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Casein Kinase 2α Enhances 5-Fluorouracil Resistance in Colorectal Cancer Cells by Inhibiting Endoplasmic Reticulum Stress

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Abstract. *Background/Aim: Anti-cancer drug resistance restricts the efficacy of chemotherapy in malignant tumors. Casein kinase 2α (CK2α) is highly expressed in 5-fluorouracil (5FU)-resistant colorectal cancer (CRC) cells. We hypothesized that inhibition of CK2α might reduce CRC resistance to 5FU. Materials and Methods: To investigate the role of CK2α in 5FU- resistant CRC cells, we assessed cell viability, apoptosis, cyclin-dependent kinase 4 (CDK4) activity, cell-cycle progression, invasion, and sphere formation in 5FU-resistant CRC cells. Results: CK2α levels were significantly increased in 5FU-resistant CRC cells compared to those in wild-type CRC cells. During exposure to 5FU, viability, CDK4 activity, cell-cycle progression, invasion, and sphere formation were enhanced, while apoptosis was decreased in 5FU-resistant CRC cells. These effects were mediated by the inhibiting effects of CK2α on endoplasmic reticulum (ER) stress. Combination of CK2α knockdown with 5FU treatment promoted apoptosis of 5FU-resistant CRC cells by inducing ER stress. Conclusion: 5FU treatment in combination with a CK2α inhibitor may exert a synergistic effect against drug-resistant cancer cells.*

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide, and the second leading cause of cancer-related mortality (1). Although anti-cancer chemotherapy is a promising treatment strategy for CRC, anti-cancer drug resistance limits its clinical outcomes, resulting in cancer-related death (2, 3). Thus, to address drug resistance to single-agent chemotherapy, polychemotherapy regimens with non-overlapping mechanisms of actions have been employed, leading to enhanced therapeutic efficacy in several tumor types, such as lymphoma, breast cancer, and testicular cancer (4). However, these polychemotherapies, as well as surgical therapy (5) and radiotherapy (6), do not address all cancer types. Novel therapeutic strategies have emerged to attack key characteristics of tumors, including targeted therapy. In particular, a combination of targeted therapy with chemotherapy is a promising strategy for overcoming drug resistance and effectively treating CRC.

Casein Kinase 2 (CK2), consisting of 2 large catalytic subunits, CK2α (44 kDa) and CK2α' (36 kDa), and 2 small non-catalytic CK2β (25 kDa) subunits, is a constitutively active protein kinase that phosphorylates hundreds of substrates. The expression and activity of CK2, particularly the CK2α subunit, are usually increased in cancer cells, where CK2 exerts anti-apoptotic, pro-migratory, and pro-proliferative effects (7, 8). In CRC, CK2 has been implicated in cancer progression and anti-cancer drug resistance (9). In addition, a previous study demonstrated that CK2 suppresses the endoplasmic reticulum (ER) stress response, leading to down-regulation of programmed cell death in cancer cells (10). These findings indicate that CK2 plays pivotal roles in CRC characteristics, including cancer cell survival, invasion, progression, and drug resistance.

In the present study, we investigated the ability of CK2α inhibition to potentiate the anti-cancer effects of 5FU *via* activation of the ER stress response. Our results showed that CK2α inhibition is a promising adjuvant therapy in combination with 5FU for CRC.

Materials and Methods

Cells and cell culture. The human colorectal cancer cell lines (wild-type SNU-C5 cells [SNU-C5/WT] and 5-fluorouracil (5FU)-resistant SNU-C5 cells [SNU-C5/5FUR]) were obtained from the Chosun University Research Center for Resistant Cells (Gwangju, Republic of Korea). The cells were maintained in RPMI 1640

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supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics (Biological Industries, Beit Haemek, Israel) at 37˚C and 5% CO_2 in a humidified incubator.

Small interference RNA transfection. In accordance with the manufacturer's protocols, LipofectamineTM2000 reagent (Thermo Fisher Scientific) was used to transfect small interference RNA (siRNA) genes into SNU-C5/5FUR. The cells were first grown to 70% confluence in culture dishes and then transfected for 48 h with SMART pool siRNAs (100 nM) specific to CK2α mRNA.

