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GSTs, MRP and Apoptototic Markers in DLD-1 Human Colon Cancer Cell Line Before and After 5-FU Treatment

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Abstract

Multidrugresistance is an important factor limiting the effect of chemotherapy on cancer treatment. Disorders of drug transport and apoptosis, deterioration of redox homeostasis are among the main mechanisms that lead to multidrug resistance. The aim of this study was to determine the effect of 5-FU on GST isozymes, drug resistance proteins and apoptotic proteins before and after 5-Flourouracil application on DLD-1 colon cancer cell line. The cytotoxic effect of 5-FU was measured by WST-1test and, the efficiency of drug application was, also, proved by double staining via Hoechst 33342 with Propidium iodide. Next, the expression levels of GST isozymes, drug resistance proteins and apoptotic proteins were determined by immunocytochemistry. The cytotoxic effect of 5-FU at different doses on DLD-1 colon cancer cell line was determined by WST-1 method. MRP-2, 3, 6, 7 of drug resistance proteins; GSTA1, GSTM1, GSTT1, GSTZ1, GSTK1 and GSTO1 of GST proteins; bcl-2, caspase-3, p38, and p53, which are apoptotic proteins, have higher expression in the drug-treated DLD-1 cell line. GSTS1, MDR-1 and MRP-1expressions were not immunocytochemically different. It was determined that there is a direct correlation between the level of cytotoxicity and applied drug concentration. The cytotoxic effect of the drug increased with the increase in the dose of the drug. In this study, as first in the literature, the expression levels of some apoptotic markers, GST isozymes and drug resistance proteinswere evaluated togetherand except GSTS1, MDR-1 and MRP-1, they were all upregulated with respect to the control group after 5-FU administration.

Key words: Multidrug resistance, GST isozymes, apoptosis, MRP, 5-FU, DLD-1

1. Introduction

Colorectal cancer (CRC) takes place in the second rank among the deaths related to cancer (Cancer today, 2021). In the treatment of colon cancer, two treatment methods are commonly applied, chemotherapy and surgery. Chemotherapy is applied especially, in advanced colon cancer patients after surgery (adjuvant chemotherapy) or preoperative (neoadjuvant chemotherapy) to shrink the tumor (Stein et al., 2011; Cunningham et al., 2010). However, drug resistance limits the effectiveness of chemotherapy applied in cancer treatment (Longley et al.,2005). It may ocurby inherited resistance or acquired resistance during chemotherapy (Gong et al., 2012; Baguley et al., 2010).

The mechanisms regarding drug resistance have been divided into two categories: cellular and non-cellular. Non-cellular mechanisms include external factors such as limited vascular accessibility and tumor microenvironment (Holohan et al., 2013).Cellular factors, however, include mechanisms such as overexpression of MDR transporters, disruptions in the apoptotic pathway, changes in drug metabolism, changes in DNA repair and drug targets (Fojo et al., 1987, Gottesman et al., 2006; Gong et al., 2012; Gottesman et al., 2002; Szakács et al., 2006; Baguley et al., 2010).

5-FU is a chemotherapy agent used primarily in the treatment of colorectal cancer (Longley et al.,2003). Today, 5-FU-based chemotherapeutic regimens such as FOLFOX (FA, 5-FU and oxaliplatin), FOLFIRI (FA, 5-FU and irinotecan), FUFA (FA and 5-FU) are used in the systematic treatment of colorectal cancer (Emilyet et al., 2016). However, although effective anticancer agents are available, treatments may become ineffective due to the development of resistance to these drugs. Therefore, it is necessary to cope with MDR to increase tumor chemosensitivity and to improve the prognosis of CRC patients (Dongxıngat et al., 2017).

The majority of ATP dependent carrier proteins with multiple drug resistance proteins and involved in the transport of xenobiotics have the MDR phenotype; Over-expression of GST in tumor cells is in line with the development of MDR (Morrow et al., 2006; Towsend et al., 2003). Several clinical studies have shown that high levels of p53 accumulation are associated with resistance to 5-FU-based chemotherapy (Elsaleh et al., 2001; Liang et al., 2002; Ahnen et al., 1998). MAPK has been associated with anti-apoptotic processes and chemo-resistance development in CRC cells (Xu et al., 2009).

