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Evaluation of Drug Resistance in the Tamoxifen-treated MKN-45 Gastric Cancer Cell Line via the Epithelial-mesenchymal Transition Signaling Pathway

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ABSTRACT

One of the major challenges in gastric cancer (GC) chemotherapy is the phenomenon of multi-drug resistance (MDR). The epithelial-mesenchymal transition (EMT) and its key molecules, transforming growth factor-β (TGFβ) and SMAD2, play a central role in MDR occurrence. Tamoxifen (TAM), a triphenylethylene derivative, can overcome MDR in human gastric cancers. The aim of this study was to investigate the effect of TAM on 5-FU resistance of GC by suppressing the TGFβ1/SMAD2 signaling pathway and EMT. The MKN-45 cell line was subjected to treatment with 5-FU, TAM and a combination of both. The MTT assay was used to investigate the cytotoxic effects of 5-FU and TAM, and the DNA laddering technique was used to assess DNA fragmentation and apoptosis. Real-time RT-PCR examined the change in gene expression in EMT-related genes (SNAI2, VIM, *TGFβ1 and SMAD2)*. The results of the present study indicated that not only TAM treatment significantly decreased the IC50 of 5-FU ($P \leq 0.05$), but also the addition of TAM to 5-FU induced apoptosis in the MKN-45 cell line. Treatment with TAM and 5-FU significantly inhibited *TGFβ1* and TGFβ1-induced expression of EMT markers (VIM and SNAI2) in MKN-45 cells ($P \leq 0.05$). The reduction of TGFβ1 targets downstream of the SMAD2 signaling pathway reversed the process of EMT and significantly increased the sensitivity of MKN-45 cells to 5-FU. The results of the present study suggested that reversal of EMT-mediated MDR via the TGFβ1/SMAD signaling pathway using TAM may be a potential new therapeutic strategy to overcome chemoresistance to 5-FU during GC chemotherapy.

Keywords: Epithelial-mesenchymal transition, gastric cancer, drug resistance; Tamoxifen, TGFβ1/ SMAD2 signaling pathway

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Introduction

Gastric cancer (GC) is the fifth most commonly detected tumor and one of the leading causes of cancer-related deaths worldwide (1). The predominant treatment strategy for GC involves a combination of surgery, chemotherapy and radiotherapy. However, more than half of patients relapse after resection and/or have advanced disease at the time of diagnosis where surgical resection is not recommended (2). Hence, the outcome of patients depends only on the success of pharmacological treatment (3). Furthermore, chemotherapy is the standard treatment option for metastatic GC, and drugs such as 5-fluorouracil (5-FU), cisplatin, paclitaxel and epirubicin are the most popular chemotherapeutic agents (4). Although chemotherapy can enhance both survival rates and patients' quality of life, it is not without its problems. One of the main obstacles is the development of drug resistance in cancer cells (5,6). Multidrug resistance (MDR) is a deceive factor that significantly impairs the success of GC treatment. It refers to the insensitivity of tumor cells to chemotherapeutic agents before and during treatment (7,8). Despite significant advances in diagnosis and therapeutic strategies and significant improvements in patient survival, GC still has a poor prognosis due to high recurrence and metastasis rates (9). Finding strategies to overcome drug resistance is particularly crucial in the GC treatment (10). Research suggests that cells exhibit three primary mechanisms of drug resistance: reduced drug uptake, enhanced energy-dependent removal of drugs, and various cellular alterations that impair the efficacy of cytotoxic drugs in killing cells (11). The epithelial-mesenchymal transition (EMT) is a critical process in which epithelial cells transform into motile mesenchymal cells, and this transition plays a pivotal role in conferring drug resistance in cancer (12). Previously, high expression of EMT-related genes was observed in the oxaliplatin-resistant cell line SCG7901 (13). The phenomenon known as EMT has been demonstrated to play a role in cancer progression, metastasis, drug resistance, and tumor recurrence (7,14). In cancer cells, EMT typically leads to decreased expression of epithelial markers like E-cadherin and increased expression of mesenchymal markers such as vimentin (15). This change in cell differentiation and behavior is mediated by the key EMT-inducing transcription factors (EMT-TFs), whose functions are finely regulated (16–18). Upstream signaling pathways respond to external cues, initiating and controlling gene expression changes during EMT. In this context, signaling of the transforming growth factor-β (TGFβ) family plays a dominant role in various cancers, including GC, and causes activation of EMT through a variety of mechanisms (19–22). TGFβ induces SMAD complexes that activate transcription of mesenchymal markers and EMT-TFs (e.g., SNAI1, SNAI2), leading to E-cadherin repression (15,23).