Western blot analysis. Total cell proteins were extracted using RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were transferred to polyvinylidene fluoride membranes (Sigma-Aldrich, St. Louis, MO, USA). Membranes were blocked with 5% skim milk and incubated with primary antibodies against CK2α (Novus Biological, Centennial, CO, USA), Bcl-2-associated X protein (BAX; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cleaved caspase-3 (Thermo Fisher Scientific), cleaved poly(ADP-ribose) polymerase 1 (PARP-1; Santa Cruz Biotechnology), phospho-protein kinase-like endoplasmic reticulum kinase (p-PERK; Novus), phospho-eukaryotic initiation factor 2 alpha (p-eIF2α; Novus), activating transcription factor 4 (ATF4; Novus), phospho-inositol-requiring enzyme 1 alpha (p-IRE1α; Novus), phospho-c-Jun N-terminal kinases (p-JNK; Santa Cruz Biotechnology), p-p38 (Santa Cruz Biotechnology), C/EBP homologous protein (CHOP; Santa Cruz Biotechnology), β-actin (Santa Cruz Biotechnology), and α-tubulin (Santa Cruz Biotechnology). After incubation of membranes with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), bands were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific) in a dark room.

Quantitative real-time PCR. Quantitative real-time PCR (qPCR) analysis was performed using the Rotor-Gene 6000 real-time thermal cycling system (Corbett Research, Mortlake, NSW, Australia) with a QuantiMix SYBR Kit (Phile Korea Technology, Daejeon, Republic of Korea). PCR was performed under the following cycling conditions: 95˚C for 15 s, annealing at 60˚C for 30 s, and extension at 72˚C for 60 s for 40 cycles. The data were analyzed by the comparative threshold cycle (CT) method and normalized against *Gapdh* controls. The primer sequences used were the following: CK2α forward, 5'-CCGAGTTG CTTCCCGATAC-3' CK2α reverse 5'-GGGCTGACAAGGTG CTGAT-3' (Bioneer, Daejeon, Republic of Korea).

Cell viability assays. Cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay, which is based on the conversion of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium to formazan by mitochondrial dehydrogenase. Formazan was quantified by measuring the absorbance of the samples at 570 nm using a microplate reader (BMG Labtech, Ortenberg, Germany).

Propidium iodide/Annexin V analysis. To determine the level of apoptosis, SNU-C5/5FUR cells were stained with propidium iodide (PI) and annexin V-FITC (Thermo Fisher Scientific) and evaluated using a Cyflow Cube 8 kit (Partec, Münster, Germany). Data were analyzed using standard FSC Express software (De Novo Software, Los Angeles, CA, USA).

Cell-cycle analysis. The cells were harvested and fixed with 70% ethanol at −20˚C for 2 h. After two washes with cold PBS, the cells were subsequently incubated with RNase and the DNA-intercalating dye PI (Sysmex) at 4˚C for 1 h. Cell cycle analysis of the PI-stained cells was performed by flow cytometry (Sysmex). Events were recorded for at least 10^4 cells per sample and the data were analyzed using the FCS express 5 software (DeNovo).

CKD4 activity. The cells were lysed with RIPA lysis buffer (Thermo Fisher Scientific). Activity assays of cyclin-dependent kinase 4 (CDK4) were performed using a CDK4 Assay Kit (Cusabio, Baltimore, MD, USA) and a total cell lysate of 30-50 μg. Activation of CDK4 was quantified by measuring absorbance at 450 nm on a microplate reader (BMG labtech, Ortenberg, Germany).

Invasion assay. Invasion of SNU-C5/5FUR cells was assessed using Matrigel-coated transwell cell culture chambers (8 μm pore size; Merck Millipore, Burlington, MA, USA). The SNU-C5/5FUR cells were first collected and resuspended in serum-free RPMI1640, and then 1×10^4 cells were seeded into the chambers of transwell inserts. Serum-containing RPMI1640 (10% FBS) was added in the lower chamber. All samples were incubated for 72 h at 37˚C. In the transwell chambers, cells were fixed with 4% paraformaldehyde (Affymetrix, Santa Clara, CA, USA) in PBS and stained with 2% crystal violet in 2% ethanol; non-invasive cells were eliminated using a cotton swab. The lower surface of the transwell chamber contained invasive cells, which were quantified and photographed using a light microscope. Microscopy images were obtained at 100× magnification, and the number of invasive cells was determined in 6 random fields; the experiment was repeated four times.

Spheroid culture. Spheroids were generated by growing SNU-C5/5FUR cells in suspension using ultra-low attachment six-well plates (Sigma-Aldrich) for spheroid generation. The SNU-C5/5FUR cells were cultured in growth media and incubated at 37˚C under a 5% CO_2 atmosphere. Spheroids were formed at day seven and identified and measured using a visual inspection microscope (Olympus, Tokyo, Japan).

