DOI: 10.25092/baunfbed.757286 *J. BAUN Inst. Sci. Technol., 22(2), 698-708, (2020)*

Multidrug resistance gene expression response to cisplatin and 5FU treatment in hepatoma, prostate and colon cancer cells

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Geliş Tarihi (Received Date): 01.11.2019
Kabul Tarihi (Accepted Date): 01.05.2020

Abstract

Variable expression of drug transporters at the plasma membrane of tumor cells contributed to drug resistance. The aim of the present study was to explore the expression levels of MRP2 and MRP3genes in Cisplatin and 5 fluorouracil (5FU) treated Hep3B, PC3 and HT29 cell lines. MTT assay was used to determine the cytotoxic effects of cisplatin and 5FU. The results from the MTT assay revealed that PC3 cell line is the most sensitive for cisplatin treatment presenting the lowest IC_{50} value (0.5 µg/ml) for 48h. 5FU treatment altered the proliferation of PC3 cells with the IC_{50} values 6.0 µg/ml for 48 h and 8.2 µg/ml for 72h. The mRNA expression levels of MRP2 and MRP3 were determined by quantitative reverse transcription polymerase chain reaction. qRT-PCR results revealed that while the expression levels of MRP3 mRNA was generally down regulated in all cell lines treated with cisplatin and 5FU, MRP2 was upregulated in PC3 and Hep3B cells.

Keywords*: cytotoxicity, cisplatin, 5FU, multidrug resistance.*

Karaciğer, prostat ve kolon kanseri hücrelerinde cisplatin ve 5FU uygulamasına çoklu ilaç direnci gen ifadesi yanıtı

Öz

Tümör hücrelerinin plazma membranında ilaç taşıyıcıların değişken ekspresyonu, ilaç direncine katkıda bulunmuştur. Bu çalışmanın amacı Hep3B, PC3 ve HT29 hücre

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hatlarında Cisplatin ve 5-florourasil (5FU) uygulamasının MRP2 ve MRP3 genlerinin ekspresyon seviyelerine etkilerini araştırmaktır. Cisplatin ve 5FU'nun sitotoksik etkilerini belirlemek için MTT metodu kullanıldı. MTT testinden elde edilen sonuçlar PC3 hücre hattının, 48 saat cisplatin uygulamasına en duyarlı hücre hattı olduğunu göstermiştir ve bu zaman aralığı için en düşük IC_{50} değerini (0.5 µg / ml) göstermiştir. 5FU uygulaması PC3 hücrelerinin çoğalmasını farklı şekillerde değiştirmiştir, 48 saat için 6.0 µg / ml ve 72 saat için8.2 µg / ml IC_{50} değerleri elde edilmiştir. MRP2 ve MRP3'ün mRNA ekspresyon seviyeleri, kantitatif ters transkripsiyon polimeraz zincir reaksiyonu ile belirlendi. qRT-PCR sonuçları, MRP3 mRNA ekspresyon seviyesinin cisplatin ve 5FU ile muamele edilmiş tüm hücre hatlarında genel olarak azalmaya neden olmasına ragmen, MRP2'nin PC3 ve Hep3B hücrelerinde genel olarak artışa neden olduğu belirlenmiştir.

