Article published online: 2021-07-14

Allicin Reduces 5-fluorouracil-resistance in Gastric Cancer Cells through Modulating MDR1, DKK1, and WNT5A Expression

Authors
Parya Khakbaz[1, 2], Reza Panahizadeh[1, 2], Mohammad Amin Vatankhah[1, 2], Nowruz Najafzadeh[2]

Affiliations
1 Student Research Committee, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran
2 Research Laboratory for Embryology and Stem Cells, Department of Anatomical Sciences, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran

Key words
Gastric cancer, chemoresistance, allicin, 5-fluorouracil, CD44, P-glycoprotein

published online 14.07.2021

Bibliography
Drug Res 2021; 71: 448–454
DOI 10.1055/a-1525-1499
ISSN 2194-9379

Georg Thieme Verlag KG, Rüdigerstraße 14
70469 Stuttgart · Germany

Correspondence
Nowruz Najafzadeh, Ph.D., Associate Professor
Research Laboratory for Embryology and Stem Cells,
Department of Anatomical Sciences, School of Medicine,
Ardabil University of Medical Sciences
5618985991 Ardabil
Iran
Tel.: + 989104075700, Fax: + 984533534703
nowruz30@gmail.com

ABSTRACT

Background & Objective 5-fluorouracil (5-FU) is approved for the treatment of gastric carcinoma (GC), but chemo-resistance limits the application of it for GC. Thus, the combination of 5-FU with adjuvants such as allicin may overcome multidrug resistance (MDR).

Methods The anticancer effects of allicin, 5-FU, and allicin/5-FU on the 5-FU resistant MKN-45 cells were evaluated by MTT assay and DAPi staining. The expression of the P-glycoprotein (P-gp) and CD44 protein were determined using immunocytochemistry. We also quantified mRNA expression levels of *WNT5A, Dickkopf-1* (*DKK1*), and *MDR1* in the GC cells.

Results Here, we found that the combination of allicin with 5-FU significantly increased apoptosis compared to 5-FU alone ($P < 0.05$). We showed that *WNT5A*, *MDR1,* and *DKK1* mRNA expression levels were down-regulated in the allicin- and allicin/5-FU-treated cells. Indeed, the combination of allicin and 5-FU significantly decreased the expression of the P-gp and CD44 proteins ($P < 0.05$).

Conclusion Our findings indicate that the combination of allicin with 5-FU could reverse multidrug resistance in the GC cells by reducing the expression of *WNT5A*, *DKK1*, *MDR1*, P-gp, and CD44 levels.

Introduction

Gastric cancer (GC) remained the most common cause of cancer mortality among men worldwide. The incidence rate is high in Western Asia countries, including Iran, Turkmenistan, and Kyrgyzstan. *Helicobacter pylori* infection, genetic changes, environmental factors, obesity, alcohol consumption, and tobacco smoking are the main risk factor for stomach cancer [1]. The highest prevalence rate has been reported in the northern region of Iran, Ardabil province [2].

GC is asymptomatic in the early stage. In advanced stages, it usually metastasizes to the lung, liver, peritoneum, and bone marrow. Although radical gastrectomy and chemotherapy are the main treatment options for GC to prolong the life of patients, the prognosis of GC patients has not been improved significantly [3]. 5-FU, as a thymidylate synthase inhibitor, blocks DNA synthesis and prevents cancer cell growth. Monotherapy with 5-FU elicits poor responses and the response rate is low (25–35 %). Thus, combination regimens with chemotherapeutics may have significant benefits in metastatic GC [4]. Although combination chemotherapy is commonly accepted for the treatment of GC, the efficacy of chemotherapeutics is limited due to chemoresistance and stem cell recurrence [5].

Prescription of 5-FU usually induces the expression of efflux pumps and the overexpression of DNA-repair mechanisms in cancer cells. The overexpression of *MDR*, *Bcl-2*, *Bcl-XL*, and *Mcl-1* genes and increased activation of thymidylate synthase and deoxyuridine triphosphatase mediates chemoresistance to 5-FU in many cancers [6]. In addition, Wnt pathway genes including *Wnt5a* and *DKK1* involved in chemoresistance in several cancers. Wnt5a regulates G1-S transition and involved in chemoresistance in pancreatic cancer cells [7]. Overexpression of ALDH1A, REPS2, and DKK1 genes causes detoxification of drug agents and induces chemoresistance in colorectal cancer [8].