Statistical analysis. The results were expressed as the mean±standard error of the mean (SEM). One-way analysis of variance, followed by Tukey's *post-hoc* test, was used for multiple comparisons. A p-value of <0.05 was considered statistically significant.

Results

CK2α is associated with 5FU resistance in CRC cells. To examine the expression of CK2α at the cellular level, we assessed the expression of CK2α in the 5FU-resistant CRC cell line (SNU-C5/5FUR) and the wild-type CRC cell line (SNU-C5/WT) using qPCR and western blot analyses. At both the mRNA and protein levels, the expression of CK2α was significantly higher in 5FU-resistant CRC cells than in WT cells (Figure 1A-C), suggesting that an increased level of CK2α may be involved in the mechanism of 5FU-resistance in CRC cells.

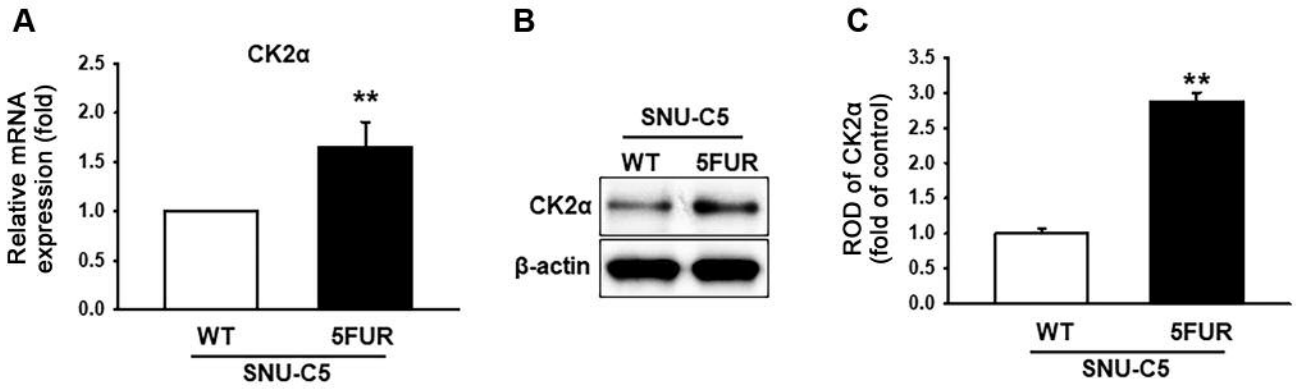

Figure 1. *Expression of CK2α is increased in CRC tissues and 5FU-resistant CRC cells. (A) mRNA expression of CK2α in the SNU-C5/WT and SNU-C5/5FUR cells (n=3). The values represent the means±SEM. **p<0.01 vs. SNU-C5/WT. (B) Western blot analysis of CK2α in the SNU-C5/WT and SNU-C5/5FUR cells. (C) Quantification of CK2α protein expression levels (n=3). The values represent the means±SEM. **p<0.01 vs. SNU-C5/WT.*

Knockdown of CK2α increases the anti-cancer drug sensitivity of 5FU-resistant CRC cells. To examine whether CK2α is associated with resistance of SNU-C5/5FUR cells to 5FU, the viability of SNU-C5/5FUR cells, with or without siRNA silencing of *CK2α*, was assessed after 5FU treatment. The 5FU was administered at concentrations below the half-maximal inhibitory concentration (IC_{50}) of 184 μM, which was determined in a previous study (11). Compared to those without CK2α inhibition, the cells in which CK2α was inhibited showed significantly lower levels of viability when administered with the same doses of 5FU (Figure 2A). On the contrary, the viability of SNU-C5/5FUR cells did not change significantly with CK2α inhibition when 5FU was not administered (Figure 2A). In addition, CK2α inhibition increased the rate of cells undergoing apoptosis by ≥50% at the same concentrations of 5FU (Figure 2B and 2C). Consistent with these results, western blot analysis demonstrated that CK2α inhibition significantly increased the expression of pro-apoptotic proteins BAX, cleaved caspase-3 (Cl-Caspase3), and cleaved PARP-1 (Cl-PARP-1) (Figure 2D-F). These findings further support the association between CK2α and CRC resistance to 5FU and suggest that CK2α inhibition may be useful for increasing the susceptibility of 5FU-resistant CRC cells to the anti-cancer effects of 5FU.