***Anahtar kelimeler**: sitotoksisite, cisplatin, 5FU, çoklu ilaç direnci.*

1. Introduction

Chemotherapy is the only systemic treatment for many malignant tumors. 5-Fluorouracil (5FU) and Cisplatin (cis-diamminedichloroplatinum (II)) are both widely used chemotherapeutic agents for variety of tumors. Cisplatin is a well-known DNA intercalating drug that builds intra-strand DNA adducts to disrupt DNA synthesis. Cisplatin is commonly used as a chemotherapeutic agent for hepatocellular carcinoma (HCC) but cisplatin cannot successfully increase the survival rate for advanced HCC patients due to intrinsic or acquired drug resistance caused by multidrug resistance-associated proteins (MRPs) [1]. Recent studies showed that cisplatin also interacts with other components of the cells, such as cytosolic proteins, mitochondrial proteins and mitochondrial DNA. Multidrug resistance (MDR) is one of the main problem for the therapy of cancer, may be come out in the first step of treatment or during the period of treatment [2,3]. Efflux drug transporters and uptake drug transporters have primary effect on drug toxicity and efficacy [4]. Multidrug resistance phenotype of cancer cells are generally composed by ATP-binding cassette transporters, breast cancer resistance protein or several MRPs contributed to extruding chemotherapeutic drugs or their metabolites from cells [5]. Altered expression of these drug transporters is another key factor for the resistance to chemotherapeutics. In human hepatocytes, there are several transporters that take up drugs through membrane and efflux them into bile [6]. Multidrug resistance-associated protein 2 (MRP2) and multidrug resistance-associated protein 3 (MRP3) are the most prominent members of these transporters and expressed in hepatocytes under physiologic conditions. As MRP2 and MRP3 are already expressed under physiologic conditions in the liver, they are generally assumed to contribute to anticancer drug resistance HCC [7]. HCC is one of the most common cancers worldwide and is a frequent cause of cancer-related deaths [8]. HCC cells are often resistant to standard chemotherapy and tumor recurrence or metastasis is quite common in HCC patients.

In this study, we determined the (i) cytotoxic effects of the drugs, cisplatin and 5FU, in hepatocellular carcinoma cell line Hep3B, as a main model system, (ii) the mRNA expression levels of MRP2 and MRP3 were evaluated following drug treatments. The results obtained from Hep3B cells were also compared to other cancer cell lines, prostate adenocarcinoma cell line PC3 and colon cancer cell line HT29. We hypothesize

that alterations in the expression of drug resistance genes leads to many changes in cancer cells. Therefore the aim of this study was to gain an overview on the significance of MRP2 and MRP3 expressions in association with the cytotoxic effects of the drugs cisplatin and 5FU in Hep3B, PC3 and HT29 cells.

2. Materials and methods

2.1.Cell lines

Hep3B cell line was supplied from Cardiff University, Dr. Dipak P. Ramji's Laboratory. PC3 cell line was supplied from Ege University, Dr. Kemal Sami Korkmaz's Laboratory. HT29 cell line was from Yeditepe University. All cell lines were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum. The cultures were maintained at 37 °C in an incubator with air containing 5% (v/v) CO_2. Attached cells were trypsinized with 0.2% trypsin containing 0.025% ethylene diamidetetraacetic acid (EDTA) dissolved in PBS, pH=7.4, and the number of viable cells was counted using the trypan blue exclusion method.

2.2. In vitro Antiproliferative Activity

Cell viability was determined by MTT (3-(4,5- dimethylthiazole–2-yl)-2,5-diphenyl tetrazolium bromide) assay [9]. 5 x 10^3 cells per well were plated on to 96-well plates in 200 µl of medium and incubated for 24, 48 and 72h. After both treatments of various dilutions of Cisplatin (0.5µg/ml, 5µg/ml, 25µg/ml, 50µg/ml, 100µg/ml) and 5FU (5µg/ml, 50µg/ml, 250µg/ml, 1000µg/ml, 2500µg/ml), the cells were fixed by adding 20µl of MTT (5mg/ml) per well and incubated for 4h at 37ºC. Subsequently, the medium containing MTT was removed, and 200µl of acidified isopropanol (0.04N HCl) was added. Absorbance of each sample was measured at 550nm using a micro plate reader (Bio-Tek, model powerwave XS). All cytotoxicity experiments were performed in at least 18 wells. % inhibition of each concentration was determined by;

% inhibition =[1-(T/C)]x100

where, T is the mean absorbance of the treated cells and C the mean absorbance of the controls. The concentration of drug required to inhibit cell proliferation by 50% (IC_{50}) was determined by plotting the percentage of cell growth inhibition vs the chemotherapeutic drug concentration.

2.3.RNA extraction and Reverse Transcriptase Reaction

5 µg/ml Cisplatin and 2500 µg/ml 5FU were applied to cells that were cultured as $2x10^6$ cells per 25 cm^2 flasks for 48 and 72h. After each time intervals cells were trypsinized with 0.2% trypsin containing 0.025% ethylene diamidetetraacetic acid (EDTA) dissolved in PBS. Pellets were collected for RNA isolation.

