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Effects of 5-Fluorouracil on Gene Expression and Clonogenesis of Gastric Cancer Stem-Like Cells

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ABSTRACT

Background: Gastric cancer (GC) is a leading cause of cancer-related mortality worldwide, and chemoresistance remains a major therapeutic challenge. Cancer stem cells (CSCs) contribute to tumor recurrence, therapeutic resistance, and disease progression. This study investigated the effects of 5-fluorouracil (5-FU) on GC stem-like cells (GCSCs) derived from MKN-45 cells, focusing on cell viability, clonogenic potential, and stemness-associated gene expression.

Methods: GCSCs were derived from MKN-45 cells under non-adherent culture conditions. Sphere formation and soft agar assays were used to assess stem-like characteristics of the cells. The cells were treated with increasing concentrations of 5-FU, and cell viability was evaluated using the Trypan Blue exclusion assay. Clonogenicity was assessed through a colony formation assay, while the expression levels of *OCT3/4*, *NANOG*, *SOX2*, *KLF4*, and *CD44* were quantified by quantitative real-time PCR.

Results: MKN-45-derived GCSCs exhibited enhanced sphere- and colony-forming capacity compared to the parental cells. Treatment with 5-FU reduced cell viability in a concentration-dependent manner, with an IC_{50} of approximately 73 µM after 24 h, and markedly suppressed colony formation. The treatment significantly downregulated *OCT3/4* (0.25-fold), *NANOG* (0.17-fold), and *CD44* (0.54-fold), while upregulating *SOX2* (5.3-fold) and *KLF4* (2.1-fold).

Conclusion: 5-FU reduces GCSC viability and clonogenic potential while inducing heterogeneous changes in stemness-associated gene expression. These findings suggest that chemotherapeutic stress triggers transcriptional adaptation in surviving GCSC populations, potentially contributing to residual drug resistance. Understanding these responses could support the development of combination strategies targeting GCSCs.
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Keywords: Fluorouracil, Gene expression, Neoplastic stem cells, Stomach neoplasms

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1. INTRODUCTION

Currently, gastric cancer (GC) remains one of the most prevalent and lethal malignancies globally[1]. Despite significant progress in its diagnostics and therapeutic strategies, the incidence rate and mortality continue to be high worldwide[2]. In 2022, there were an estimated 968,350 newly diagnosed cases of GC, accounting for around 659,853 deaths[2]. Although various diagnostic

techniques and therapeutic strategies have been recently developed, the clinical outcomes of GC patients are still dismal. This situation might be attributed to the highly malignant biological behaviors of gastric tumors, such as detection at late stages and chemoresistance to anticancer agents[3]. Chemotherapy is currently one of the pivotal therapeutic options for treating patients suffering from GC.

The 5-fluorouracil (5-FU)-based regimen alone or combined with other anticancer drugs is considered the gold standard strategy for this malignancy over previous decades[3,4]. However, despite initial responses from most patients to this therapy, relapse and chemoresistance develop frequently during the treatment course, which leads to tumor progression and worse survival outcomes[5]. Until now, numerous studies have investigated the molecular mechanisms behind drug resistance in GCs, but few comprehensive findings on this topic have been achieved[5].

Over recent years, increasing interest has focused on the function of cancer stem cells (CSCs) involved in promoting tumor progression and therapeutic resistance[6,7]. As compared to normal cells, CSCs, representing a unique population of tumor cells, demonstrate greater potential to differentiate into different lineages while maintaining their ability to undergo self-renewal and initiate tumors[7]. Accumulated data have shown that CSCs possess high resistance to chemotherapeutics through activating various cellular defense mechanisms, such as enhancing DNA repair, activating cell survival pathways, resisting apoptosis, and promoting drug efflux via transporter upregulation[6,8]. Non-adherent sphere cultures are frequently applied to increase the frequency of CSC populations while evaluating their functionally relevant attributes, such as spheroid or colony-forming abilities, which are considered crucial hallmark characteristics of CSCs[9]. The expression levels of specific stemness-related transcription factors (e.g., *OCT3/4*, *NANOG*, *SOX2*, *KLF4*) and/or surface antigens (e.g., *CD44*) play decisive roles in both determination and maintenance of the stem-like features of CSCs[10]. Dysregulated expression of these genes is correlated with heightened tumor malignancy, chemoresistance, and relapsing events[10]. Previously, several reports have established a lesser sensitivity to 5-FU exhibited by gastric stem-like cells (GCSCs) when compared to the bulk of the tumor cell mass, thus delineating a critical role for CSCs in acquiring chemoresistance, which leads to tumor recurrence[11-13]. An earlier study has illustrated that *CD44*-positive CSCs display augmented resistance to various kinds of chemotherapy drugs due to their significantly increased ability to initiate tumors under xenotransplantation conditions[11]. Also, *SOX2* has been shown to play a critical role in controlling the degree of tumorigenesis and resistance to 5-FU in gastric CSCs[12]. As a result, targeting *SOX2*-positive CSC subsets could become a successful strategy for therapy[13].

KLF4, a transcription factor playing an important part in regulating stem cell biology, has lately been indicated to regulate the progression of GCs and affect the prognosis of patients[14-16]. However, the significance of *KLF4* in governing the chemoresponsiveness of GCSCs to 5-FU seems context-dependent and still poorly defined[15,16].

Despite some convincing evidence describing the implication of CSCs in mediating 5-FU resistance in GCs, there is a need for detailed molecular investigations into the integrated effect of 5-FU treatment on the expression of various stemness-related transcriptional factors, including *OCT3/4*, *NANOG*, *SOX2*, *KLF4*, and *CD44*, together with the clonogenic features of the cancer cells[6,17-19]. The results obtained from such an extensive evaluation could shed some light on the molecular mechanisms underlying chemoresistance. To achieve this aim, we obtained GC stem cells from the MKN45 cell line under specific non-adherent growth conditions. The stem-like nature of these isolated cells was verified by their ability to form spheres and colonies. Afterwards, we explored the impact of treatment with 5-FU on cell viability and the expression levels of several stemness-related factors, like *Oct3/4*, *Nanog*, *Sox2*, *KlF4*, and *CD44*. For assessment of cell viability, the Trypan blue test was applied, and the gene expression profile was analyzed via quantitative real-time polymerase chain reaction (qRT-PCR).