Several toxicities and side effects of 5-FU such as loss of appetite, hair loss, and skin inflammation [9] urged researchers to evaluate natural remedies against cancer.

Several garlic-derived compounds such as allicin, ajoene, diallyl trisulfide, and S-allylmercaptocysteine have potential anticancer activity. Allicin, the major component of garlic, has antibacterial, antiviral, and anticancer properties [10]. Previous studies have shown that allicin can sensitize chemotherapeutics against hepatocellular carcinoma [11], melanoma [12], osteosarcoma [10], and colorectal carcinoma [13] by inhibiting cell growth. Allicin inhibits proliferation in many cancers by activation of caspases, overexpression of *Bax,* and *Fas* induction of cytochrome C release [14]. When the human lung, colorectal, and hepatocellular cancer cells were treated with both allicin and 5-FU, synergistic antitumor effects were observed [13, 15].

However, whether allicin can sensitize chemoresistant GC to 5-FU is not clear. An innovative treatment perspective might be the use of allicin, particularly in combination with 5-FU, to re-sensitize cancer cells in the 5-FU resistant MKN-45 GC cell line. Here, we investigated whether the low doses of allicin could enhance the cytotoxicity of 5-FU and reduce the resistance of the cancer cells by regulating *WNT5A, DKK1, MDR1*, CD44, and P-gp expression level.

Material & Methods

Drugs

5-FU (F6627) was purchased from Sigma-Aldrich. Dr. Mohsen Arzanlou (Ardabil, Iran) kindly provided allicin.

Cell culture

The human 5-FU resistant gastric cancer cell line MKN-45 was previously established by Pouremamali et al.[16] The GC cells were grown in RPMI1640 (Gibco, UK) medium supplemented with 10 % fetal bovine serum (FBS, Gibco, UK), and 1 % antibiotics.

MTT assay

The cell proliferation was evaluated with a standard MTT method. Briefly, 10^4 cells/well were incubated overnight. The different concentrations of allicin (2–64 µg/ml), 5-FU (10–480 µg/ml), and allicin/5-FU were used to treat the GC cells. Then, the cells were incubated in RPMI1640 medium containing 5 mg/ml MTT (Sigma, M2128). The MTT solution was then replaced with 150 µl of DMSO (Scharlau Chemie). The absorbance values were determined by an ELISA reader.

Nuclear morphology assay

Briefly, the cells (5×10^3 cells/well) were grown in a 6-well plate. Treated MKN-45 cells were fixed with 4 % paraformaldehyde (PFA) at 4 ° C for 30 min. Finally, the cells were stained with 1 µg/ml DAPi in a dark room.

Immunocytochemistry

Immunofluorescence staining was used to determine the protein expression levels of P-gp and CD44 in the cancer cells. The cells were fixed with 4 % PFA followed by incubation in normal goat serum and bovine serum albumin for 30 min. Then, the cells were stained with the primary antibody against mouse anti-P-gp (1:150; sc-390883, Santa Cruz Biotechnology, Inc) for 2 h in dark. Next, cells were incubated with rat anti-mouse FITC secondary antibody (Thermo Fisher, 1: 200). The CD44 positive cells were detected with PE-conjugated mouse anti-human CD44 (1:150, Miltenyi Biotec, 130–095–180).

Real-time PCR (RT-PCR)

The mRNA level of DKK1, WNT5A, MDR1, and GAPDH was determined using RT- PCR as previously stated by Mokabber et al. [17]

Briefly, total RNA was extracted from the MKN-45 cell line using 1 ml TRIzol (Invitrogen). First-strand complementary DNA (cDNA) was produced from 1 µg total RNA using oligo (dT) primer and M-MLV reverse enzyme (Vivantis, USA). oligo(dT, 1 µl) primer and nuclease-free water were mixed with mRNA, incubated at 65 °C for 5 min and placed on ice for at least 1 min. M-MuLV enzyme (100 u) and buffer (10 ×) were added and incubated at 42 °C for 60 min and then 85 °C for 10 min. Finally, qPCR was done with the SYBR Green PCR Master Mix (EURx, Ltd, Gdañsk, Poland). Real-time PCR reaction was performed in 3 steps: 95 °C for 20 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 5 s.