Total RNA was extracted from the human cancer cell lines using an RNAeasy Mini Kit (ROCHE), following the manufacturer's instructions and quantified by spectrophotometry. Total RNA (1µg) from each cell line was reverse-transcribed in 20 µL containing 3.5 µM of oligo(dT), 1U/µl of ribonuclease inhibitor, 1U/µl of reverse transcriptase, 1X buffer RT and 10mM of dNTP. Initially, RNA and oligo(dT) were mixed together and heated at 65˚C for 5 min. Other reagents were then added and incubated for 60 min at 42˚C and 10 min at 70˚C. Real-time PCR amplification was performed using specific primers for the genes of interest.

2.4.Quantitative Real Time reverse transcription polymerase chain reaction (qRT-PCR)

Drug transporters gene expression was quantified by qRT-PCR. Briefly, 1 µl cDNA, 0.5 µl of each pairs of primers (50 ng/µl) and 5 µl of Light Cycler-Fast Start DNA Master SYBR Green I mix (Roche) was get involved at the total volume of 20 µl. Reaction was run on the Light Cycler 485 instrument (Roche Applied Science, Mannheim, Germany). Primer sequences used for MRP2 [F-5'GCCAGATTGGCCCAGCAAA3';R-5'AATCTGACCACCGGCAGCCT3'], for MRP3 [F-5'GGGACCCTGCGCATGAACCTG3';R- 5'TAGGCAAGTCCAGCATCTCTGG3'] [10] and for the housekeeping gene human beta 2 microglobulin (Hβ2micro) [F-5'TTTCTGGCCTGGAGGCTATC3';R-5'CATGTCTCCATCCCACTTAACT3']

Samples were studied triplicate and the Ct value was defined automatically by the instrument. The relative change in gene expression was calculated by 2(-ΔΔC(T) method[11].

2.5. Statistical analysis

Standard deviations and p values were calculated using Mini Tab 14 software. Statistical differences were evaluated by one-way ANOVA analysis. $P < 0.05$ was accepted as significant difference.

3. Results

PC3 cells were treated with 0.5, 5, 50, 100 µg/ml of 5FU for 48 and 72h, the relative cell proliferation progressively decreased in a dose-dependent manner, as shown in Fig. 1.