Lately, there have been recommendations for combination therapy as a strategy to combat MDR and enhance treatment efficacy (24). Tamoxifen (TAM), a selective estrogen receptor modifier (SERM), inhibits the transcriptional function of the ER by competitively binding to it and serves as the main hormonal therapy for breast cancer in both adjuvant and metastatic contexts (25,26). To date, the antiproliferative effect of this chemopreventive agent has been reported in human breast and gastric cancer (27,28). TAM exhibits novel effects in various malignancies that are independent of its anti-estrogen mechanisms, ranging from inhibition of sphingolipid acid ceramidase to blockade of protein kinase C. Both target proteins are upregulated in malignancies and may be associated with tumor resistance to chemoradiotherapy (29). Numerous researchers have documented the biological effects of this triphenylethylene derivative on both

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the α-estrogen receptor (ER) and non-ER-mediated signaling pathways in human tumors (29,30). Preliminary studies have also shown that TAM can effectively counteract multidrug resistance of colorectal cancer, gastrointestinal cancer, and cholangiocarcinoma cells by directly binding to and inhibiting P-glycoprotein (P-gp) (31–33). Therefore, it is possible that the combination of 5-FU and TAM may result in increased efficacy and overcome MDR in gastric cancer. The aim of the current study was to investigate the effect of TAM on 5-FU resistance of GC and increased 5-FU sensitivity by suppressing of TGFβ1/SMAD2 signaling pathway and EMT in GC.

Materials and methods

Reagents and chemicals

5-Fluorouracil (5-FU) was obtained from KOREA UNITED PHARM (South Korea). Tamoxifen (TAM) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company (UK). Glacial acetic acid, isopropanol, chloroform, Tris base, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), NaCl, KCl, KH_2PO_4, Na_2HPO_4, glycerol and agarose were purchased from Merck (Germany). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), trypsin, streptomycin, penicillin G, L-glutamine, ethidium bromide, bromophenol blue, trypan blue, were provided by Sigma (USA). Proteinase K and RNAase A were purchased from Invitrogen (USA). DMEM/F12 and FBS were purchased from Caisson (USA) and BIO-IDEA (Iran), respectively.

Cell culture

The MKN-45 gastric cancer cell line was purchased from the Pasteur Institute (Tehran, Iran) and cultured in Dulbecco's Modified Eagle/F12 (DMEM/F12) medium containing 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C and 5% CO2. The medium was refreshed twice a week.

Cell viability assay

MKN-45 cells were cultured at 5 x 10^3 in 96-well until 80% confluence was reached and then the cells were treated with different concentrations of 5-FU (PBS as vehicle, 0.2, 0.4, 0.6, 0.8, 1, 2 µg/ml) and TAM (DMSO as vehicle, 2,4,6,8,10,12 µg/mL) for 48 hours. Moreover, the combination treatment (5-FU+TAM) was performed to investigate the effect of TAM treatment on the IC50 of 5-FU. After 48 hours, 100 µL MTT (5 mg/mL) was added to each well and incubated for 4 hours. Subsequently, 100 µl DMSO was added to dissolve the formazan crystals. Finally, absorbance was measured at 570 nm using a plate reader (BioTek Instruments, Iran) and viability was calculated in comparison to control cells. All experiments were performed in triplicate.