The aim of this study was to determine the effect of 5-FU on GST isozymes, drug resistance proteins and apoptotic proteins before and after 5-FU treatment in DLD-1 colon cancer cell line. Thus, by revealing the relationship of 5-FU with the resistance mechanism proteins in the study, it was aimed to determine the effects of these proteins on the development of resistance in colon cancer.

2. Material and Methods

2.1. Cell line

Human DLD-1 cell line was obtained from American Type Culture Collection. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37 ˚C in a humidified atmosphere of 95% air and 5% CO_2 with medium changes every 2 days.

2.2. Chemical reagents and antibodies

5-FU was obtained from DEVA**.** MDR-1, MRP-1,2,3,6,7,GSTZ1,GSTO1 and p38 antibodies were purchased from Bioss. p53, GSTP1, bcl-2 and caspase-3 from Boster.GSTS1 and GSTA1 antibodies from Santa Cruz.GSTM1,GSTK1 and β-actin antibodies from Cell Signaling, GSTT1 from ProteinTech.

2.3. WST-1 assay for testing cytotoxicity

Cell viability was determined by WST-1 assay as described in Çiftçi et al., 2013. Cells were cultured in 48-well plates for 24 h before the treatment. DLD-1 cells were incubated in four different concentrations of 5-FU (5 µg/ mL, 2.5 µg/ mL, 1.25 µg/ mL, 0.625 µg /mL).

WST-1 reagent (10 µL) was added into each well and incubated for 4 h. The plates were immediately read in an ELISA Microplate Reader (BioTek, USA) at 440-nm and 630-nm reference wavelengths.

2.4. Analysis of apoptotic and necrotic cells (live/dead double staining)

The Double Staining Detection Kit (Serva, ISRAEL), containing Hoechst 33342 and Propidium iodide (PI), was used to identify of apoptotic and necrotic cells in culture. DLD-1 cells ($10x10^3$ cells/well) were grown in DMEM with RPMI 1640 supplemented with 10% fetal calf serum and 1% penicillin-streptomycin at 37 °C in 5% CO_2 humidified atmosphere, in 48-well plates. DLD-1 cells were treated with different concentrations of 5-FU (0.625-5 µg/ mL) for 24h. The control group was treated with cell medium only. At the end of the incubation period, apoptotic cells and FITCH (480-520 nm), necrotic cells were evaluated by using a DAPI filter in fluorescence microscope (Fluorescence Inverted Microscope, Leica DMI600, Germany).

2.5. Immunocytochemical staining

DLD-1 colon cancer cells were treated by a 5-FU dose of 2.5 µg mL-1 every two days for 6 times. Cells were removed by trypsin and preparations with cytospin were completed. MDR-1, MRP1,2,3,6-7 drug resistance proteins, GST isozymes (GSTA1, GSTP1, GSTM1, GSTT1, GSTO1, GSTZ1, GSTK1, GSTS1) and apoptotic proteins(p38,p53,Bcl-2,caspase-3) expressions were determined by immunocytochemical stainingas described by Kocdogan et al., 2020. For an indirect immunocytochemical procedure, cytology specimens were treated with 3% H2O2 for 10 minutes, taken to water, and then rinsed in PBS (pH: 7.4) for 5minutes. Nonspecific protein binding was blocked on specimen by incubating with blocking solution for 10 minutes. The primary antibodies, GSTA, GSTP, GSTM4, GSTT1and p53 were used at GSTA1,1:50; MDR-1 andGSTT1 1:100; p53, 1:200; Bcl-2 and

GSTM1 1:250; GSTS1and GSTO1 1:300; MRP1,2,3,6-7, GSTK1,GSTZ1 and p38,1:500;GSTP1 and caspase-3 1:1000 dilutions, respectively, and incubated for 1 hour at roomtemperature. Specimens were washed with PBS buffer (pH: 7.4) and incubated in biotinylated secondary antibody solution for 10 minutes. Diaminobenzidine (DAB) served as the chromagen and Mayer's hematoxylin as the counterstain. The staining intensity was quantitated in each case. A scoreof 0 to 3 positivity was given to each cell. The radar plots of the changes in immunocytochemical expression levels of tested proteins were prepared by the licensed software SigmaPlot13.0.