2. MATERIALS AND METHODS

2.1. Cell culture of the MKN-45 cell line

The human GC cell line MKN-45 was obtained from the National Cell Bank of Iran (NCBI), affiliated with the Pasteur Institute of Iran, Tehran. The cells were cultured in DMEM/F12 medium (Sigma-Aldrich, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Belgium). The cells were maintained under standard culture conditions in a humidified atmosphere containing 5% CO_2 and 95% humidity at 37°C. The culture medium was replaced twice weekly.

2.2. Enrichment of GCSCs from the MKN-45 cell Line

Following dissociation into a single-cell suspension, MKN-45 cells were seeded at a density of 5×10^4 cells in 100-mm^2 culture plates, previously coated with a thin layer of agarose. The cells were cultured in DMEM/F12 medium supplemented with 10% FBS to enrich spheroid-forming stem-like cells[19]. Fresh spheroid culture medium was added to the cell-containing plates three times per week.

2.3. Assessment of self-renewal and colony-forming capacity using the soft agar assay

Anchorage-independent clonogenic potential of the isolated GCSCs was analyzed through a soft agar colony formation assay. Following cell counting using the Trypan Blue exclusion method, 5×10^3 viable cells/well were used. For the preparation of base agar layers, an equal volume of 1% agarose solution was mixed with complete culture medium. Then 1 mL of the mixture was poured into the wells of a six-well plate and left undisturbed (~45 min) until solidification. To prepare the top layer of agar, we suspended 5×10^3 cells in 500 µL of complete culture medium (with 10% FBS) and then mixed it with 500 µl of 0.7% agarose. This mixture was overlaid on the top of the previously set agar base. After solidification, 1 mL of complete culture medium supplemented with 10% FBS and 1% penicillin-streptomycin antibiotic was added to each well. Finally, parental MKN45 cells maintained under similar conditions were regarded as the control group. All colonies were kept in a humidified atmosphere at 5% CO_2 at 37°C. After 14 days of growth, the culture medium was renewed twice per week. Colonies having more than 50 cells were considered positive.

2.4. Preparation of 5-FU and cell viability assessment

Stock solutions of 5-FU (Sigma-Aldrich) were prepared by dissolving 5-FU (Sigma-Aldrich, USA) in dimethyl sulfoxide (DMSO), which was then diluted to working concentrations (0-100 µM) using culture media. The final percentage of DMSO used for each treatment group was kept less than 0.1%. Untreated and vehicle controls (containing an equal amount of DMSO) were included in the experiment. Cell viability was measured by the Trypan Blue exclusion method at 24 h post-treatment. Cells were trypsinized and washed twice prior to counting. Treated and control GCSCs were pelleted by centrifugation at 200 ×g for five minutes before being resuspended in culture media. Viability was determined by mixing equal volumes of cell suspensions with 0.4% Trypan blue solution for five minutes at room temperature, followed by loading onto a hemacytometer. After the determination of live/dead cell numbers on a light microscope, cell viability was calculated as the ratio of live cells to the total number of cells. The half-maximal inhibitory concentration (IC_{50}) was determined using non-linear regression of the four-parameter logistic (4PL) equation against cell viability results at the 24 h time point[20].

2.5. Assessment of 5-FU effect on colony formation ability

The effect of the drug on the colony formation ability of GCSCs was assessed after treatment with the IC_{50} concentration of 5-FU using the soft agar colony formation assay. At the end of the incubation period, the culture medium from each well was cautiously removed. Following this step, all the wells were rinsed two times with PBS to remove any leftover medium and debris. Subsequently, the colonies were fixed using methanol at room temperature for 10 min. Then, 5% crystal violet solution was added to each well, and the wells were kept at room temperature for a further 30 min to stain all the colonies formed. Lastly, extra dyes were gently eliminated, and all wells were washed with distilled water. Further, the plates were allowed to fully air-dry at room temperature before being viewed and photographed using an inverted light microscope.

2.6. Primer design and synthesis

Primers targeting *GAPDH*, *OCT3/4*, *NANOG*, *SOX2*, *KLF4*, and *CD44* were designed using Primer-BLAST (NCBI) and Primer3 software based on human reference mRNA sequences obtained from the NCBI database (Table 1). Primer design parameters included a length of 18–24 nucleotides, GC content of 40–60%, and melting temperature of 58–62°C, with expected amplicon sizes of 100–200 bp. Primer specificity was verified using BLAST analysis, and those spanning exon-exon junctions were preferentially selected to avoid genomic DNA amplification. All primers were commercially synthesized and prepared as 10 µM working solutions.

2.7. RNA extraction and qRT-PCR

Total RNA was isolated using a RNeasy Plus Mini kit (Qiagen, Germany) after being treated with 5-FU at the IC_{50} value of 5-Fu for the GCSCs. For each sample, total RNA was isolated according to the manufacturer's instruction manual. Then, we used a NanoDrop spectrophotometer to assess the concentration and purity of the RNA samples. To carry out qRT-PCR analysis, we generated complementary DNA using the PrimeScript™ RT reagent kit (TaKaRa Bio Inc., Japan). After that, the amplification reactions were run using SYBR Premix Ex TaqII (TaKaRa Bio Inc.) and performed in triplicate on a Rotor Gene3000 System (Corbet Research, Australia). Relative expression of the genes related to CSCs such as *Oct3/4*, *Nanog*, *Sox2*, *KLF4*, *CD44*, and *GAPDH* was analyzed by employing the $2^{-\Delta\Delta CT}$ method. *GAPDH* was chosen as the endogenous control because this housekeeping gene is commonly used in GC gene expression studies.