Cell apoptosis analysis

MKN-45 cells ($2.5 * 10^6$) were cultured in T25 flasks and then treated with drugs (5-FU and TAM). Apoptotic DNA fragmentation was determined as described by Nagata et al. with some modifications (34,35). In summary, MKN-45 cell pellets were lysed with a cell lysis buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% SDS, and 10 µg/mL proteinase K). The RNA was then digested with RNase A at 37 °C for 60 minutes. Then, the cell lysate was treated with NaCl (6 mol/L) and centrifuged at 8000 g for 5 minutes to precipitate the proteins. Isopropanol (500 µL) was then added and incubated overnight on ice at -20 °C and then centrifuged again at 12000 g for 10 minutes until the DNA was precipitated. In the next step, 70%

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alcohol (300 µL) was added and centrifuged at 7000 g for 6 minutes. Finally, TE II buffer (40 uL, 2h) was added to dissolve the DNA. The sample was then electrophoresed on a 2% agarose gel to detect DNA fragments.

Genes expression analysis by Real-time quantitative RT-PCR

Total RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN, USA) according to the kit manufacturer's protocol. The integrity and purity of total RNA samples were assessed by electrophoresis on 1% agarose gel in TAE buffer and staining with ethidium bromide (EtBr). RNA from the cell pellets was stored at -80°C for further experiments. Then, cDNA was synthesized using the PrimeScript™ RT Master Mix according to the instructions of the cDNA synthesis kit manufacturer (AddBio, South Korea). In the present study, the SYBR green-based polymerase chain reaction (PCR) Master Mix from Amplicon Kit (Denmark) was used to perform real-time RT-PCR protocols, and the ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA, USA) was used to evaluate quantitative gene expression. To perform real-time RT-PCR, the cDNA of the experimental groups was mixed with primers and PCR Master Mix (2X). The mixture was then incubated in an RT-PCR device with a reaction program of 40 predefined cycles (each cycle consisted of denaturation at 92°C for 20 seconds, annealing at 50°C for 20 S, and extension at 72°C for 30 seconds). Real-time analysis of RT-PCR data was performed using the $2^{-\Delta\Delta Ct}$ method. Threshold cycle (Ct) values were measured for both target genes and *GAPDH*, an endogenous control gene. Each experiment was performed in triplicate. The primer sequences are listed in Table 1.

Table 1. Primer sequences

Genes Name	Primers	Sequences	Primers Size (bp)	Annealing (°C)	Product size (bp)
GAPDH	forward	5'-ACTCTGGTAAAGTGGATATTGTTGC -3'	25	50	162
	reverse	5'-GGAAGATGGTGATGGGATTTC -3'	21		
TGFβ1	forward	5'- GCAAGTGGACATCAACGGGTTC -3'	22	50	192
	reverse	5'- CGCACGCAGCAGTTCTTCTC -3'	20		
SMAD2	forward	5'- AAAGGGTGGGGAGCAGAATAC -3'	21	50	135
	reverse	5'- TGAGCAACGCACTGAAGGG -3'	19		
SNAI2	forward	5'- ATTCGGACCCACACATTACC -3'	20	50	123
	reverse	5'- GCAGTGAGGGCAAGAAAAAG -3'	20		
VIM	forward	5'- GGCGAGGAGAGCAGGATTTC -3'	20	50	172
	reverse	5'- CGTGATGCTGAGAAGTTTCGTTG -3'	23		

Statistical analysis

All experiments were performed in triplicate. Data were reported as mean ± SD. Statistical analyses were performed using one-way ANOVA and Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). GraphPad Prism 8.0 software (San Diego, CA, USA) was used for all statistical analyses.

Results

TAM effect on cell viability and IC50 of 5-FU

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As shown in Figure 1, the viability of MKN-45 cells decreased in a dose-dependent manner when treated with TAM and 5-FU. The IC50 value was calculated as 0.8 µg/mL for 5-FU and 8 µg/mL for TAM. The results also indicated that simultaneous treatment with 5-FU and TAM significantly reduced the IC50 of 5-FU in MKN-45. Finally, based on the findings, a dose of 0.4 µg/mL for 5-FU and a dose of 4 µg/mL for TAM were selected as the optimal concentration for the treatment experiments of MKN-45 cells, as these concentrations have minimal cytotoxicity to the cells and maximal efficacy for cell function (Figure 1).