3. Results

According to the findings, the cytotoxic effect of the drug increased due to the increase in the drug dose and decreased the% viability value in cancer cells. As the 5-FU concentration applied to DLD-1 cells increases, cell viability decreases (Fig. 1). The IC_{50} value of 5-FU, which is the amount of drug component that causes 50% of the cells to undergo apoptosis, was calculated as 7.85 µg/mL.

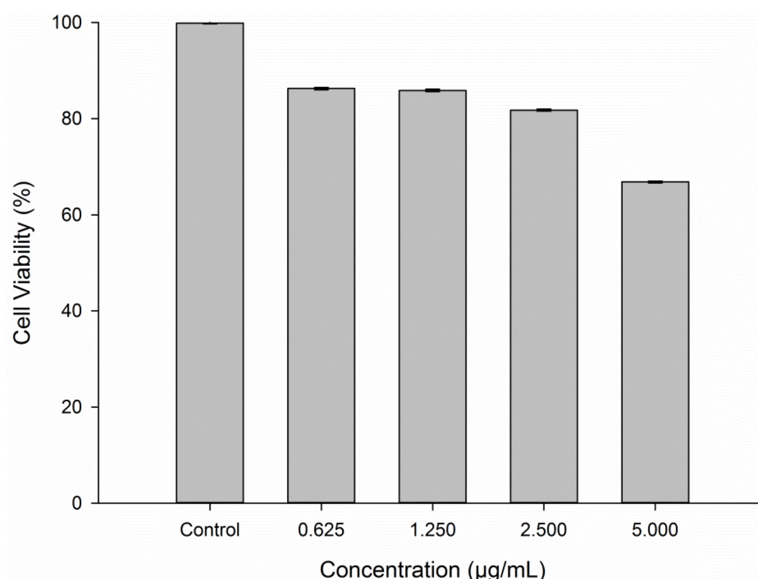

Figure 1. The results of WST-1 assay used to measure cell viabilities of DLD-1 at different concent rations of 5-FU. Error barsindicate ± standard error.

According to double staining results, it was shown that as the 5-FU dilution applied in DLD-1 cell line increased, the percent apoptotic and necrotic values increased (Table 1**).** Cytotoxicity test and double staining results are compatible with each other and those, clearly, put forth the functioning and efficiency of 5-FU.

Table 1. Apoptotic / necrotic index results in DLD-1 cell lines treated with different doses of 5-FU. Data are expressed as mean ± standard error as calculated from 3 separate experiments.

Concentration (µg/mL)	% Apoptosis	% Necrosis
5	5,90 ±2,4	2,55 ±1,2
2,5	2,57 ±1,6	1,92 ±1,0
1,25	2,74 ±1,6	0,73 ±0,8
0,625	1,56 ±0,8	0,69 ±0,6
Negative control	0,74 ±0,7	1,99 ±0,6
Positive control	9,64 ±2,4	32,14±4,1

At the microscope images, arrows show some of the apoptotic cells in Fig 2. Cell nuclei that have undergone apoptosis look bright, fragmented, and those that do not undergo apoptosis appear pale blue.

