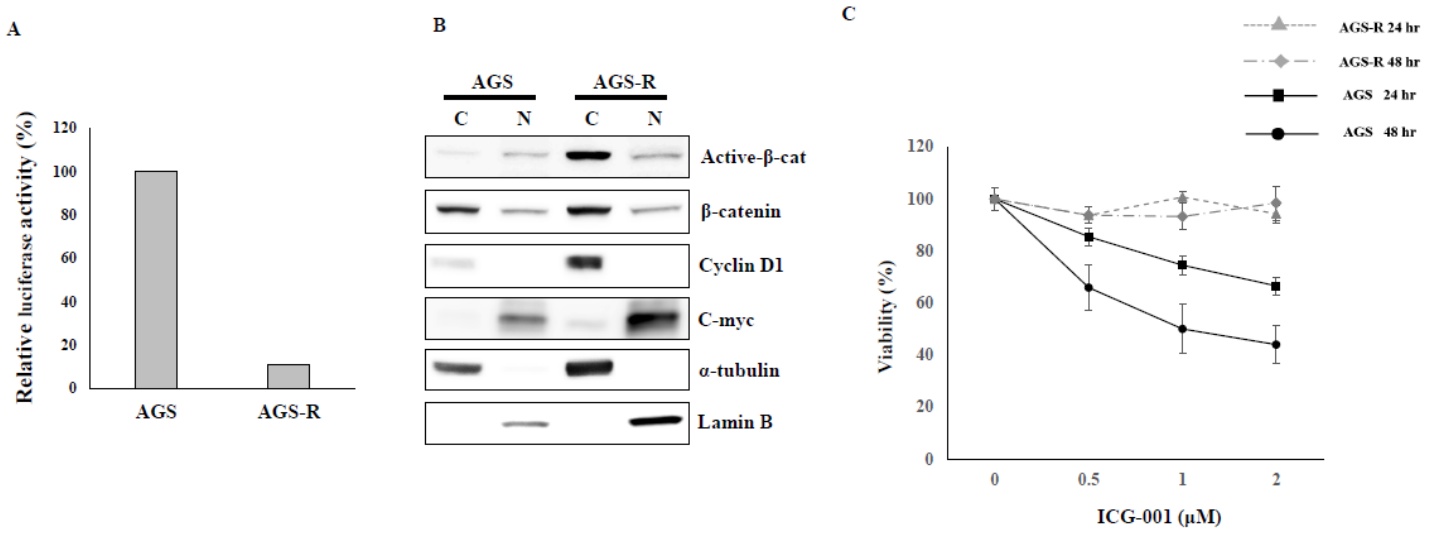

Figure 2

Changes in the characteristics of 5-fluorouracil (5-FU)-resistant gastric cancer (AGS-R) cells. (a) Comparison of the transcriptional activity of β-catenin between parental gastric cancer (AGS) cells and AGS-R cells. (b) Isolation of cytoplasmic and nuclear proteins from AGS and AGS-R cells, and subsequent analysis of the changes in the expression of β-catenin, cyclin D1, c-myc, α-tubulin, and lamin-B in each fraction by western blotting. [C: cytoplasm, N: nucleus] (c) Change in the viability of AGS and AGS-R cells, after treatment with ICG-001, which is a specific inhibitor of β-catenin. AGS and AGS-R cells were cultured with different concentrations (0, 0.5, 1, and 2 μM) of ICG-001 for 24 and 48 h. Data are representative of more than three independent experiments