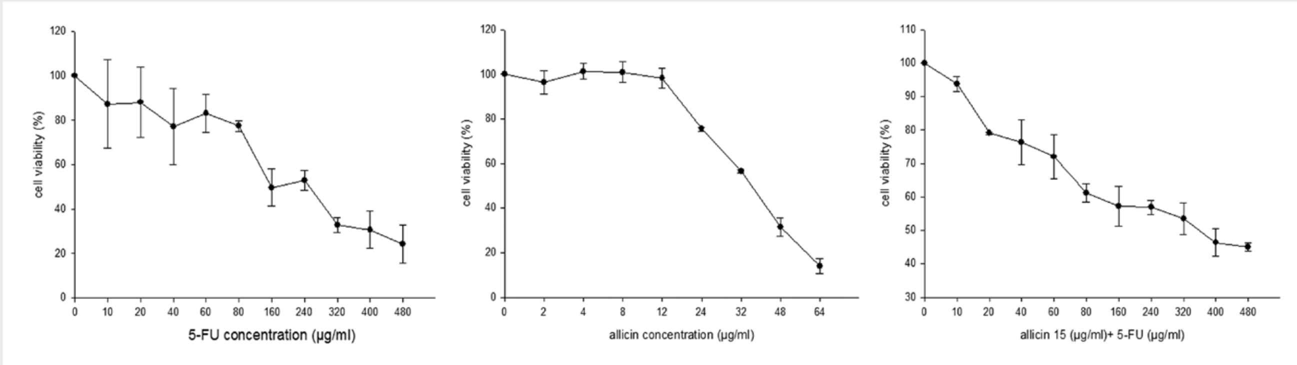
The RT-PCR System (Roche Applied Science) was used to analyze gene expression. Specific human primers DKK1, WNT5A, MDR1, and GAPDH were designed using OLIGO 7.0 software. GAPDH gene was used as a reference gene and relative gene expression was calculated using the CT method [18, 19]. Primer sequences were shown in ▶ **Table 1**.

Statistics

Data evaluation was performed using the SPSS software ver. 21. Statistical comparisons were made using the unpaired one-way ANOVA and Student's *t*-test.

▶ **Table 1** List of primer sequences and product sizes used for RT-PCR analysis.

Genes	Forward	Reverse	Product size
DKK1	TAGCACCTTGGATGGGTATT	ATCCTGAGGCACAGTCTGAT	110
WNT5A	CGCCCAGGTTGTAATTGAAG	GCATGTGGTCCTGATACAAGT	164
MDR1	AGAGGGGATGGTCAGTGTTGA	TCACGGCCATAGCGAATGTT	138
GAPDH	ACATCATCCCTGCCTCTACTG	CCTGCTTCACCACCTTCTTG	180



▶ **Fig. 1** Cell growth inhibitory curves after treatment of cells with different concentrations of 5-FU (**a**), allicin (**b**), and their combination treatments (**c**).

▶ **Table 2** IC_{50} values represent the concentration of each drug that inhibits cell viability by 50 %.

Groups	IC_{50}
5-FU (µg/ml)	164.52 ± 1.92
allicin (µg/ml)	35.39 ± 0.05
allicin (µg/ml) + 5-FU	49.46 ± 10.61 *
*$P<0.05$ compared to 5-FU alone.	

Results

Low doses of allicin enhance the cytotoxicity of 5-FU against 5-FU resistant gastric cancer

The anticancer activities of allicin, 5-FU, and their combination were investigated using MTT assay in the 5-FU resistant MKN-45 gastric cells. According to our results, allicin and 5-FU exerted anticancer effects in a dose-dependent manner. The pretreatment with low-dose allicin has significantly increased the sensitivity of the chemoresistant GC cells to 5-FU. Importantly, the IC_{50} value of co-treatment of allicin with 5-FU was 49.46 ± 10.61 µg/ml which lower than the IC_{50} values of 5-FU alone (164.52 ± 1.92) (▶ **Fig.1**, ▶ **Table 2**) ($P<0.05$).