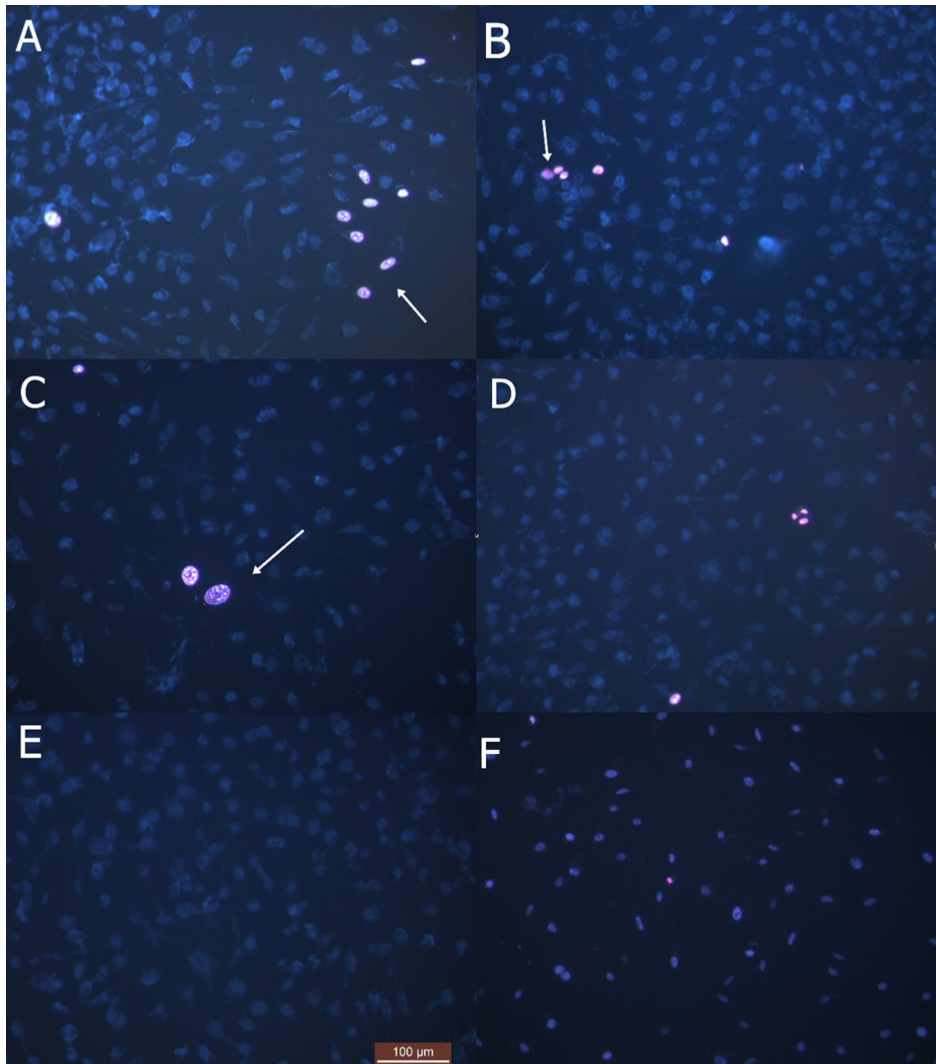

Figure 2. The images of apoptotic cells in DLD-1 cell culture, after 5-FU treatment at different concentrations.

Photos were taken with a Leica inverted fluorescent microscope at 200X magnification.

A: 5 -FU, 5 µg / mL apoptotic result;B: 5-FU, 2.5 µg / mL apoptotic result;

C:5- FU, 1,25 µg/mL apoptotic result; D: 5-FU, 0,625 µg/mL apoptotic result;

E: Negative control apoptotic result ;F: Positive control apoptotic result

Necrotic cells are stained red with propodium iodide. Arrows show some of the necrotic cells in Figure 3.