2.8. Statistical analysis

The data shown herein were generated from experiments conducted in triplicate. All statistical calculations have been performed with SPSS Software version 16.0 (SPSS Inc., Chicago, IL, USA). For all

Table 1. Real-time PCR primer sequences

Genes name	Primers	Sequences	Annealing (°C)
OCT3/4	Sense Antisense	5'- GCTTCAGGGTTTCATCCA -3' 5'- GGCGGCAATCATCCTCTG -3'	54
CD44	Sense Antisense	5'- TAACAGTTCCTGCATGGGCGGC -3' 5'- CGTGCAAATTCACCAGAAGGC -3'	53
SOX2	Sense Antisense	5'- CAACATCACAGAGGAAGTAGACTG -3' 5'- CCTTGGCATGAGATGCAGG -3'	54
NANOG	Sense Antisense	5'- AACTCTCCAACATCCTGAACC -3' 5'- GTGGTAGGAAGAGTAAAGGCTG -3'	59
KLF4	Sense Antisense	5'- GAACCCACACAGGTGAGAAAC -3' 5'- TGTGTAAGGCGAGGTGGTC -3'	59
GAPDH	Sense Antisense	5'-ACTCTGGTAAAGTGGATATTGTTGC-3' 5'- GGAAGATGGTGATGGGATTTC -3'	54

experiments, we first used 'Shapiro-Wilk' test to find out whether our sample data follows a normal distribution pattern. If the data did not follow a normal distribution, Student's t-test was used to compare two groups. For comparisons of more than two groups, we performed one-way analysis of variance (ANOVA), followed by Post Hoc tests (Tukey's). *P* value less than 0.05 was considered to be statistically significant.

3. RESULTS

3.1. Formation and expansion of cancer stem-like cell colonies

Single-cell suspensions of the MKN-45 cell line were prepared by gently passing the cells through a 1 mL syringe and subsequently cultured under non-adherent three-dimensional conditions in 25 cm^2 culture flasks. During the first three days of culture, some cells underwent apoptosis, whereas the remaining viable cells proliferated and gradually formed multicellular spherical colonies suspended in the culture medium. By day seven, the resulting colonies appeared as compact and flattened cell aggregates. With prolonged culture, the size and area of these colonies progressively increased. In addition, darker regions became visible in the central areas of the colonies, indicating the presence of apoptotic cells. At this stage, the cellular colonies were dissociated, and the cells were transferred to new culture flasks for further propagation (Fig. 1).

3.2. Confirmation of stem-like properties of the enriched cells via soft agar colony formation assay

Results of this study indicate that when MKN-45 cells were cultured in non-adherent conditions, they gain more ability to grow in a clonal fashion, as from the results illustrated in the soft agar colony formation assay (Fig. 2). These findings confirmed an increased clonogenicity as well as anchorage-independent growth capability of the cells. The data also showed an increased number of colonies with large size, which indicates that these cells acquire stem cell-like properties during the enrichment process (Fig. 2).

3.3. Effect of 5-FU on the viability of GCSCs

The effect of 5-FU on GCSC viability was determined using the Trypan Blue exclusion assay, following treatment for 24 h with increasing concentrations of 5-FU (0–100 μM). There was a progressive decrease in cell viability as the concentrations of 5-FU increased, relative to the untreated control group. Non-linear regression analyses of the dose-response curves utilizing a 4PL model resulted in an estimated IC_{50} of ~73 μM over 24 h of exposure. We observed a significant reduction ($p < 0.05$) in viability when concentrations reached or exceeded the IC_{50} value, showing a clear concentration dependency in the inhibitory action of 5-FU on the viability of GCSCs (Fig. 3).

3.4. Inhibitory effect of 5-FU on colony-forming ability of GCSCs

The effect of 5-FU on the clonogenic capacity of GCSCs was evaluated. In brief, MKN-45-derived GCSCs were seeded onto soft agar and then exposed to 5-FU at the IC_{50} concentration for 14 days. Colony formation was assessed microscopically after crystal violet staining. As shown in Figure 4, treatment with 5-FU significantly reduced the colony-forming ability of GCSCs when compared to the untreated control. Both the number and size of colonies visibly decreased after exposure of the cells to 5-FU. Microscopic observations demonstrated that colonies formed in the 5-FU-treated cells were fewer and smaller than those in the untreated controls.