Co-treatment of allicin with 5-FU reduces the viability of gastric cancer cells

Morphological assessment of apoptosis was carried out using DAPi. Pretreatment with allicin significantly increased apoptosis in 5-FU resistant GC cells. The formation of chromosomal DNA fragments and chromatin condensation was evident in the gastric cancer cells upon combination treatments. As presented in ▶ **Fig. 2**, when the cells were grown in 5-FU for 48 h, the apoptotic percentage of MKN-45 cells was 9.76 ± 3.54, whereas in allicin/5-FU treated cells the apoptotic rate was 19.11 ± 5.19 ($P<0.05$).

Combination of allicin with 5-FU can alter the expression of P-gp and CD44 proteins

To confirm the results of allicin/5-FU induced apoptosis in the MKN-45 cells, we further investigated whether allicin/5-FU can change P-gp and CD44 protein expression by immunocytochemistry. Here, we found that P-gp expression was significantly decreased after treatment with allicin and allicin/5-FU when compared to control or 5-FU alone (▶ **Fig. 3c–f**) ($P<0.05$). Interestingly, in the 5-FU resistant MKN-45 cell line where CD44 is overexpressed, allicin and allicin/5-FU decreased the CD44 expression compared to 5-FU and control (▶ **Figure 4c, d**) ($P<0.05$). In 5-FU-treated cells, the percentage of CD44 expression was 42.29 ± 6.8, whereas, in allicin- and allicin/5-FU-treated groups were 25.70 ± 5.59 and 25.06 ± 1.8, respectively.

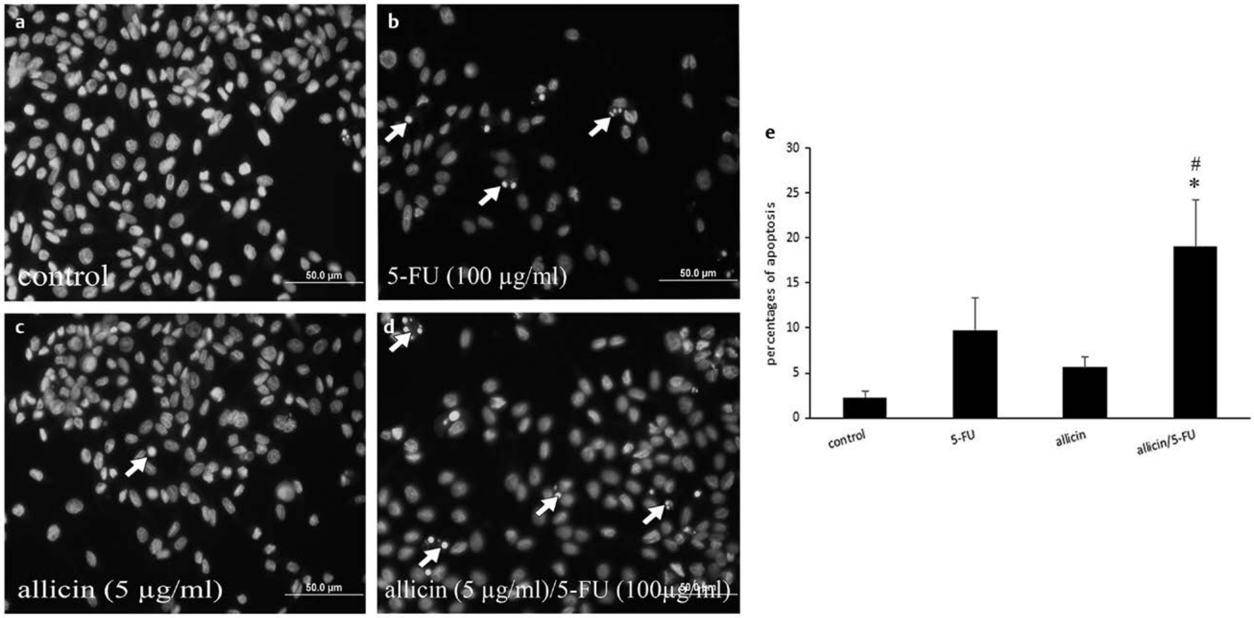
Efficacy of allicin, 5-FU, and their co-treatments on the expression of *WNT5A* and *DKK1* mRNA level