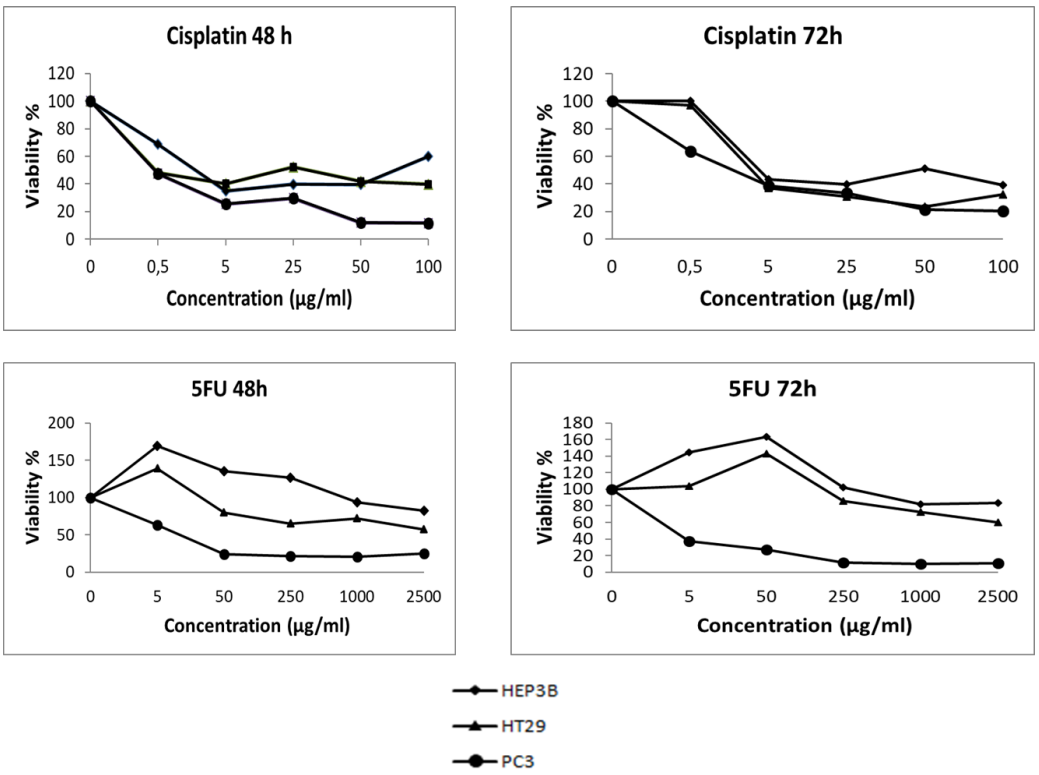

Figure 1. Diagrams of survival expressed as inhibition % after 48 and 72 h exposure to the drugs against the cell lines Hep3B, PC3 and HT29.

The most significant decrease of proliferation for PC3 prostate cells were obtained with the lowest IC_{50} value, 6.0 µg/ml for 48h. Treatment with 5FU also decreased the cell viability of HT29 and Hep3B cells for 48 and 72h; however, the reduction was not greater than 50% for the concentrations used in this study (Table 1).

Table 1. IC_{50} values of the cisplatin and 5FU drugs for Hep3B, PC3 and HT29 cell lines after 48- and 72-hour treatment periods (A and B, respectively).

(A) 48h	**IC_{50}(µg/ml)**		
Drug	***HEP3B***	***HT29***	***PC3***
Cisplatin	3,68	13,33	0,5
5FU	>2500	>2500	6,009

(B) 72h	**IC_{50}(µg/ml)**		
Drug	***HEP3B***	***HT29***	***PC3***
Cisplatin	12,96	3,23	0,5
5FU	>2500	>2500	8,24

*The IC50 values are averages of two independent determinations

As indicated in Fig. 1, in the aftermath of 48 and 72 h administration, cisplatin significantly decreased cell proliferation of all cell lines. The data in Table 1 represents, cisplatin effected the proliferation of PC3 prostate cells more drastically compared to other cell lines, with the lowest IC_{50} values for 48 and 72h.

Real time PCR results for endogenous MRP2 and MRP3 are shown for Hep3B, PC3 and HT29 cells in Fig.2. The mRNA expression of MRP3 was found significantly different among the cell lines. HT29 cells showed 2.3 fold higher expression of MRP3 compared to Hep3B and 12.4 fold higher expression compared to PC3. MRP2 mRNA level in Hep3B was determined 1.4 fold higher than HT29 cells and 3.2 fold higher than PC3 cells.

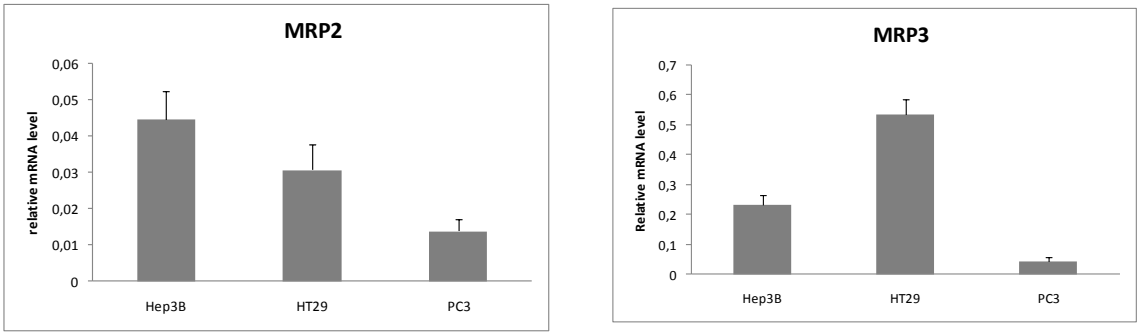

Figure 2. Relative mRNA levels of MRP2 and MRP3 in cell lines, Hep3B, HT29 and PC3.

Differential mRNA expression levels of drug transporters MRP2 and MRP3 were evaluated by qRT-PCR from cisplatin and 5FU treated cells for 48 and 72h. (Fig.3 and Fig.4).As the results of qRT-PCR analysis, comparing controls with cisplatin and 5FU treatment groups, generally MRP3 gene showed lower expression in treatment groups (Fig.3 and Fig.4).