CK2α suppresses 5FU-induced ER stress in 5FU-resistant CRC cells. To investigate whether CK2α regulates the ER stress response in SNU-C5/5FUR cells, we assessed the activation and expression of the ER stress markers PERK, eIF2α, ATF4, IRE1α, JNK, p38, and CHOP after treatment with various concentrations of 5FU. At the same doses of 5FU administration, the phosphorylation of PERK and eIF2α and the expression of ATF4 were significantly increased in the CK2α knockdown SNU-C5/5FUR cells compared to the control cells (Figure 3A-C). In addition, CK2α inhibition significantly increased the phosphorylation of IRE1α, JNK, and p38, and the expression of CHOP (Figure 3D-G). These results suggest that CK2α inhibition hyperactivates ER stress response signaling in the 5FU-resistant CRC cells after treatment with 5FU.

CK2α inhibition suppresses proliferation of 5FU-resistant CRC cells. To investigate the effect of CK2α inhibition on 5FU-resistant CRC cell proliferation, CDK4 assay and cell-cycle analysis were conducted. When the expression of CK2α was not inhibited, the CDK4 activity did not change significantly after administration of 5FU (Figure 4A). However, after CK2α silencing, CDK4 activity was significantly decreased during treatment with 5FU (Figure 4A). In addition, cell-cycle analysis revealed that knockdown of CK2α assisted the interference of 5FU with cell-cycle progression, as exhibited by the reduced percentages of CK2α-inhibited cells in S phase compared to the CK2α-intact group at the same levels of 5FU administration (Figure 4B and C). These results indicate that co-administration of CK2α inhibition and 5FU is significantly more effective in suppressing the proliferation of 5FU-resistant CRC cells than the administration of 5FU alone.

CK2α is involved in 5FU-resistant CRC cell invasion and spheroid formation. The involvement of CK2α in the cell invasion and spheroid formation capacity of 5FU-resistant CRC cells was assessed after knockdown of CK2α in SNU-C5/5FUR cells. Silencing of CK2α alone drastically inhibited the invasion of 5FU-resistant CRC cells, and this effect persisted at different concentrations of 5FU (Figure 5A and B). This shows that the expression of CK2α is positively