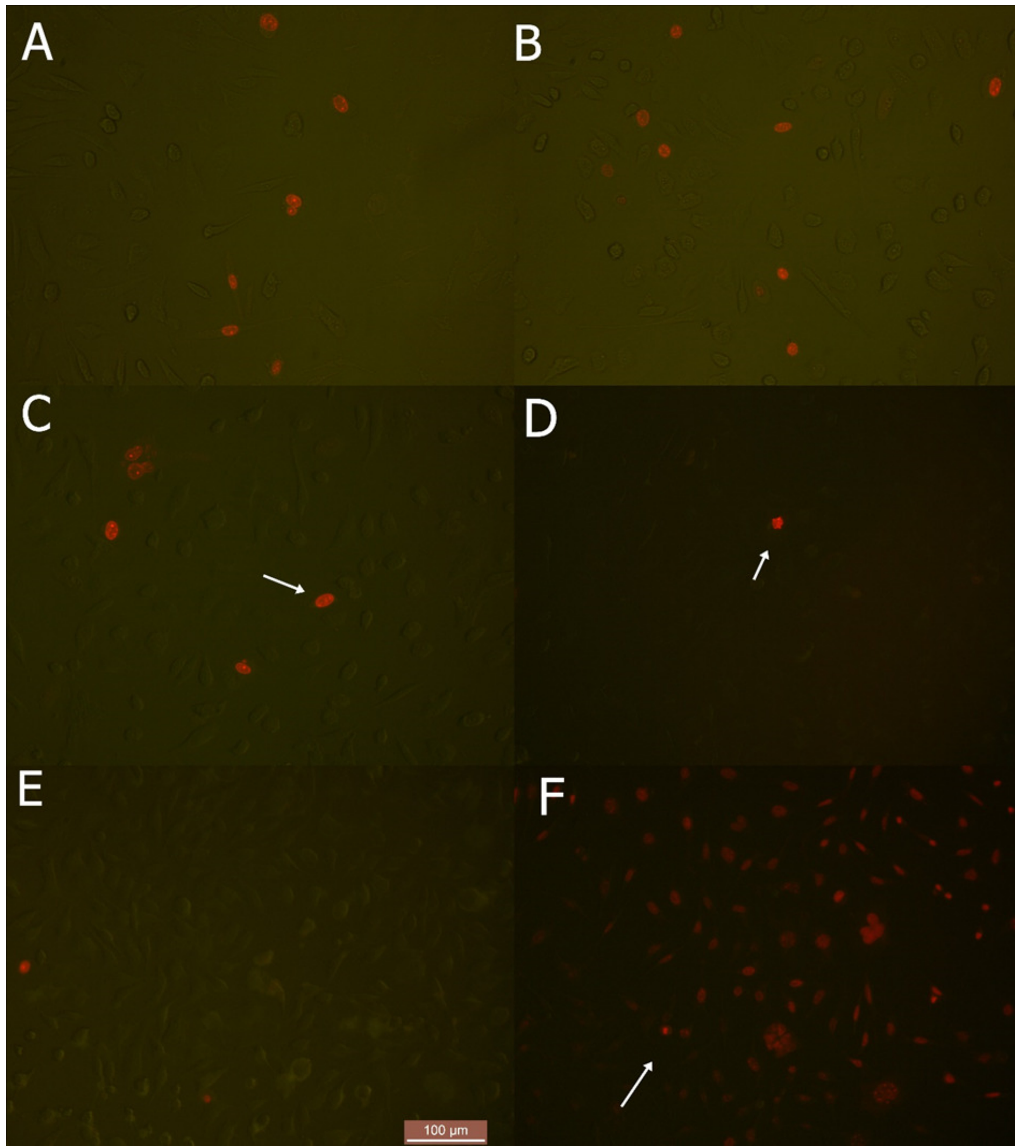

Figure 3. The images of necrotic cells in DLD-1 cell culture, after 5-FU treatment at different concentrations. Photos were taken with a Leica inverted fluorescent microscope at 200X magnification.

A: 5- FU, 5 µg / mL necrotic result; B: 5- FU, 2,5 µg/mL necrotic result;

C: 5 -FU, 1,25 µg/mL necrotic result; D: 5- Fu, 0,625 µg/mL necrotic result;

E. Negative control necrotic result; F: Positive control necrotic result

The immunocytochemical staining demonstrated the upregulation of drug-resistant proteins, apoptotic markers and GST isozymes upon application of 5-FU on DLD-1 cancer cell line (Fig. 4). MRP-2, MRP-3, 6, 7 have higher expression in the drug-treated DLD-1 cell line with respect to untreated cell line. GSTA1, GSTM1, GSTT1, GSTZ1, GSTK1 and GSTO1 proteins have higher expression in the drug-treated DLD-1 cell line than in the untreated cell line. Bcl-2, caspase-3, p38, and p53, which are apoptotic proteins, have higher expression in the

drug-treated DLD-1 cell line than the untreated cell line. MDR-1, MRP-1, GSTS1 proteins have a negative expression in the drug-treated DLD-1 cell line than the untreated cell line.