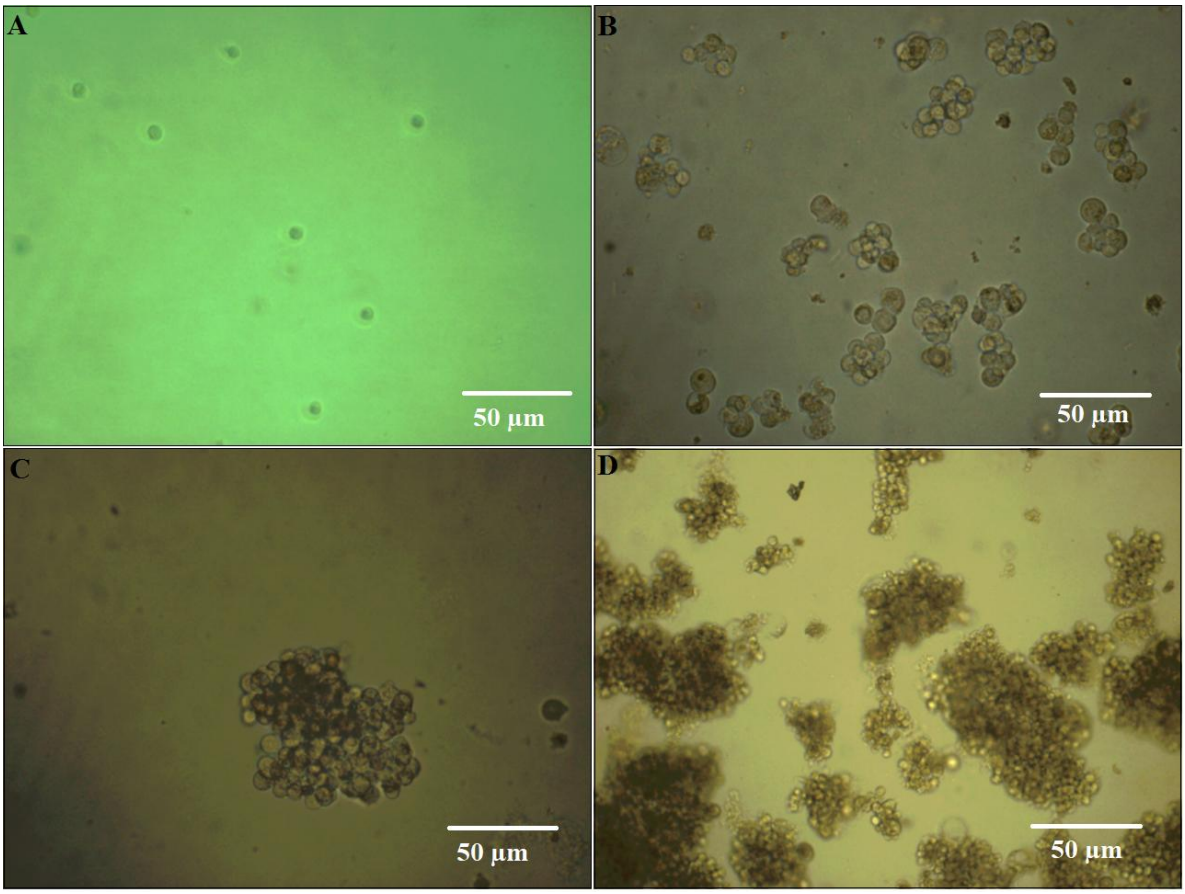


Fig. 1. Formation of GCSC spheroids under non-adherent culture conditions. Representative phase-contrast images of MKN-45-derived GCSCs cultured under non-adherent conditions at days (A) 0, (B) 3, (C) 7, and (D) 14 (scale bar = 50 µm).

Together, these data suggest that 5-FU exerts negative effects on the anchorage-independent growth and clonogenic potential of MKN-45-derived GCSCs under non-adherent culture conditions.

3.5. The effect of 5-FU on the expression of stemness-associated genes in GCSCs

The expression levels of stemness-associated genes in GCSCs were analyzed following treatment with the IC_{50} concentration of 5-FU for 24 h using qRT-PCR. The results demonstrated a differential regulation of stemness-related genes compared to untreated controls. *SOX2* and *KLF4* were significantly upregulated, with fold changes of 5.3 and 2.1, respectively. In contrast, *OCT3/4*, *CD44*, and *NANOG* were downregulated to 0.25-, 0.54-, and 0.17-fold, respectively (Fig. 5).

4. DISCUSSION

Recently, CSCs have gained more attention for being responsible for drug resistance, recurrence, and treatment failure in various cancers, including GC[8,17,21]. Unlike the majority of tumor-cell populations, CSCs exhibit unique abilities such as self-renewal, activation of various survival signaling pathways, and high cellular plasticity, allowing these cells to overcome adverse effects of chemotherapy and disease recurrence[21].

GCSCs were successfully enriched using non-adherent culture conditions similar to previous reports on sphere enrichment methods[22]. This enrichment from the MKN-45 cell line showed enhanced spheroid-forming capabilities and a significant increase in colony-forming units (CFUs) compared to parental cells, suggesting that a self-renewing and anchorage-independent population of GCSCs was successfully isolated.

Our results demonstrated that after exposing the GCSC population to different concentrations of 5-FU, there was a concentration-dependent decline in cell viability, along with a considerable loss in their colony-forming ability. This observation indicates their susceptibility to the anticancer agent, even though they were relatively resistant. The antitumor properties of 5-FU are mainly attributed to its inhibitory effect on the