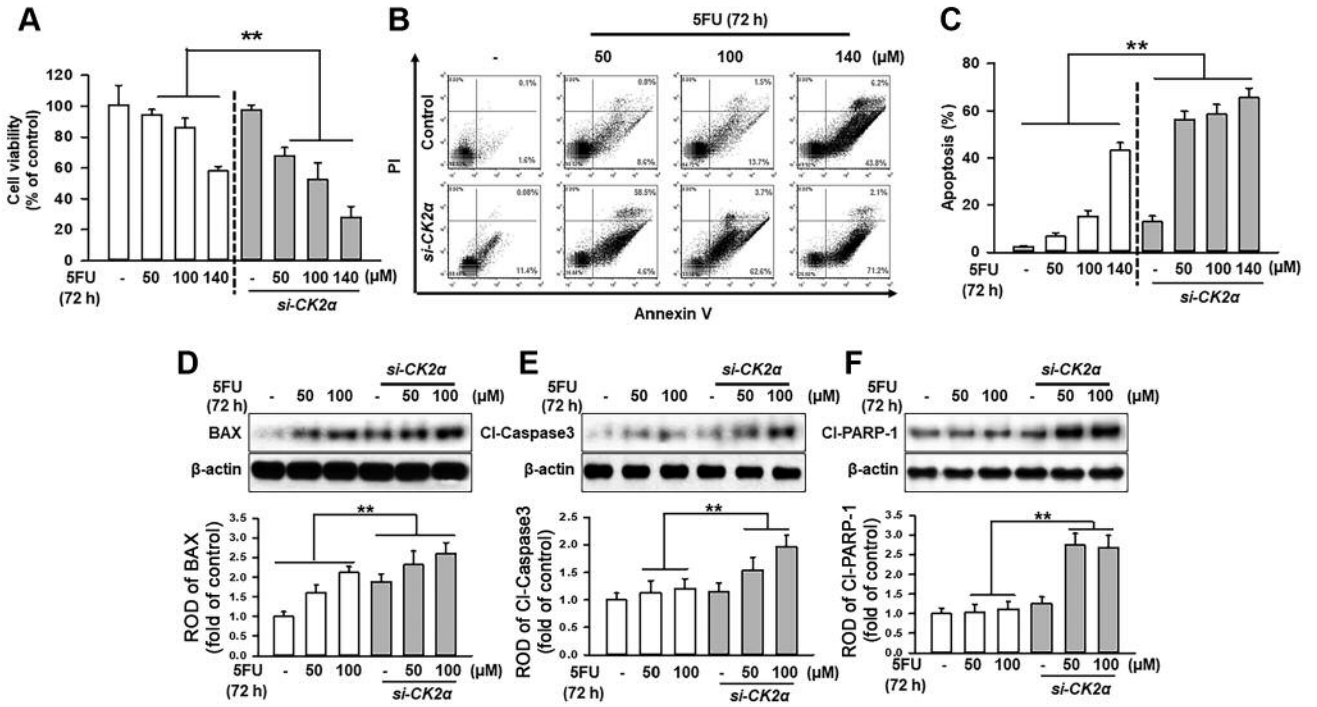

Figure 2. *CK2α protects 5FU-resistant CRC cells against 5FU-mediated apoptosis. (A) Cell viability analysis of control SNU-C5/5FUR cells (SNU-C5/5FUR) and CK2α-knockdown SNU-C5/5FUR cells (si-CK2α-treated SNU-C5/5FUR) after treatment with 5FU (0, 50, 100, and 140 μM) for 72 h (n=3). Values represent mean±SEM. **p<0.01 vs. SNU-C5/5FUR. (B) Flow cytometry analysis of PI and Annexin V staining in SNU-C5/5FUR and si-CK2α-treated SNU-C5/5FUR after treatment with 5FU (0, 50, 100, and 140 μM) for 72 h (n=5). (C) Percentage of apoptotic cells in SNU-C5/5FUR and si-CK2α-treated SNU-C5/5FUR after treatment with 5FU (0, 50, 100, and 140 μM) for 72 h (n=5). Values represent mean±SEM. **p<0.01 vs. SNU-C5/5FUR. (D-E) Western blot analysis of Bax (D), cleaved caspase-3 (Cl-Caspase 3; E), cleaved-PARP-1 (Cl-PARP-1; F) in SNU-C5/5FUR and si-CK2α SNU-C5/5FUR after treatment with 5FU (0, 50, and 100 μM) for 72 h. Quantification of protein expression levels (n=3). The values represent the means±SEM. **p<0.01 vs. SNU-C5/5FUR.*

associated with the invasion ability of 5FU-resistant CRC cells. In addition, CK2α inhibition in combination with 5FU treatment significantly suppressed the spheroid formation ability of 5FU-resistant CRC cells (Figure 5C). The 5FU-resistant cells formed a significantly reduced number of spheroids, which were smaller in size, when co-administered with CK2α inhibition and 5FU compared to 5FU treatment alone (Figure 5D and E). These results suggest that the reduced metastatic and tumorigenic capacities elicited by CK2α inhibition may drastically improve the susceptibility of 5FU-resistant CRC cells to the anti-cancer effects of 5FU.

Discussion

In this study, we demonstrated that high levels of CK2α promoted cancer cell survival following exposure to the anti-cancer drug 5FU *via* modulation of the ER stress response and apoptotic signaling pathways. Previous studies have shown that CK2α is important for cancer cell proliferation, ER stress response, and anti-cancer drug-resistance of malignant cells (10, 12-14). Increased levels of CK2 in tumors affect cancer cell proliferation, as well as tumor progression (12). In addition, suppression of CK2 triggers cancer cell apoptosis through activation of ER stress (10). In particular, overexpression of CK2α shows significant resistance to anti-cancer drug-induced apoptosis in cancer cells (13). Our results showed that treatment of 5FU-resistant SNU-C5 cells with 5FU in combination with CK2α inhibition drastically up-regulated the ER stress response and the induction of apoptosis in these cells. Moreover, CK2α inhibition reduced the invasion and spheroid formation capacities of 5FU-resistant SNU-C5 cells.

We observed an increased expression of CK2α in 5FU-resistant SNU-C5 cells, indicating that the augmented expression of CK2α is associated with 5FU resistance in these cells. A previous study demonstrated that CK2 inhibition resulted in up-regulated apoptosis events in cancer cells (10). In our study, we confirmed that CK2α inhibition leads to decreased viability and increased apoptosis of 5FU-resistant CRC cells at concentrations of 5FU well below the