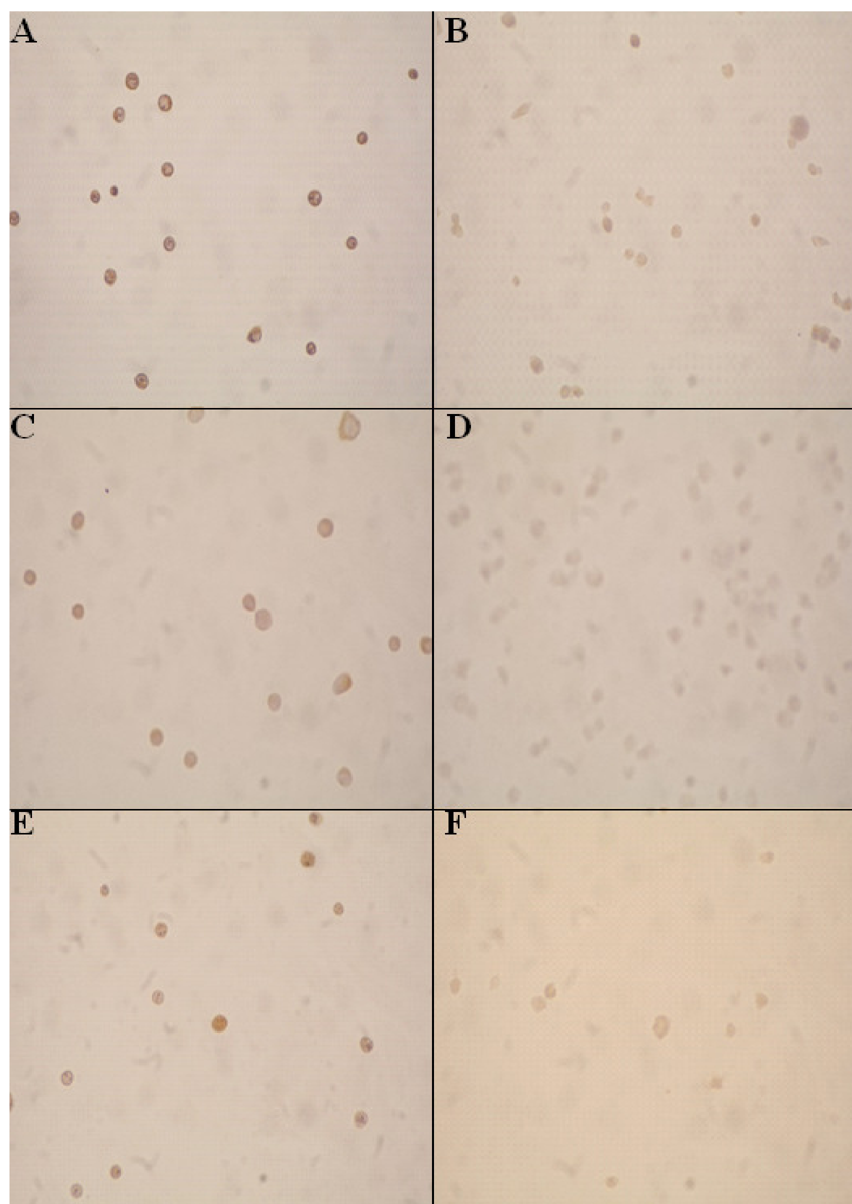

Figure 4. Immunocytochemical detection of expression levels of some markers subjected in the study.

A: Immunocytochemistry of Bcl-2 showing positive (+3) staining in the 5-FU-treated DLD-1 cell line.

B: Immunocytochemistry of Bcl-2 showing positive (+1) staining in the untreated DLD-1 cell line.

C: Immunocytochemistry of GSTZ1 showing positive (+3) staining in the 5-FU-treated DLD-1 cell line.

D: Immunocytochemistry of GSTZ1 showing (-) staining in the untreated DLD-1 cell line.

E: Immunocytochemistry of p53 showing positive (+2) staining in the 5-FU-treated DLD-1 cell line.

F: Immunocytochemistry of p53 showing (-) staining in the untreated DLD-1 cell line.