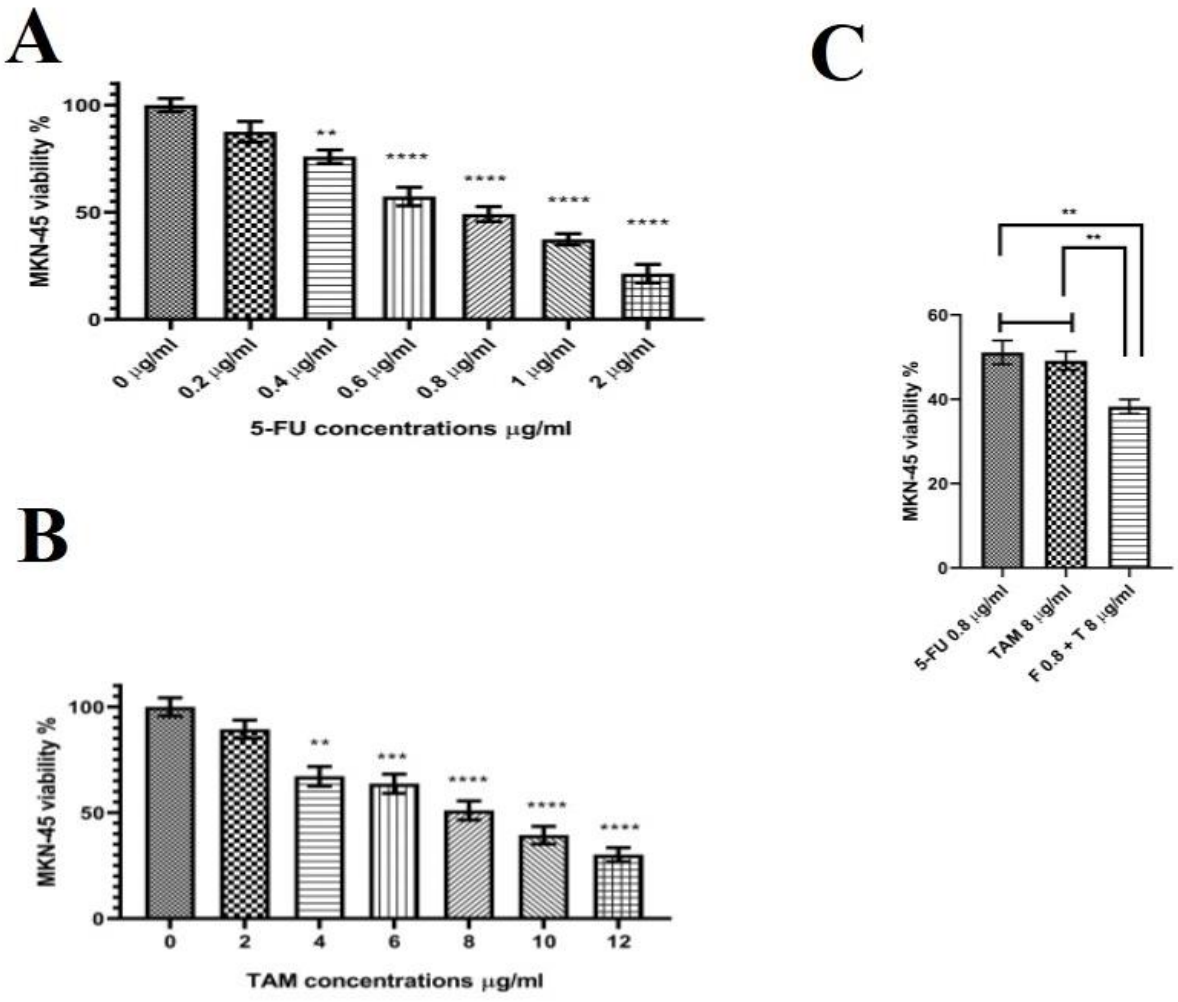

Fig. 1. Dose-dependent growth inhibition and modulation IC50 of 5-FU by TAM in MKN-45 gastric cancer cells. MKN-45 gastric cancer cell line was treated with different concentrations of TAM and 5-FU drugs for 48 hours. The viability of MKN-45 cells treated with 5-FU (1-A) and TAM (1-B) drugs alone and simultaneously (1-C) for 48 hours of incubation was analyzed using the MTT method. The result showed that with TAM and 5-FU treatment, cell viability decreased in a dose-dependent manner and TAM treatment remarkably lowers the IC50 of MKN-45 cells to 5-FU. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to untreated control.

[DOI: 10.22088/IJMCM.BUMS.12.4.361]

Effect of TAM on apoptosis induction

Examination of extracted DNAs in agarose gel suggested that treatment with 5-FU and TAM and with 5-FU + TAM resulted in DNA fragmentation compared to the control group and that FU + TAM increased DNA fragmentation in MKN-45 cells compared to 5-FU and TAM (Figure 2).