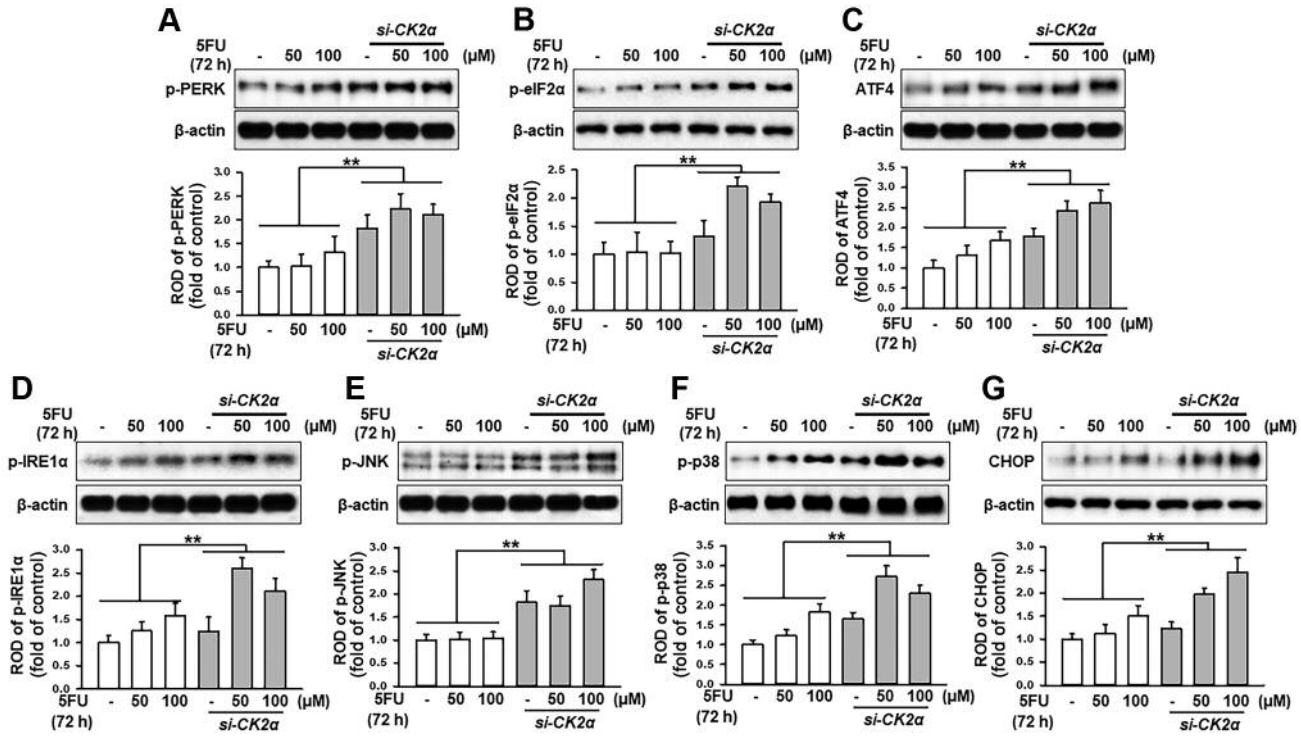

Figure 3. *CK2α suppresses the 5FU-mediated ER stress response. (A-G) Western blot analysis of p-PERK (A), p-eIF2α (B), ATF4 (C), p-IRE1α (D), p-JNK (E), p-p38 (F) and CHOP (G) in SNU-C5/5FUR and si-CK2α-treated SNU-C5/5FUR after treatment with 5FU (0, 50, and 100 μM) for 72 h. Quantification of protein expression levels (n=3). The values represent the means±SEM. **p<0.01 vs. SNU-C5/5FUR.*

IC_{50} of 184 μM (11). These results indicate that CK2α inhibition mediates the increased apoptosis observed in 5FU-resistant CRC cells treated with 5FU, suggesting that CK2α inhibition may be a promising combination therapy with chemotherapy for improving patient outcomes.

Increasing evidence has demonstrated that CK2α plays an important role in the ER stress response of cancer cells (15, 16). It is known that CK2 and its substrates localize to the ER. The transcription factor CHOP, which is a substrate of CK2, is involved in ER stress-induced apoptosis (17). Phosphorylation of CHOP by CK2 suppresses its transcriptional activity, resulting in inhibition of apoptosis (18). Moreover, the transcription and stability of ATF4, an essential component of the ER stress signaling, is directly regulated by CK2α (15, 19). The ATF4 pathway allows cell adaptation to stress conditions and is initiated by stress-activated protein kinases, such as PERK, which phosphorylate eIF2α leading to a general inhibition of protein synthesis but selective translation of ATF4 (20, 21). The sustained ATF4/CHOP signaling is responsible for initiation of the apoptotic signaling cascade (22). A previous study showed that CK2α inhibition leads to induction of ER stress and activation of PERK and eIF2α (23). Consistent with these findings, we confirmed that CK2α inhibition induced ER stress in response to 5FU treatment and that the combination of CK2α inhibition with 5FU administration in 5FU-resistant CRC cells increased the expression of ATF4 and CHOP in a dose-dependent manner. ER stress-induced apoptosis was mediated by the increased expression of apoptosis-associated proteins BAX, Cl-Caspase3, and Cl-PARP-1, and the reduced viability of 5FU-resistant CRC cells. The co-administration of 5FU with CK2α inhibition markedly increased the concomitant induction of apoptosis in 5FU-resistant CRC cells. These results suggest that CK2α inhibition increased the sensitivity of 5FU in drug-resistant CRC cells by inducing ER stress.