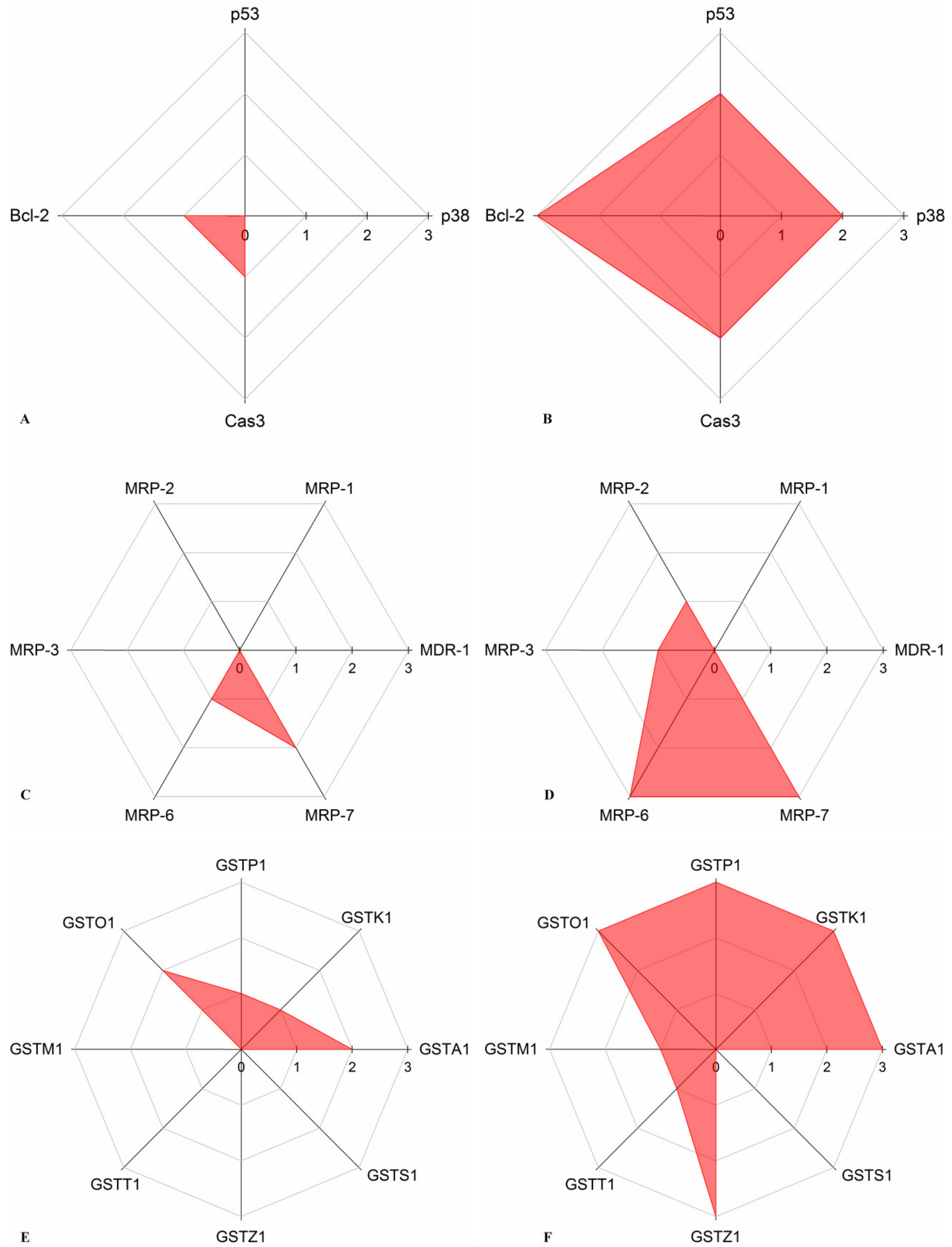

Figure 5. The radar plots of the changes in immunocytochemical expression levels of tested proteins.

The scores of apoptotic markers in control (A) and 5-FU-treated (B) groups; the scores of drug resistance proteins in control (C) and 5-FU-treated (D) groups; and, the scores of GST isozymes in control (E) and 5-FU-treated (F) groups were represented. The radar plots were prepared by the licensed software SigmaPlot 13.0.

All of the apoptotic markers were upregulated in 5-FU-treated groups with respect to control (Fig. 5) resulting from a considerable increase in the stained area of the radar plot. There was a selective increase in the expression of drug resistance proteins; such that, MDR-1 and MRP-1 showed negative immunostaining as in the control group. All GST isozymes, except GSTS1, were upregulated and the total stained area of the radar plot for the treatment group has become larger.

4. Discussion

The studies on cancer generally use genetically modified lab organisms, xenografts, primary tumors, paraffin-embedded samples, tumour primary cell cultures or basically cancer cell lines (Van et al., 2008). Among those, especially for drug research, cell lines emerge as a better alternative because of their being easy to use, manipulate and molecularly characterise. The characterisation is vital because it enables us to get information on the complexity of cancer in the aspect of cellular metabolism. By this way, it would be possible to develop novel anticancer drugs, to understand the action mechanism of existing ones and to uncover their resistance/sensitivity patterns in different cancer types (Nakatsu et al., 2005).