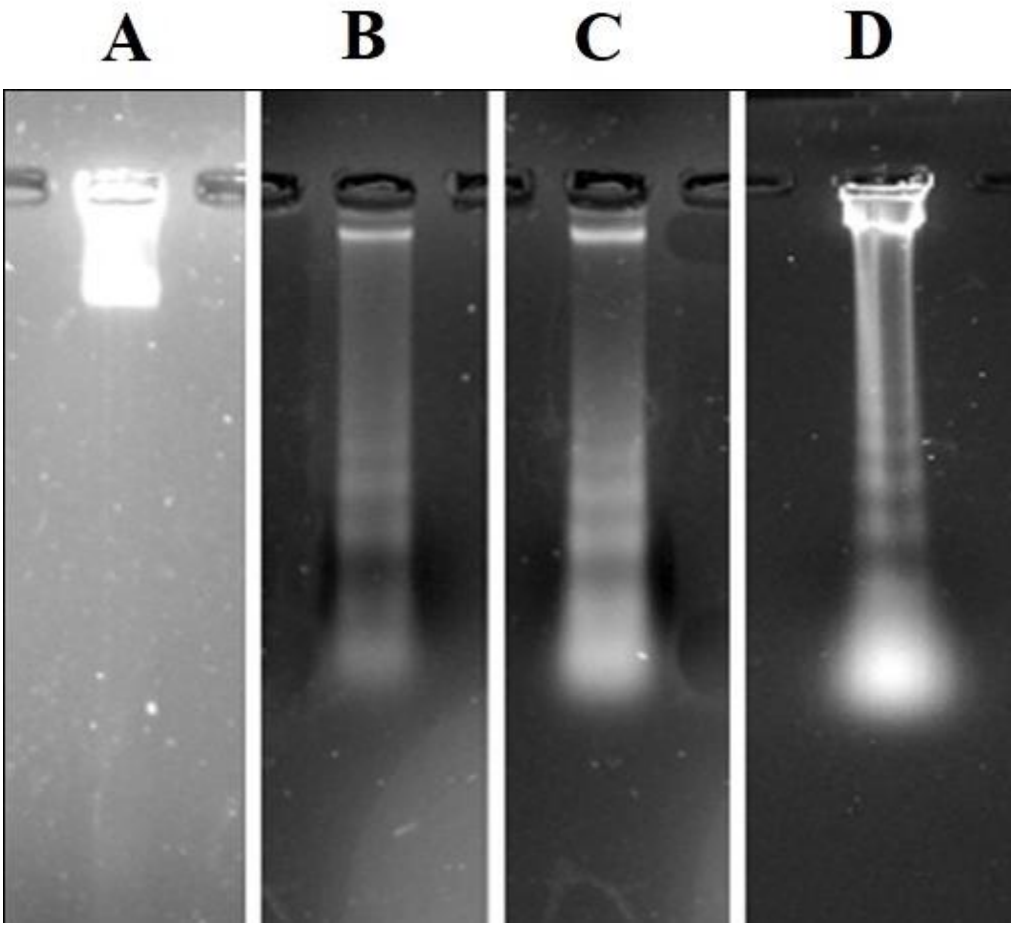

Fig. 2. Apoptosis induction by TAM in MKN-45 gastric cancer cells; Apoptosis of MKN-45 treated with TAM(C) and 5-FU (B) drugs alone and 5-FU+TAM (D) for 48 hours of incubation was assayed using DNA laddering. The results showed DNA fragmentation in GC cells treated with TAM or 5-FU alone or combined, but not in untreated cells (A). In addition, TAM treatment remarkably intensifies the DNA fragmentation of 5-FU-treated MKN-45 cells.

TAM effect on the expression of EMT-related genes (*SNAI2, VIM, TGFβ1, and SMAD)*.

Real-time RT-PCR results demonstrated that *TGFB1* mRNA levels were significantly downregulated in cells treated with TAM and 5-FU alone and in combination compared to untreated MKN-45 cells. On the other hand, treatment with TAM and 5-FU significantly inhibited TGFβ1-induced expression of EMT markers (*VIM* and *SNAI2*) in MKN-45 cells ($P \leq 0.05$). In addition, treatment with TAM and 5-FU led to a slight decrease in the expression of SMAD2. Interestingly, the results showed that TAM treatment more significantly reduced the transcription of the study target genes in MKN-45 cells treated with 5-FU. These

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results indicated that TAM treatment regulated the EMT process and inhibited the MDR of GC cells by downregulating the activity of the TGFB1 signaling pathway (Figure 3).