Drug-resistant cancer cells display high cell proliferation and tumorigenic potential during chemotherapy (11, 24, 25). In addition, drug-resistant cancer cells exhibit greater invasiveness and metastatic ability compared to their non-resistant counterparts, and are partly responsible for rendering the anti-cancer therapy ineffective (26). A phase I study has shown that inhibition of CK2 by a CK2 peptide-based inhibitor resulted in a beneficial clinical outcome in patients with cervical malignancies (27). Phase II clinical trials have also shown positive clinical results, indicating that the inhibition of CK2 could be tolerated in humans (28). We observed that CK2α inhibition significantly inhibited the

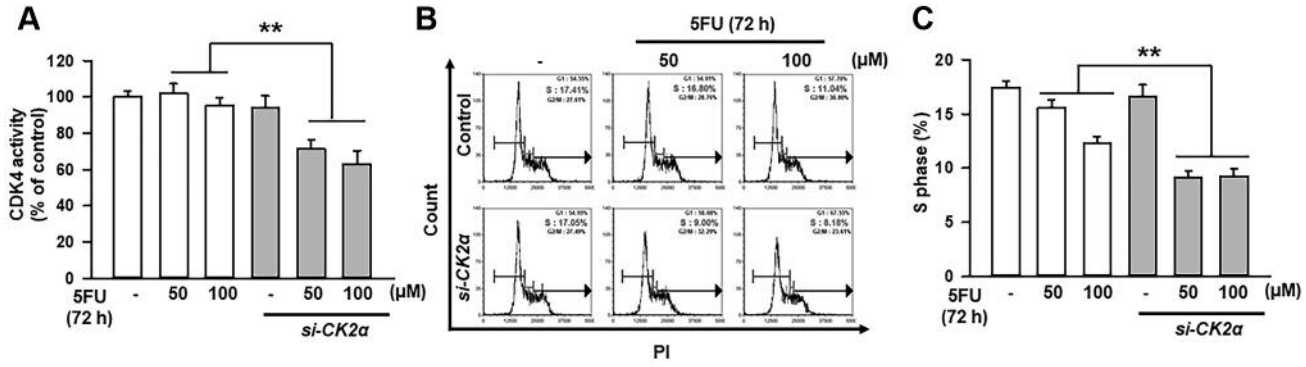

Figure 4. *CK2α maintains cell cycle progression in 5FU-resistant CRC cells under 5FU treatment. (A) Assessment of CDK4 activity in SNU-C5/5FUR or si-CK2α-treated SNU-C5/5FUR after treatment with 5FU (0, 50, and 100 μM) for 72 h (n=3). Values represent mean±SEM. **p<0.01 vs. SNU-C5/5FUR. (B) Flow cytometry analysis of PI staining in SNU-C5/5FUR and si-CK2α-treated SNU-C5/5FUR after treatment with 5FU (0, 50, and 100 μM) for 72 h (n=5). (C) Percentage of S phase in SNU-C5/5FUR or si-CK2α-treated SNU-C5/5FUR after treatment with 5FU (0, 50, and 100 μM) for 72 h (n=5). Values represent mean±SEM. **p<0.01 vs. SNU-C5/5FUR.*