Cancer cell lines have diverse responses and levels of resistance against chemotherapeutical agents, as colon cancer cell lines are more susceptible to drugs having the ability to prevent cell proliferation by inhibiting enzymes functioning in DNA synthesis, such as 5-Fluorouracil.5-FU has been used as an important chemotherapeutic for patients with colorectal cancer over the last 50 years (Bahremi et al., 2018).

The resistance or sensitivity of some types of cancer to different drugs forces researchers to discover and test new biomarkers, or it makes them study on various signalling pathways associated with the therapeutic response (Neve et al., 2006). The common and popular apoptotic markers such as p53, p38, Bcl-2, Bax and caspase family are the benchmarks of the evaluation of the state of the cells and their response against a drug active component. Apoptotic proteins Bcl-2, p53, p38 and caspase-3 showed higher expression in DLD-1 colon cancer cells exposed to 5-FU, with respect to control cells. Similar results were reported in the literature for Bcl-2 (Nita et al., 1998), p53 (Choi et al., 2012), p38 (Fiore et al., 2016) and caspase-3 (Gong et al., 2022) for 5-FU treated DLD-1 cell line.

It has been found that the inhibition of p38 MAPK could increase the effectiveness of 5-FU to kill colorectal cancer cells (Yang et al., 2011).

In the 5-FU-induced apoptosis resistance study in 8 different colon cancer cell lines with different p53 and Bax states, it has been noted that levels of Bcl-2, Bcl-xL and Bax could contribute to the determination of the resistance of most colon tumor cells to long-term 5-FU therapy, regardless of their p53 status (Violette et al., 2002). Over-expression of Bcl-2 has been shown to be associated with loss of efficacy of chemotherapy drugs like 5-FU, ADM and MMC in patients with gastric cancer (Geng et al., 2013).

The characterization of cancer cell lines for the presence and diversity of Multidrug Resistance Domains (MDRs) and Multidrug Resistance Proteins (MRPs) is essential in anticancer drug testing. There are reports stating

the upregulation of those proteins in 5-FU treated colorectal cancer cell lines, such as for MDRs (Ghafouri-Fard et al., 2021) and for MRPs (Reipsch et al., 2021; Namwat et al., 2008; Jung et al., 2020).

Glutathione S-transferases are important members of drug-metabolizing enzymes and having roles in cancer progress and chemoresistance. With the entrance of the anticancer agent into the tumor cell, GSH level, the physiological substrate of the GST enzyme, and GST expression, generally, increase. There are records in the literature stating that they develop resistance against some chemotherapeutic agents as a result of a coordinated increase in GST isozymes with specific MRPs, in tumor cells (Sau et al., 2010; Aksoy, 2010). In the current study, 5-FU application on DLD-1 cells led a concurrent increase in GSTA1, GSTT1, GSTM1, GSTO1, GSTZ1, GSTK1, GSTP1, MRP2,3,6,7 expressions, compared with the control group. As mediators of signalling pathways in cell proliferation and cell death, GST isozymes, especially GSTP1, are of considerable importance (Laborde et al., 2010). Silencing GSTP-1 increased the sensitivity of SNU-407 colon cells to anticancer agent 5-fluorouracilindicating that GSTP-1 might be a clinically useful biomarker for colon cancer and a target for anti-colon cancer drugs (Zhang et al., 2014). The overexpression of some GST isozymes were reported in studies on colon cancer cell lines (Very et al., 2017; Dang et al., 2005; Chao et al., 1992; Hoban et al., 1992; Yang et al., 2016). Some of those studies, also, aimed to determine both drug resistance mechanisms and their relations with GST isozymes. In this way, it would be possible to reveal new mechanisms which might reverse the resilience and increase the success of chemotherapy in colon cancer.

5. Conclusion

As a conclusion, it was determined that the expression of apoptotic proteins, GST isozymes (except GSTS1), drug resistance proteins (except MRP-1) after 5-FU administration in DLD-1 colon cancer cell line were higher than the group that did not receive 5-Flourouracil.To our knowledge, this is the first study presenting the changes in expressions of some major apoptotic markers and drug resistance proteins in correlation with all cytoplasmic GST isozymes, in the short term response of colorectal cancer cells to highly effective chemotherapeutic drug of 5-Fluorouracil. We suggest that further studies with different cell lines are needed in order to fully determine the roles of those proteins on the development of 5-FU drug resistance in colorectal cancer.

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Conflicts of Interests

The authors declare no conflict of interest, financial or otherwise.

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