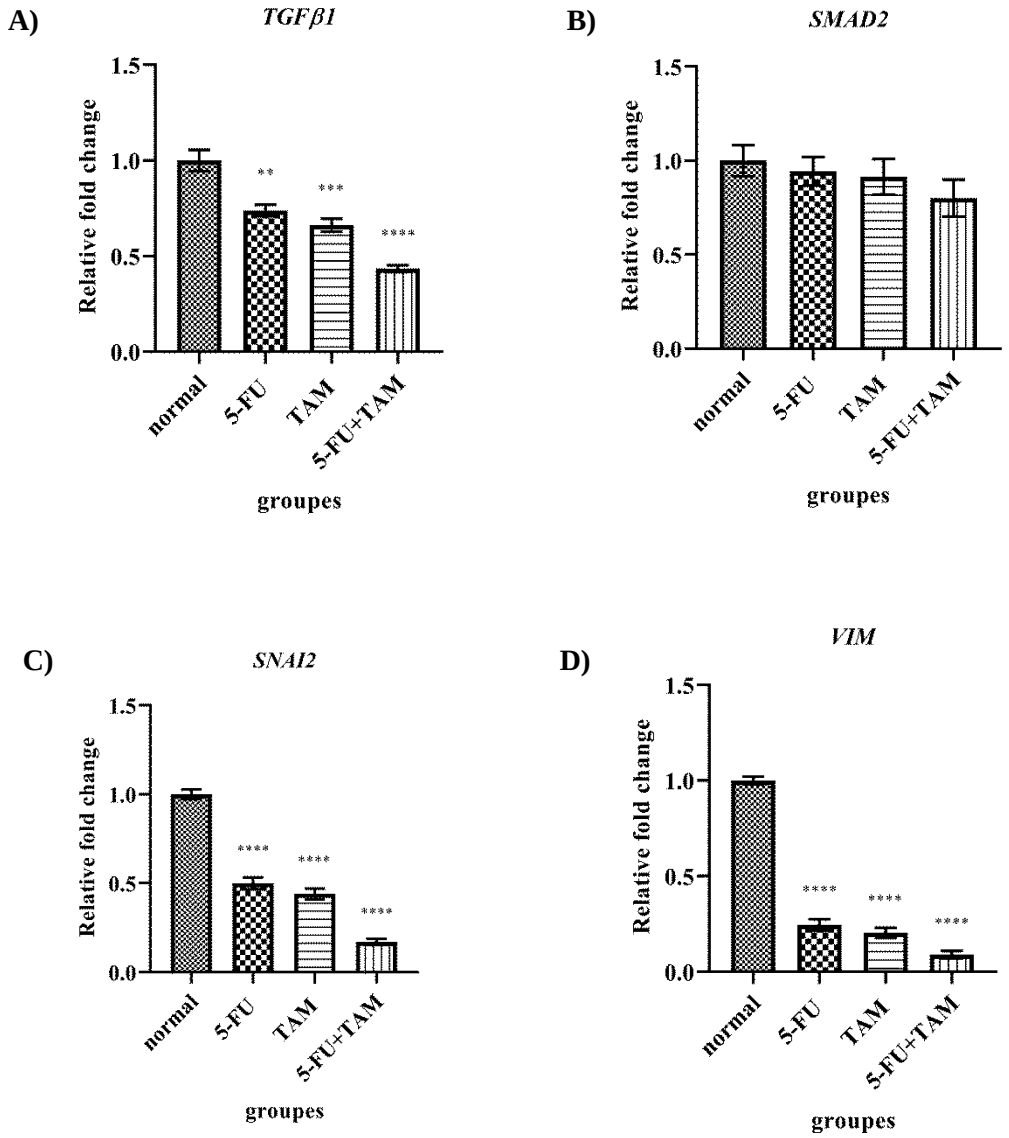

Fig. 3. The levels expression of TGFβ1 (A), SMAD2 (C), SNAI2 (B), and VIM (D) genes in MKN-45 treated with TAM and 5-FU drugs alone and in combination. The results showed down-regulating of TGFB1, SNAI2, and VIM genes in GC cells treated with TAM or 5-FU alone or in combination, but not in the SMAD2 gene. In addition, the combination treatment of TAM and 5-FU significantly reduced transcription of these genes in MKN-45 cells compared to the treatment of each drug alone. *P < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to untreated control; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene.

Discussion

The present study demonstrated that TAM significantly decreased the IC50 of 5-FU in the GC cancer cell line (MKN-45). Moreover, it strongly inhibited cell proliferation and increased apoptosis. In addition,

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the data represented that TAM significantly reduced the expression of *TGFB1* and SMAD2 transcriptional targets including the transcription factors *SNAI2* and the mesenchymal marker protein *VIM* in MKN-45 in these cells treated with 5-FU. TAM treatment led to a significant decrease in *SNAI2*, *VIM*, and *TGFβ1* mRNA levels. However, the gene expression of *SMAD2* was not affected by TAM treatment (probably due to the difference in sensitivity). The researchers found that SMAD2 expression levels remained consistent across the different treatments, while notable alterations were observed in the protein levels of phosphorylated SMAD2—the transcriptionally active complex (36–39).