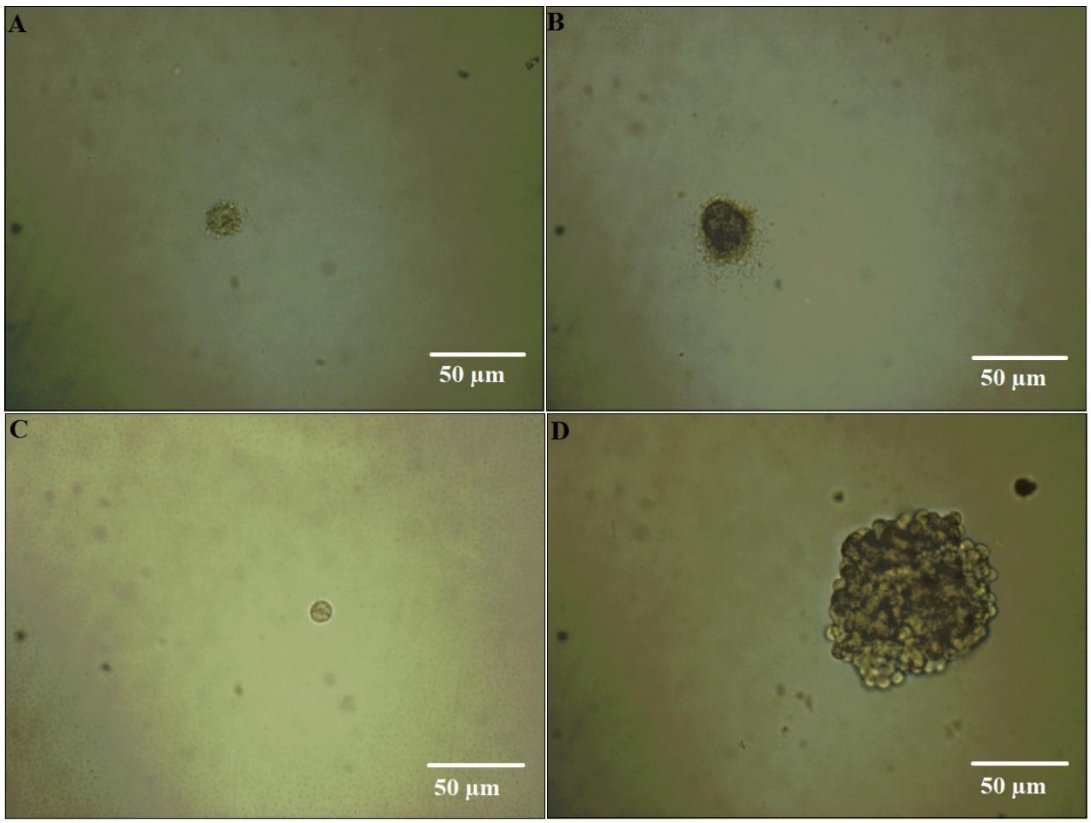


Fig. 2. Soft agar colony formation assay of parental MKN-45 cells and GCSCs. Representative images show colony formation by parental MKN-45 cells at days (A) 0 and (B) 14 and by GCSCs at days (C) 0 and (D) 14 (scale bar = 50 µm).

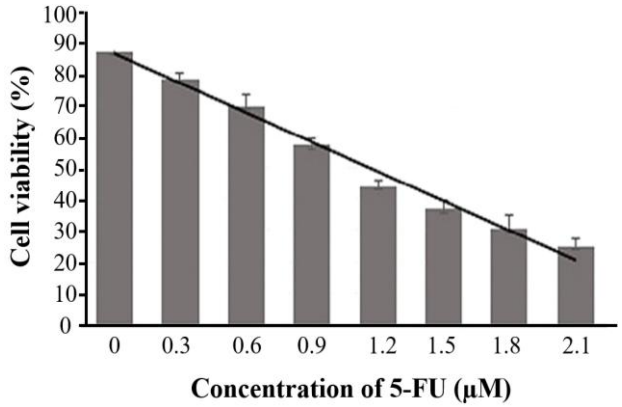


Fig. 3. Dose-response curve of 5-FU in GCSCs. Cell viability was assessed by the Trypan Blue exclusion assay after 24 h of treatment with increasing concentrations of 5-FU (0–100 µM). The line represents nonlinear regression using a 4PL model for estimating the IC_{50}. Data are presented as mean ± SEM from three independent experiments[20].

thymidylate synthase enzyme, which causes the impairment of DNA synthesis. Furthermore, the active metabolite of 5-Fu is involved in the formation of both RNA and DNA, hence disturbing many vital cell activities[23,24].

The calculated concentration yielding an IC_{50} of approximately 73 µM was somewhat higher than the values typically reported for bulk GC cell lines[25,26]. Cancer stem-like cells are resistant to chemotherapy due to increased activity of ATP-binding cassette (ABC) drug efflux transporters, enhanced DNA damage repair, and their relatively quiescent state[8,17]. Therefore, higher drug concentrations may be needed to achieve a similar level of growth inhibition in these cells compared to the bulk tumor cells[27,28]. It is important to note that the maximum concentrations applied in our experiments are several orders of magnitude greater than the clinically achievable plasma levels of 5-FU (~1-5 µM). Therefore, we cannot directly translate our results to therapeutic applications in patients. Nonetheless, the substantial reduction in colony formation after treatment with 5-FU clearly indicates an impairment of both initial survival rate and long-term clonogenicity of these GCSCs.

As previously noted, analysis of the differential expression profiles revealed considerable heterogeneity within the stemness-associated transcriptome upon exposure to 5-FU. This observation can at least partially be explained by the fact that each of these genes