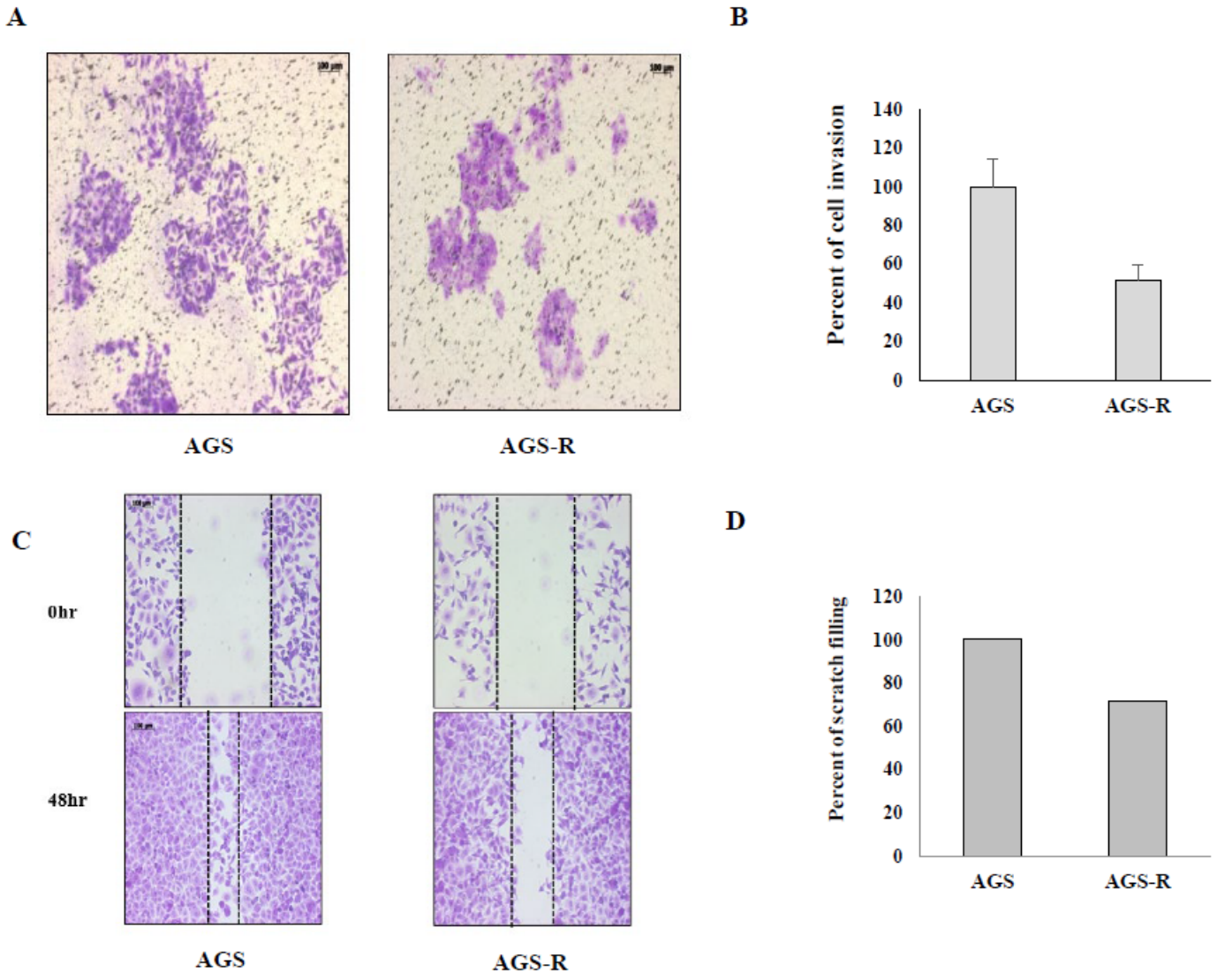

Figure 3

Changes in migratory and invasive activities of 5-fluorouracil (5-FU)-resistant gastric cancer (AGS-R) cells. (a) and (b) Migratory and invasive activities of parental gastric cancer (AGS) cells (left) and AGS-R (right) cells were examined using a cell migration assay with a two-chamber system. Migratory cells in both chambers were counted, calculated as a percentage of cell invasion, and presented in a graphical format. (c) and (d) Scratch assay was performed to examine the proliferative activity of AGS-R cells compared with that of AGS cells. (c) depicts observations at 0 and 48 h, and (d) depicts the percentage of scratch filling. Data are representative of more than three independent experiments