Epithelial-mesenchymal transition (EMT) signaling is one of the most important processes in cancer invasion and metastasis through multidrug resistance (MDR) (7). Targeting the EMT is considered a novel way to overcome MDR of cancer (7). From a molecular perspective, transforming growth factor-β (TGFβ) family signaling is involved in EMT regulation as an upstream pathway of EMT via EMT-related genes such as *VIM*, *TGFB1*, *SMAD2* and *SNAI2* (23). Agents that effectively suppress EMT could therefore inhibit drug resistance and lead to promising effects in GC treatment. Despite the widespread use of 5-FU in cancer therapy as an antimetabolite that disrupts key biosynthetic pathways, it faces significant challenges in clinical use due to drug resistance (40). TAM as a selective estrogen receptor modulator in hormonal therapy of breast cancer exerts its biological effects via both ER- and non-ER-mediated mechanisms, leading to upregulation of apoptotic pathways and association with autophagic processes in human tumors (29). The promising effect of combination therapies, especially other drugs with TAM, in chemotherapy of GC and other cancers has been reported in the past. In this regard, Zhang et al. reported that the combination of 5-FU/celecoxib increased the efficacy of chemotherapy in GC cell line SGC7901 and suggested a significant synergistic effect on tumor growth inhibition by MTT method (41). Furthermore, Miyake et al. discovered that co-administration of TAM and the fusicoccin derivative (ISIR-042) together with 5-FU demonstrated significant efficacy in overcoming drug resistance in pancreatic cancer (42). In patients with advanced non-small cell lung cancer (NSCLC), the combination of TAM and docetaxel has been shown to improve both median progression-free survival and overall survival. This effect is achieved by reversing the P-glycoprotein-mediated MDR (43). Khanipouyani *et al*. investigated the effects of tamoxifen on gene expression in MKN-45, a gastric cancer cell line. The treatment with tamoxifen decreased the viability of MKN-45 cells in a dose-dependent manner and significantly downregulated mRNA levels of Notch1 and DLL1 genes compared to untreated cells. They reported that the anticancer effects of tamoxifen in GC are related to disruption of the Notch signaling pathway (27). Liu ZH *et al*. investigated the potential of TAM to overcome MDR in cholangiocarcinoma. The researchers observed enhanced effects of TAM, including growth inhibition, apoptosis, increased intracellular ADM concentration and downregulation of P-gp expression. Ultimately, their findings suggested that TAM could reverse MDR in QBC939/ADM cells and improve the efficacy of chemotherapy in cholangiocarcinoma by competitively inhibiting overexpressed P-gp (33). Moreover, Shen LZ *et al*. studied the effects of TAM on the MDR of doxorubicin (DOX) in colorectal carcinomas in living organisms. They investigated the relationship between TAM and the estrogen receptor (ER). Their findings revealed that TAM can effectively reverse MDR in colorectal carcinomas in nude mice independent of ER expression (32). In a separate study led by Hotta *et al*. the effect of TAM on DOX sensitivity in fresh human gastrointestinal cancer cells was investigated. They found

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a significant correlation between the effect of TAM on cytotoxicity and the expression of P-glycoprotein. When TAM was combined with DOX, the concentration of DOX increased in the tumor cells compared to DOX alone. These findings suggest that TAM may be able to overcome DOX resistance in cancer treatment (31). Mao *et al*. stated that TAM significantly reduced the IC50 of ER-negative SGC7901/CDDP human gastric cancer cells when treated with cisplatin, 5-FU and adriamycin (ADM). Additionally, at the molecular level, TAM significantly decreased the expression of P-glycoprotein (P-gp), phosphorylated Akt (p-Akt) and other downstream effectors regulated by Akt. These results demonstrate that TAM effectively reverses P-gp-mediated MDR in gastric cancer cells by inhibiting the PI3K/Akt signaling pathway (30). Thus, inhibition of different upstream mechanisms appears to reduce MDR in various cancer types. In the context of the scientific literature, the present study reveals that TAM effectively suppresses the induction of EMT by targeting the TGFβ1/SMAD signaling pathway, thereby modulating drug resistance of GC cells.

The results of the current study indicate that treatment of GC with TAM improves sensitivity to 5-FU and inhibits invasion in 5-FU-resistant GC cells by regulating the EMT process via blocking the TGFβ1/SMAD signaling pathway. This provides new evidence that TAM may serve as a novel therapeutic agent to overcome 5-FU resistance in GC treatment. The results of the present study suggest that reversal of EMT-mediated MDR via the TGFβ1/SMAD signaling pathway by TAM may be a potential novel therapeutic strategy to overcome chemoresistance to 5-FU in GC chemotherapy.

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Ethical considerations

This study was supported by Babol University of Medical Sciences, Babol, Iran. The Ethics Committee of Babol University of Medical Sciences approved this study (IR.MUBABOL.REC.1400.193).

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