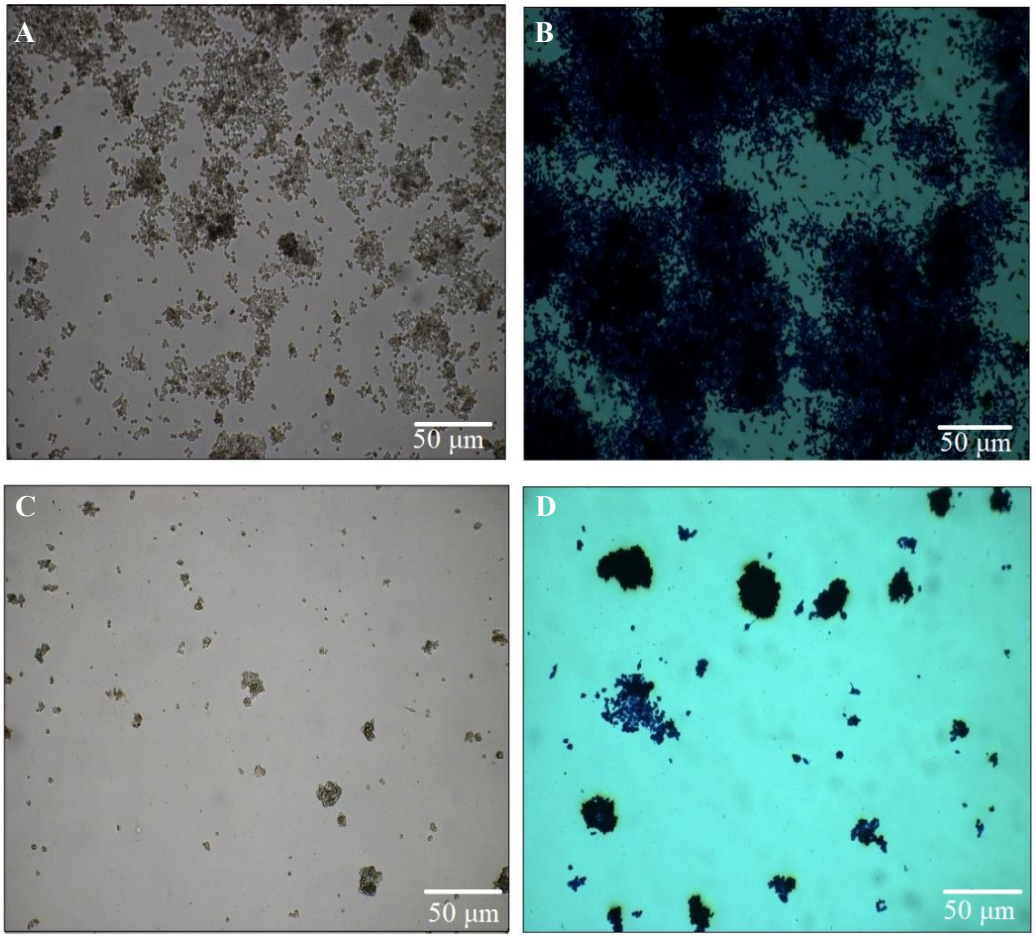


Fig. 4. Effect of 5-FU on colony formation of GCSCs. Representative images of untreated control cells (A and B) and cells treated with the IC_{50} concentration of 5-FU (C and D) in the soft agar colony formation assay following crystal violet staining (scale bar = 50 µm).

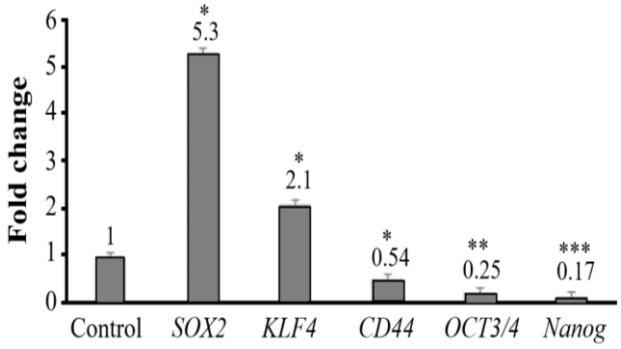


Fig. 5. Relative mRNA expression of stemness-associated genes in GCSCs following treatment with the IC_{50} concentration of 5-FU for 24 h. Expression of *OCT3/4*, *CD44*, *SOX2*, *NANOG*, and *KLF4* was quantified by qRT-PCR using the $2^{-\Delta\Delta Ct}$ method and normalized to *GAPDH*. Data are presented as mean ± SEM from three independent experiments ($^{*}p < 0.05$ versus untreated control).

represents a central regulator of distinct biological processes. On one hand, *OCT3/4*, *SOX2*, and *NANOG* form a core transcriptional network that regulates the maintenance of pluripotency and stem cell identity[29-31]. On the other hand, *KLF4* acts as a context-dependent transcription factor controlling several aspects of stem cell maintenance, differentiation, and cellular stress responses[14,15,32]. On the contrary, *CD44* acts mainly as an adhesive molecule, mediating cell-cell and cell-extracellular matrix interactions through different ligands while regulating cell migration and providing signals for cell survival[33].

In our experiments, we found significant downregulation of *OCT3/4* and *NANOG* transcripts after 5-FU administration. Increased levels of these transcription factors have shown correlation with enhanced tumorigenicity, self-renewal capability, and metastatic potential, along with decreased survival rates in GC patients[32]. Hence, downregulation of *OCT3/4* and *NANOG* may suggest some sort of partial suppression of the transcriptional program responsible for certain stem-like features in these cells. Only mRNA level changes were monitored; thus, we lack evidence for any actual functional loss of stemness at this time.

Decreased *CD44* expression may reflect an impairment in the related survival-signaling cascade in the left-over cell fraction, paralleling the dramatic reduction in clonogenic ability observed after 5-FU treatment. Indeed, *CD44* is a well-recognized marker of GC stem cells whose activity is associated with extracellular matrix interactions, activation of the PI3K/Akt pathway, evasion of apoptotic death, and maintenance of tumor-initiating capacity[11,32]. Moreover, several clinical investigations have demonstrated an association between elevated *CD44* levels and increased tumor recurrence rates, progression in staging, and incomplete efficiency of therapeutic outcomes in GC cases[34,35]. It should be noted that, unlike *OCT3/4* and *NANOG*, the transcript abundance of *SOX2* elevated more than five-fold due to 5-FU application. These non-uniform regulatory outcomes under similar conditions have already been documented in other reports addressing CSCs in response to pharmacological agents[10,17]. Additionally, it likely implies the dynamic and changeable nature of these cells instead of uniform gene expression control over all stemness-related genes. In addition to preserving stem cell status, *SOX2* is also implicated in developmental signal transduction processes, including Wnt/beta-catenin, Hedgehog, and Notch pathways, known to keep the CSC phenotype and resistance to anti-tumor treatments[36-38]. Previous studies have indicated that overexpression of *SOX2* confers the increased resistance to 5-FU in GC cells and helps preserve their stem-like properties[12,13,39]. Thus, the substantial upregulation of *SOX2* expression could suggest a transcriptional response related to drug resistance/stress adaptation. However, owing to limitations in assessing the function of *SOX2* (as we measured only mRNA expression), our results cannot demonstrate whether *SOX2* mediates the survival of residual cells after treatment with these agents. Further investigations are warranted to define the exact mechanism by which *SOX2* contributes to drug resistance in colorectal cancers.

The transcription factor *KLF4* was significantly upregulated after exposure to 5-FU and/or oxaliplatin. As one of the Yamanaka transcription factors, *KLF4* exerts various anti-oncogenic effects in many tumors, but it can simultaneously promote stem-like traits under specific conditions[14]. Beyond playing important roles in embryonic stem cell differentiation and maintenance, *KLF4* participates in multiple regulatory networks that include diverse physiological processes such as DNA damage response, cell cycle arrest, and stress adaptation, via its interactions with different signaling pathways like STAT3, tumor necrosis factor-β, and Wnt[40,41]. Hence, its elevated expression might reflect an adaptive transcriptional reaction to cellular stress induced by 5-FU without indicating increased stemness per se. Considering all the above points about *KLF4* and given that we assessed transcriptional events at just one time-point in this study, future studies should focus on precisely defining the functional impact of *KLF4* upregulation in colorectal cancer cells.