To investigate the inhibitory role of allicin/5-FU on the expression of *MDR1, WNT5A,* and *DKK1*, we pretreated the cells with 15 µg/m allicin, then the cells treated with a low dose 5-FU. According to our results, treatment with 5-FU, allicin, and allicin/5-FU down-regulated the expression of the *DKK1* and *MDR1* mRNA levels (▶ **Fig. 5a, b** and ▶ **Fig. 6**). Moreover, we observed the down-regulation of *WNT5A* after treatment with allicin and allicin/5-FU.

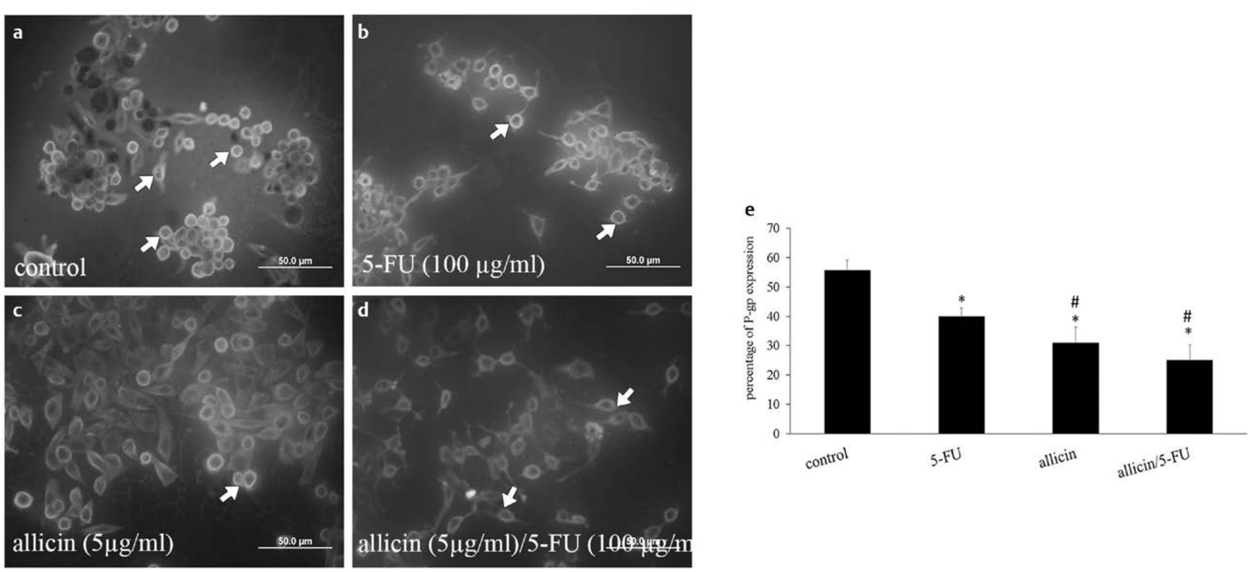
Discussion

Our results demonstrated that a combination of allicin and 5-FU effectively decreased the growth of 5-FU resistant gastric cancer which is consistent with studies of osteosarcoma [20], hepatocellular cancer cells [15], and neuroblastoma cells [13]. Previously, we also demonstrated that allicin in combination with methylsulfonylmethane increased apoptosis and inhibited cell cycle in CD44 ± breast cancer cells [21]. Jiang et al. found that the combined treatment of allicin with artesunate decreases the viability of osteosarcoma cells and suppresses the metastasis and colony formation ability through the overexpression of caspase-3/9 [20]. In another study, Gao et al. showed that the combined treatment of cyclophosphamide with allicin can improve T cell-mediated immunity and inhibit VEGF in neuroblastoma cells [11]. In a similar study, Zou et al. found that allicin enhanced anticancer activity of 5-FU by increasing reactive oxygen species (ROS) level and down-regulation of Bcl-2 [15].