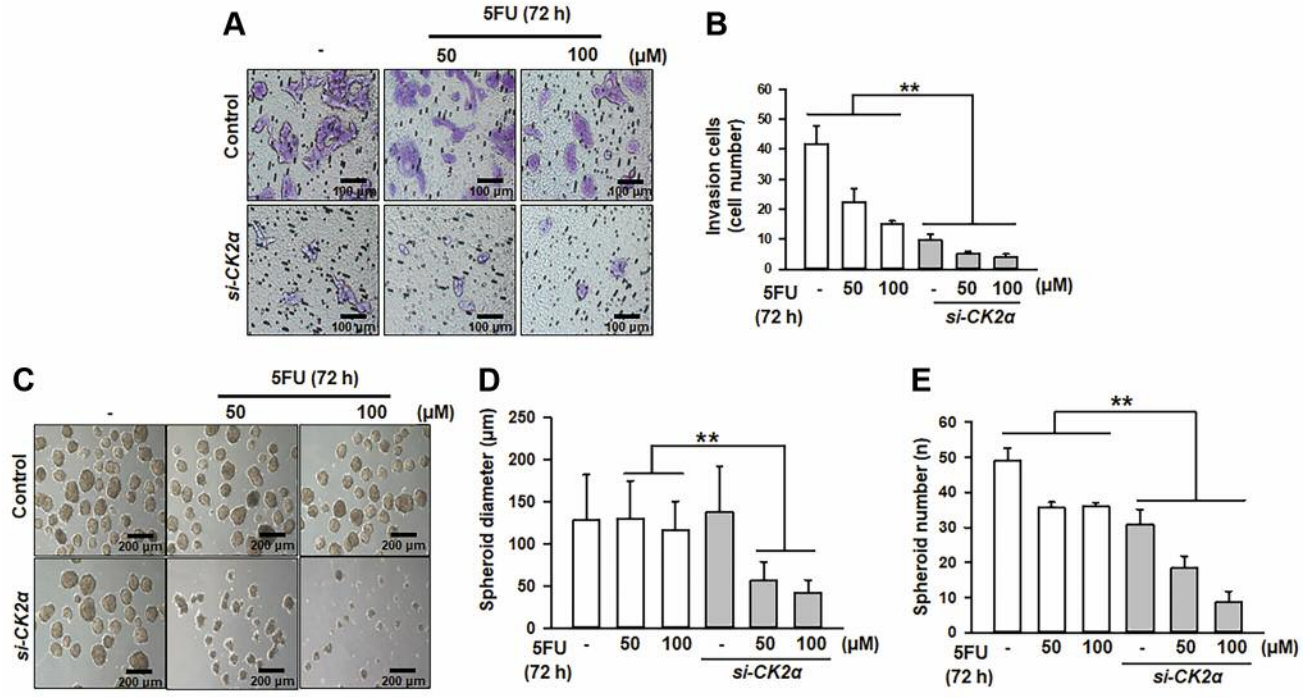

Figure 5. *CK2α plays a critical role in invasion and tumorigenesis of 5FU-resistant CRC cells. (A) Invasion assay of SNU-C5/5FUR or si-CK2α-treated SNU-C5/5FUR cells after treatment with 5FU (0, 50, and 100 μM) for 72 h (n=3). (B) Number of invasive cells (n=3). Values represent mean±SEM. **p<0.01 vs. SNU-C5/5FUR. (C) Spheroid formation assay of SNU-C5/5FUR or si-CK2α-treated SNU-C5/5FUR cells. For each group, 500 cells were seeded per well in a 6-well culture plate. Representative samples of tumor spheroids formed 10 days after plating from four independent experiments are shown (n=3). (D) Spheroid diameter (n=3). Values represent mean±SEM. **p<0.01 vs. SNU-C5/5FUR. (E) Spheroid number (n=3). Values represent mean±SEM. **p<0.01 vs. SNU-C5/5FUR.*

proliferation, invasion, and spheroid formation capacities of 5FU-resistant CRC cells. It is important to note that the co-administration of CK2a with 5FU exerted marked anti-proliferative effects in 5FU-resistant CRC cells, while CK2α inhibition alone did not alter the proliferation rate of these cells. Similarly, the combination of CK2α inhibition with 5FU administration suppressed the invasion and spheroid formation of 5FU-resistant CRC cells, indicating that

combining CK2 inhibition with 5FU treatment is much more effective than either treatment alone. These results further confirm that inhibition of CK2α significantly potentiates the anti-cancer effects of 5FU.

Conclusion

Taken together, our results reveal that CK2α contributes to the resistance of CRC cells to 5FU therapy through regulation of the ER stress response and maintenance of the invasive and tumorigenic capacities of cancer cells. Our results showed that CK2α inhibition is involved in 5FU-mediated ER stress, leading to up-regulation of apoptosis and reduced proliferation of 5FU-resistant CRC cells. In addition, inhibition of CK2α level significantly reduced the cell invasion and spheroid formation capacities of the 5FU-resistant CRC cells. These findings suggest that a combination of CK2α inhibition with 5FU administration may be a powerful therapeutic strategy for patients with CRC.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

Hyung Joo Kim: data collection and drafting of manuscript; Yong-Seok Han and Jun Hee Lee: data analysis and drafting of manuscript; Sang Hun Lee: organizing the structure of the manuscript, drafting, and editing of the manuscript, procurement of funding.

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