Our results showed that 5-FU induced a differential rather than uniform molecular response in GCSCs. While the expression of the pluripotency-associated transcription factors *OCT3/4* and *NANOG*, as well as the functional CSC marker *CD44*, is downregulated by this agent, we observed an upregulation of both *SOX2* and *KLF4* expression. Interestingly, a similar non-uniform pattern of stemness-associated gene expression has been reported in CSCs exposed to pharmacological agents. Indeed, despite reduced clonogenicity and viability, these cells exhibited simultaneous activation of distinct stress-responsive transcriptional programs, suggesting that a certain degree of cellular plasticity exists within this particular subpopulation[10,17,42]. Our findings could thus represent just one aspect of a heterogeneous transcriptional response to 5-FU. However, to circumvent such heterogeneity, suppression of all genes involved in maintaining the stem-like phenotype would presumably occur. As shown in the results, even if some of the mentioned genes remain active, certain ones are modulated at least at the transcriptome level following chemotherapy.

In summary, while many interesting points have emerged during this investigation, the current work presents various limitations. First, molecular changes were analyzed only at the transcriptome level, and protein levels were not validated accordingly. Second, analysis of gene expression was performed only once on the cells cultured for exactly 24 hours after treatment with 5-FU. Third, the stability of the reference gene (*GAPDH*) following treatment with 5-FU has not been confirmed. Finally, the assessment of cell viability only relied on the Trypan Blue exclusion assay, which merely estimates membrane integrity and does not discriminate whether the cells underwent apoptosis or another form of cell death. Lastly, we tested this hypothesis using a unique set of stem-like GC cells derived from one cell line, MKN-45. Incorporating analyses at the protein level as well as testing more models of gastric cancers would help strengthen and expand the conclusions drawn from the present study.

5. CONCLUSION

Our findings reveal that treatment with 5-FU reduces the cell viability and clonogenic potential of MKN-45-derived gastric CSCs, resulting in a concomitant heterogeneity in the expression of certain key stemness-related factors. Specifically, the data indicate that the decreased levels of *OCT3/4*, *NANOG*, and *CD44* genes are counterbalanced by upregulated expression of both

SOX2 and *KLF4* following administration. While there was no direct correlation between the specific alterations in gene expression observed herein and any particular functional consequence, our findings are consistent with those provided previously. This observation suggests that at least some degrees of molecular flexibility are retained by cancer stem-like cells even when confronted with chemotherapy. In keeping with this hypothesis, we believe that further investigations could help clarify how targeting adaptive signaling pathways simultaneously might enhance therapeutic effectiveness against gastric CSC populations. Our data can be helpful in enhancing our comprehension of the role of CSCs in chemotherapy resistance and will pave the way towards developing much more efficient therapeutic modalities against GC.

DECLARATION

Acknowledgments

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Generative AI and AI-assisted technologies

The authors declare that no AI tools were used in the preparation of this manuscript.

Ethical approval

This study was conducted based on the Ethics Committee Guidelines of Shiraz University of Medical Sciences, Shiraz, Iran (ethical code IR.SUMS.REC.1401.118).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Authors' contributions

YN: conducted experimental work, contributed to writing the manuscript; NO: contributed to quantitative PCR; FM: wrote the manuscript and contributed to analyzing the results; RE: contributed to writing the manuscript; SAS: contributed to cell culture experiments; MZ: contributed to cell viability assessment and quantitative PCR; HA: designed, directed the investigation, analyzed the results. All authors contributed to revising the manuscript.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version does not contain supplementary material.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209-49.
2. Patel AK, Sethi NS, Park H. Gastric cancer: A review. JAMA. 2026;335(5):439-50.
3. Smyth EC, Nilsson M, Grabsch HI, Van Grieken NC, Lordick F. Gastric cancer. Lancet. 2020;396(10251):635-48.
4. Joshi SS, Badgwell BD. Current treatment and recent progress in gastric cancer. CA Cancer J Clin. 2021;71(3):264-79.
5. Ilie V-C, Minciuna C-E, Andras C, Turcu SN, Herlea V, Dima SO, et al. Mechanisms of chemoresistance in gastric cancer: Interplay between microRNAs and the tumor microenvironment. Clin Transl Oncol. 2026;28(10):4328-45.
6. Lee H, Kim B, Park J, Park S, Yoo G, Yum S, et al. Cancer stem cells: Landscape, challenges and emerging therapeutic innovations. Sig Transduct Target Ther.2025;10(1):248.
7. Mahmoodi F, Akrami H. PlGF knockdown decreases tumorigenicity and stemness properties of spheroid body cells derived from gastric cancer cells. J Cell Biochem. 2017;118(4):851-9.
8. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer. 2005;5(4):275-84.
9. Weiswald LB, Bellet D, Dangles-Marie V. Spherical cancer models in tumor biology. Neoplasia. 2015;17(1):1-15.
10. Batlle E, Clevers H. Cancer stem cells revisited. Nat Med. 2017;23(10):1124-34.
11. Takaishi S, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, et al. Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells. 2009;27(5):1006-20.
12. Tian T, Zhang Y, Wang S, Zhou J, Xu S. Sox2 enhances the tumorigenicity and chemoresistance of cancer stem-