Our data showed a lower expression of P-gp, CD44 protein, and *MDR1* mRNA expression after the combined treatment of allicin with 5-FU in GC. These reductions in CD44 and P-gp expression may suggest that pretreatment with allicin can reduce chemoresistance in gastric cancer cells. *MDR* genes and P-gp usually overexpress in $CD44^{+}/CD24^{-}$ cancer cells. CD44 can interact with P-gp

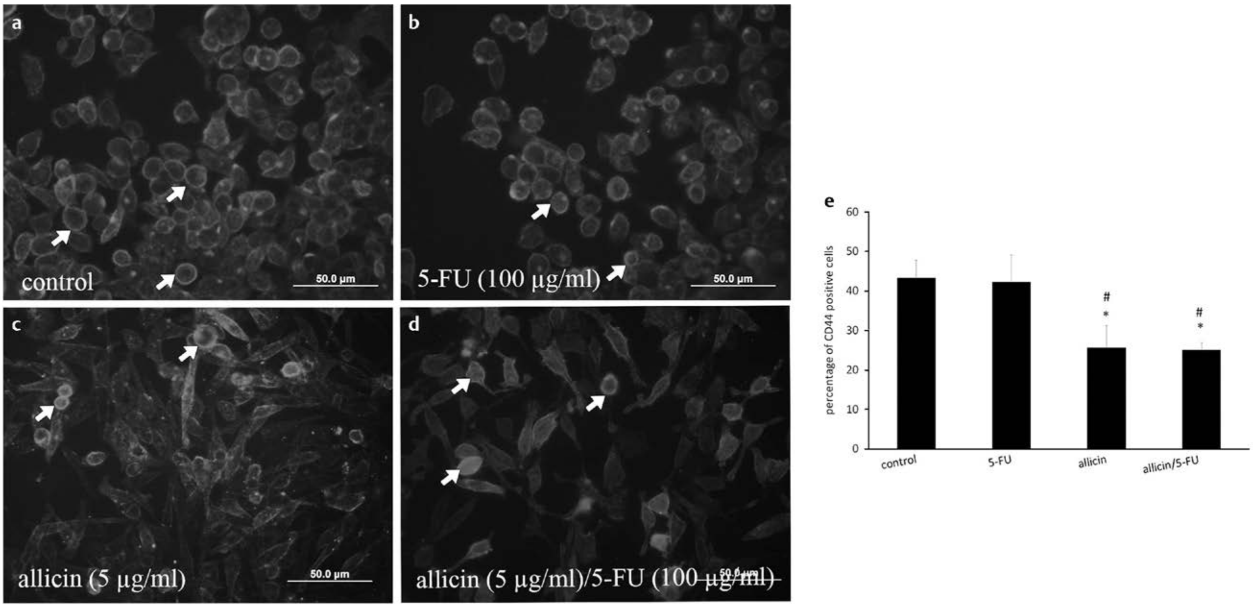


▸ **Fig. 2** DAPi staining of 5-FU resistant gastric cancer treated with 5-FU (**b**), allicin (**c**), and allicin/5-FU. More apoptotic bodies (arrows) were seen after the combination treatments (**d**). $^{*}p<0.05$ as compared to control cells. $^{\#}p<0.05$ as compared to 5-FU treated cells. Scale bar 50 μM (40X magnification).