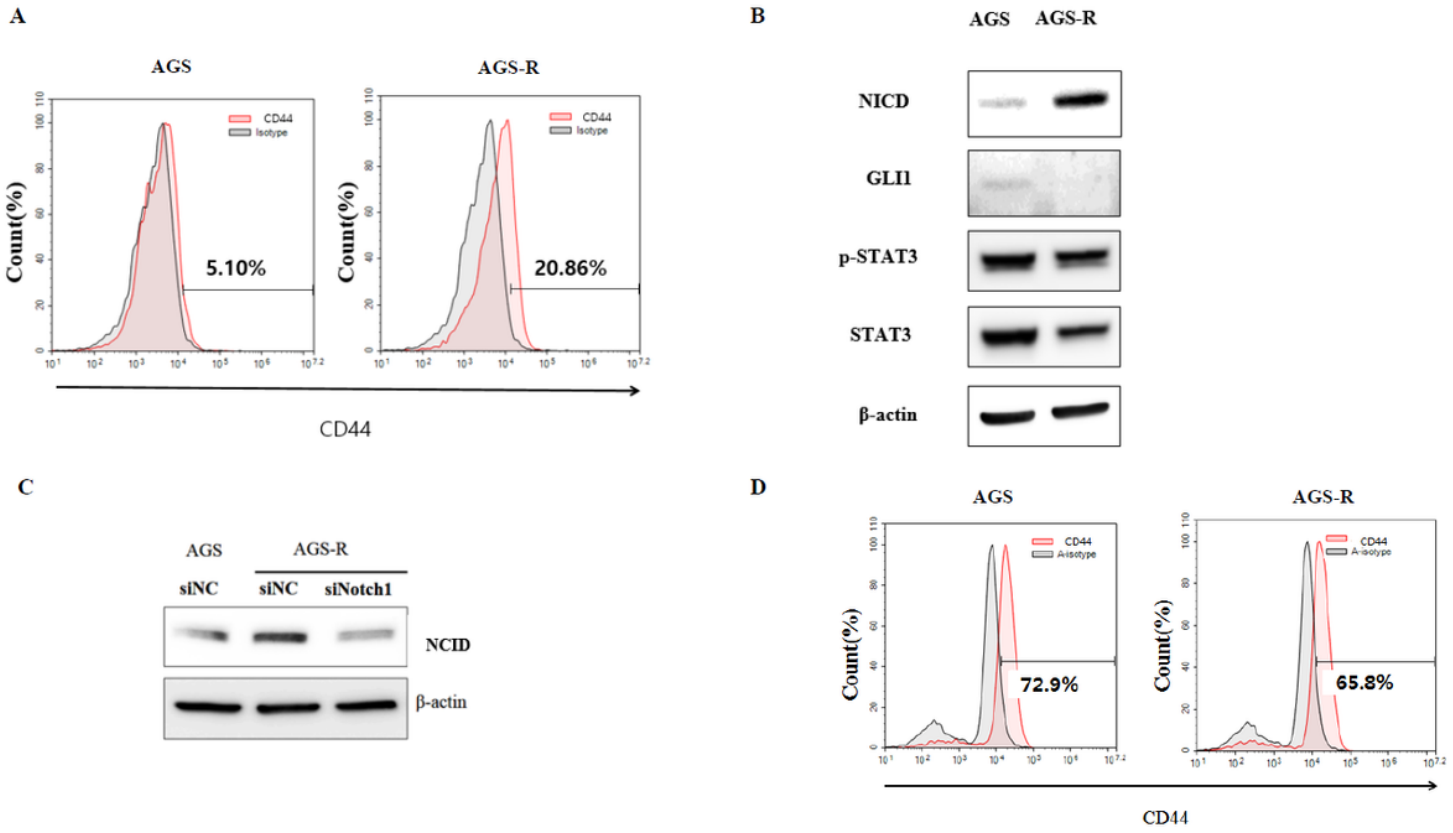

Figure 4

Aggravated stemness in 5-fluorouracil (5-FU)-resistant gastric cancer (AGS-R) cells. (a) Analysis of CD44 expression by Flow cytometry. The percentage of CD44 (+) cells in AGS was 5.1% (left), but in AGS-R, it was 20.9% (right). (b) Changes in several molecular pathways of AGS and AGS-R cells, as analyzed by western blotting. (c) The transfection of *Notch1*-specific siRNA (siNotch1) and non-targeting siRNA (siNC) was performed by jetPRIME transfection. After siRNA transfection, the changes in the expression of Notch Intra Cellular Domain (NICD), as confirmed by western blotting. (d) Decreased CD44 expression in *Notch1* knock-down AGS-R cells compared with that in AGS cells. Data are representative of more than three independent experiments