like cells derived from gastric cancer. J Biomed Res. 2012;26(5):336-45.
13. Pádua D, Barros R, Amaral AL, Mesquita P, Freire AF, Sousa M, et al. A SOX2 reporter system identifies gastric cancer stem-like cells sensitive to monensin. Cancers (Basel). 2020;12(2):495.
14. Rowland BD, Peeper DS. KLF4, p21 and context-dependent opposing forces in cancer. Nat Rev Cancer. 2006;6(1):11-23.
15. Zeng L, Zhu Y, Moreno CS, Wan Y. New insights into KLFs and SOXs in cancer pathogenesis, stemness, and therapy. Semin Cancer Biol. 2023:90:29-44.
16. Hashimoto I, Nagata T, Sekine S, Moriyama M, Shibuya K, Hojo S, et al. Prognostic significance of KLF4 expression in gastric cancer. Oncol Lett. 2017;13(2):819-26.
17. Phi LTH, Sari IN, Yang Y-G, Lee S-H, Jun N, Kim KS, et al. Cancer stem cells (CSCs) in drug resistance and their therapeutic implications in cancer treatment. Stem Cells Int. 2018:2018:5416923.
18. Longley DB, Harkin DP, Johnston PG. 5-Fluorouracil: Mechanisms of action and clinical strategies. Nat Rev Cancer. 2003;3(5):330-8.
19. Akrami H, Shamsdin SA, Nikmanesh Y, Fattahi M. Effect of mir-4270 inhibitor and mimic on viability and stemness in gastric cancer stem-like cells derived from MKN-45 cell line. Iran Biomed J. 2023;27(2-3):100-7.
20. Setter W, Schmiade P, Schmidt T, Jed G. PB2528 comparison of different SYK-inhibitors in platelet-rich plasma. Res Pract Thromb Haemost. 2026; 10:105268.
21. Fu L, Bu L, Yasuda T, Koiwa M, Akiyama T, Uchihara T, et al. Gastric cancer stem cells: Current insights into the immune microenvironment and therapeutic targets. Biomedicines. 2020;8(1):7.
22. Chen SF, Chang YC, Nieh S, Liu CL, Yang CY, Lin YS. Nonadhesive culture system as a model of rapid sphere formation with cancer stem cell properties. Plos One. 2012;7(2):31864.
23. Peters GJ, Van Der Wilt CL, Van Groeningen CJ, Smid K, Meijer S, Pinedo HM. Thymidylate synthase inhibition after administration of fluorouracil with or without leucovorin in colon cancer patients: Implications for treatment with fluorouracil. J Clin Oncol. 1994;12(10):2035-42.
24. Zhang N, Yin Y, Xu S-J, Chen W-S. 5-Fluorouracil: Mechanisms of resistance and reversal strategies. Molecules. 2008;13(8):1551-69.
25. Wang X, Zou F, Deng H, Fu Z, Li Y, Wu L, et al. Characterization of sphere-forming cells with stem-like properties from the gastric cancer cell lines MKN45 and SGC7901. Mol Med Rep. 2014;10(6):2937-41.
26. Liu J, Ma L, Xu J, Liu C, Zhang J, Liu J, et al. Spheroid body-forming cells in the human gastric cancer cell line MKN-45 possess cancer stem cell properties. Int J Oncol. 2013;42(2):453-9.
27. Nakamura A, Nakajima G, Okuyama R, Kuramochi H, Kondoh Y, Kanemura T, et al. Enhancement of 5-fluorouracil-induced cytotoxicity by leucovorin in 5-fluorouracil-resistant gastric cancer cells with upregulated expression of thymidylate synthase. Gastric Cancer. 2014;17(1):188-95.
28. Sasada S, Miyata Y, Tsutani Y, Tsuyama N, Masujima T, Hihara J, et al. Metabolomic analysis of dynamic response and drug resistance of gastric cancer cells to 5-fluorouracil. Oncol Rep. 2013;29(3):925-31.
29. Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, et al. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell. 2005;122(6):947-56.
30. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126(4):663-76.
31. Fayazi M, Salehnia M, Ziaei S. Characteristics of human endometrial stem cells in tissue and isolated cultured cells: An immunohistochemical aspect. Iran Biomed J. 2016;20(2):109-16.
32. Saravi OE, Naghshvar F, Torabizadeh Z, Sheidaei S. Immunohistochemical expression of nanog and its relation with clinicopathologic characteristics in breast ductal carcinoma. Iran Biomed J. 2019;23(3):184-9.
33. Ghaleb AM, Yang VW. Krüppel-like factor 4 (KLF4): What we currently know. Gene. 2017:611:27-37.
34. Senbanjo LT, Chellaiah MA. CD44: A multifunctional cell surface adhesion receptor is a regulator of progression and metastasis of cancer cells. Front Cell Dev Biol. 2017:5:18.
35. Matsuoka J, Yashiro M, Sakurai K, Kubo N, Tanaka H, Muguruma K, et al. Role of the stemness factors sox2, oct3/4, and nanog in gastric carcinoma. J Surg Res. 2012;174(1):130-5.
36. Chen S, Hou JH, Feng XY, Zhang XS, Zhou ZW, Yun JP, et al. Clinicopathologic significance of putative stem cell marker, CD44 and CD133, in human gastric carcinoma. J Surg Oncol. 2013;107(8):799-806.
37. Wang W, Dong L-P, Zhang N, Zhao C-H. Role of cancer stem cell marker CD44 in gastric cancer: A meta-analysis. Int J Clin Exp Med. 2014;7(12):5059-66.
38. Wuebben EL, Rizzino A. The dark side of SOX2: Cancer -a comprehensive overview. Oncotarget. 2017;8(27):44917-943.
39. Rahimi-Tamandegani H, Marashi SA, Ghavami G, Mehrmohamadi M, Sardari S. Genome-scale metabolic modeling identifies synergistic metabolites that enhance 5-fluorouracil efficacy in colon cancer. Iran Biomed J. 2025;29(5):321-34.
40. Kumar V, Vashishta M, Kong L, Wu X, Lu JJ, Guha C, et al. The role of Notch, Hedgehog, and Wnt signaling pathways in the resistance of tumors to anticancer therapies. Front Cell Dev Biol. 2021:9:650772.
41. Yuan L, Meng Y, Xiang J. KLF4 induces colorectal cancer by promoting EMT via STAT3 activation. Dig Dis Sci. 2024;69(8):2841-55.
42. Ghasemzadeh N, Pourrajab F, Firoozabadi AD, Rahnama M. Liposome-mediated microRNA delivery: An additional layer of gene network regulation and nuclear reprogramming. Iran Biomed J. 2024;28(5-6):245-54.