▸ **Fig. 3** Immunocytochemical images show the expression of P-gp proteins in the gastric cancer cells treated with 5-FU (**b**), allicin (**c**), and co-treatment of allicin/5-FU (**d**). The arrows show cells expressing the P-gp protein. The expression of P-gp significantly decreased in all treated groups compared to control. Indeed, allicin and allicin/5-FU were more effective in decreasing the expression of P-gp compared to 5-FU alone (**e**). Data are expressed as mean ± stdev. $^{*}P<0.05$ as compared to the control group. $^{\#}P<0.05$ as compared to the 5-FU alone. Scale bar 50 μM (40X magnification).

to promote the invasion of cancer cells [22]. There is a link between the Wnt/β-catenin pathway and P-gp. Recent studies have revealed that modulating of the Wnt/β-catenin pathways may downregulate P-gp in cholangiocarcinoma [23]. The overexpression of MDR genes is associated with chemoresistance and a higher rate of systemic recurrence in GC patients [24]. In another study, Cha et al. showed that allicin can induce cell death in human glioma cells through inhibition of the MAPK/ERK-dependent pathway [25]. In



▶ **Fig. 4** Immunocytochemical images show the expression of CD44 proteins in 5-FU resistant MKN-45 cells treated with 5-FU (**b**), allicin (**c**), and allicin/5-FU (**d**). Allicin and co-treatment of allicin and 5-FU were significantly decreased the expression of CD44 in gastric cancer (**e**). The arrows show the cells expressing CD44 protein. Data are expressed as mean ± stdev. * $P<0.05$ as compared to the control. # $P<0.05$ as compared to the 5-FU. Scale bar 50 µM (40X magnification).

a similar study, Wang et al. reported that diallyl trisulfide can increase cytotoxic effects of Adriamycin by downregulation of P-gp and NF-κB in human osteosarcoma [26]. The treatment of vinblastine-resistant leukemia K562 with a low dose of diallyl sulfide promotes the anticancer activity of vinblastine and reduces the expression of P-gp in chemoresistant leukemia cells [27]. Diallyl sulfide also suppresses *MDR1* gene expression by targeting the HOXC6-mediated ERK1/2 signaling pathway [28]. In our previous study, we reported that the combination of allicin with all-trans retinoic acid inhibits CD44$^+$ and CD117$^+$ melanoma cells at S phases and induces the overexpression of *cyclin D1* mRNA [12]. Therefore, these results demonstrated that co-treatment of allicin and 5-FU may inhibit the proliferation of chemoresistant gastric cancer cells by suppressing CD44 expressing cells.

Here, we observed the down-regulation of *WNT5A* and *DKK1* after combined treatment with allicin and 5-FU. The Wnt signaling pathway has crucial functions during the development and metastasis of GC. It activates the non-canonical Wnt signaling pathway. Abnormal expression of the *WNT5A* gene was reported in 30 % of GC cases. High expression of WNT5A is associated with a poor prognosis. WNT5A may behave as a tumor suppressor or a tumor-promoting agent in cancers [29, 30]. DKK1 may act as a tumor suppressor or an oncogene in different cancers. Recently, Xu et al. have revealed that S-allyl cysteine, a garlic derivative, decreases the protein expression of *WNT5A* in human ovarian cancer cells [31]. Wang et al. demonstrated that overexpression of DKK1 suppresses the tumor-forming ability of CD44$^+$ GC cells by modulating Wnt signaling [32]. In contrast, Lee et al. showed that increased levels of DKK1 in the serum of GC patients may act as an oncogene [33]. Moreover, Xia et al. reported that S-allyl-mercapto cysteine elevates DKK1 protein expression and decreases TCF/β-catenin expression in both Hep3B and Huh-7 hepatic cancer cell lines [34]. We conclude that allicin may alter the expression of *DKK1* and *WNT5A* to restore the anticancer activity of 5-FU in chemoresistant GC.

In conclusion, our results demonstrated that the combined use of allicin with 5-FU could overcome chemoresistance by inhibiting the expression of *MDR1, DKK1, WNT5A,* CD44, and P-gp. Therefore, these *in vitro* observations strongly support that the use of lower dose allicin-based combinatorial chemotherapy has medical significance in GC patients.

Author’s Contributions

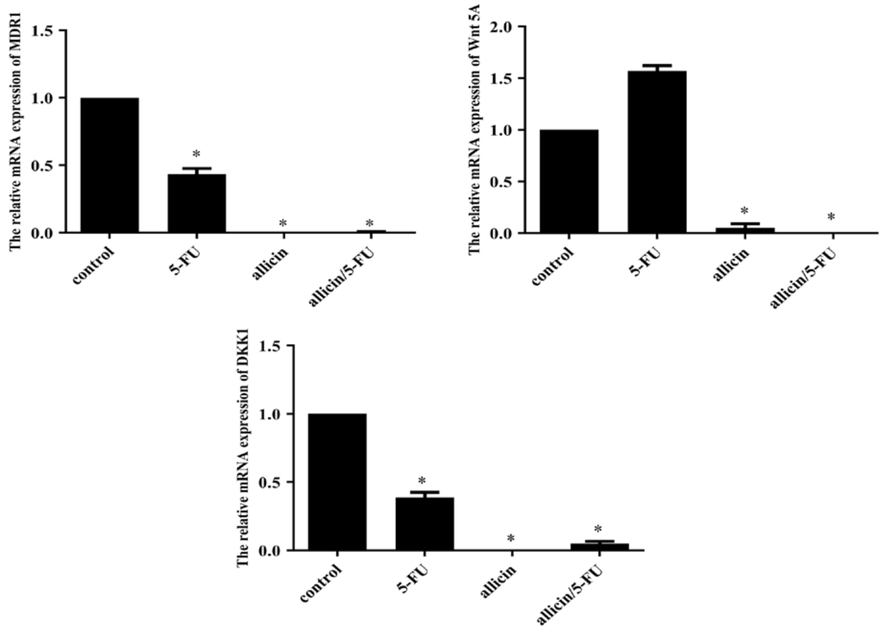
NN conceived the idea and supervised the study. PK, MAV, and RP wrote the article and performed all the experiments, all authors contributed to final approval of the manuscript.

Acknowledgment

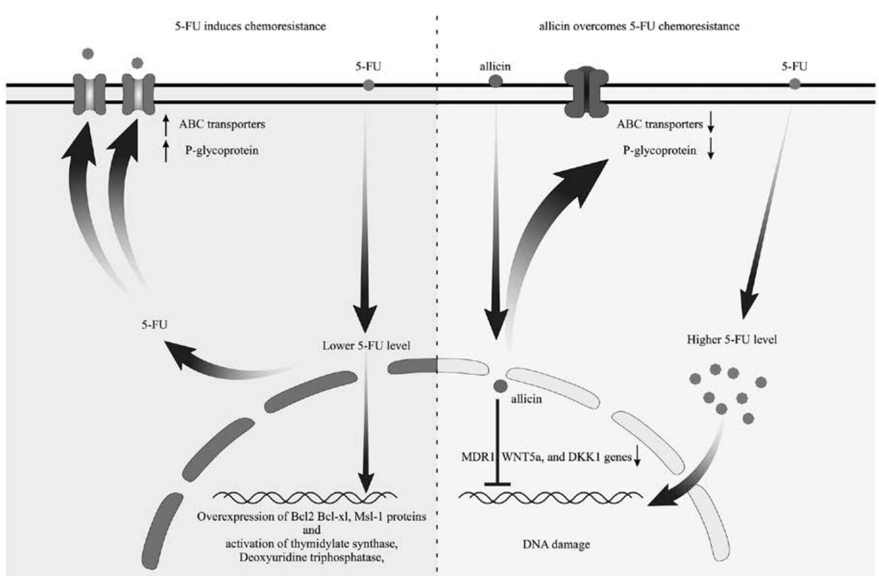
This study is a part of MSc thesis (IR.ARUMS.REC.1398.490) and a medical student research grant (IR.ARUMS.REC.1398.300) supported by Ardabil University of Medical Sciences. The authors would like to thank Mr. Farhad Pouremamali for gifting a 5-FU-resistant MKN-45 gastric cancer cell line.

Conflict of Interest

All authors declare that they have no conflict of interest.



▶ **Fig. 5** The mRNA expression level of *MDR1*, *DKK1*, and WNT5A determined in 5-FU (μg/ml), allicin (μg/ml), and allicin/5-FU treated 5-FU resistant MKN-45 gastric cancer cells. $^{*}P < 0.05$ as compared to control.



▶ **Fig. 6** Schematic diagram showing 5-FU-chemoresistance and possible action of allicin on gastric cancer.

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