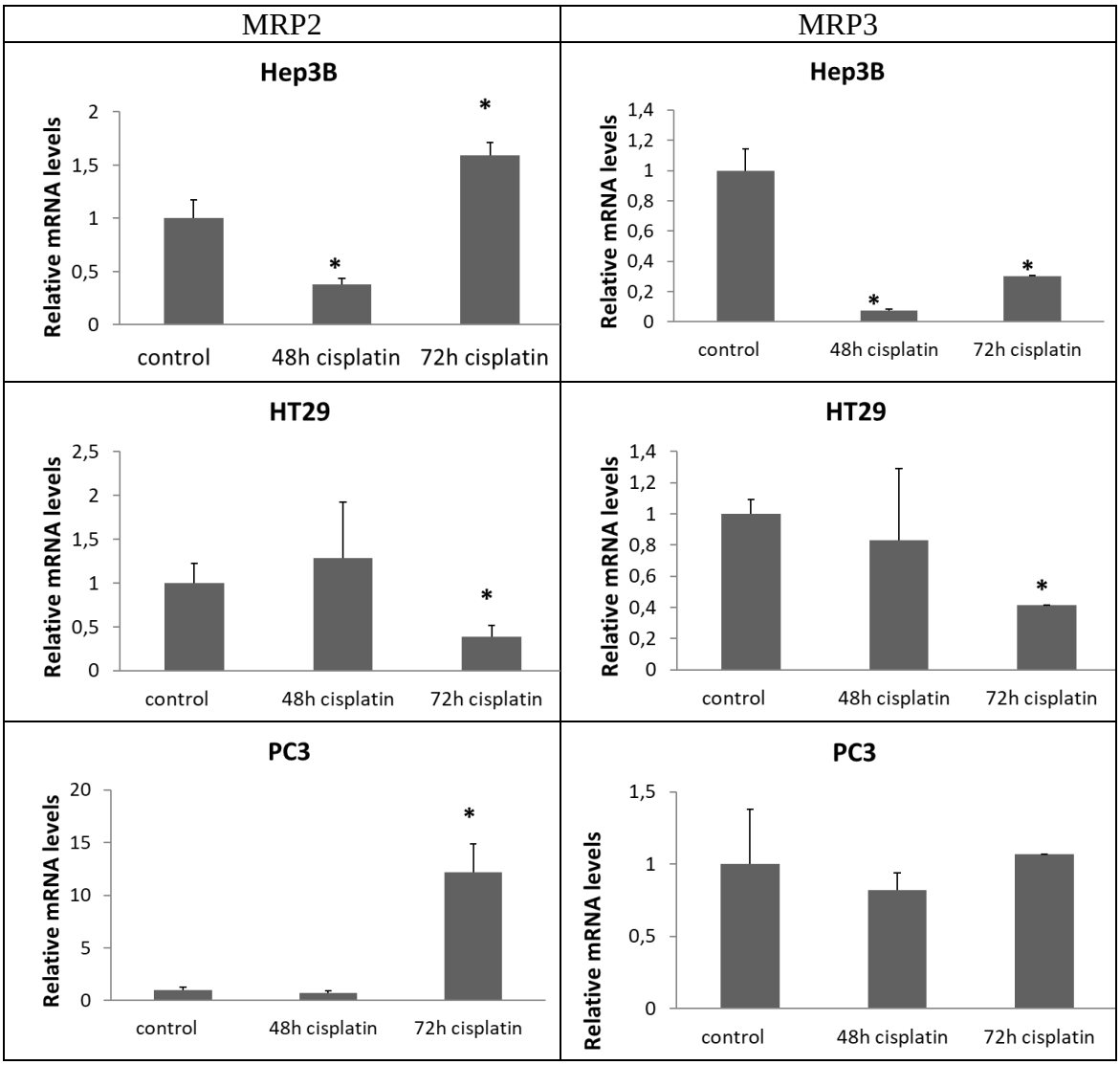

Figure 3. Changes in relative mRNA levels of MRP2 and MRP3 in cisplatin treated PC3, Hep3B and HT29 cell lines for 48 and 72h. (*p <0.05)

MRP2 mRNA level was found significantly upregulated in PC3 and Hep3B cell lines after cisplatin treatment (Fig.3). MRP2 mRNA also exhibited statistically higher expression in 5FU treated groups compared to controls in PC3 and Hep3B cells (Fig.4).

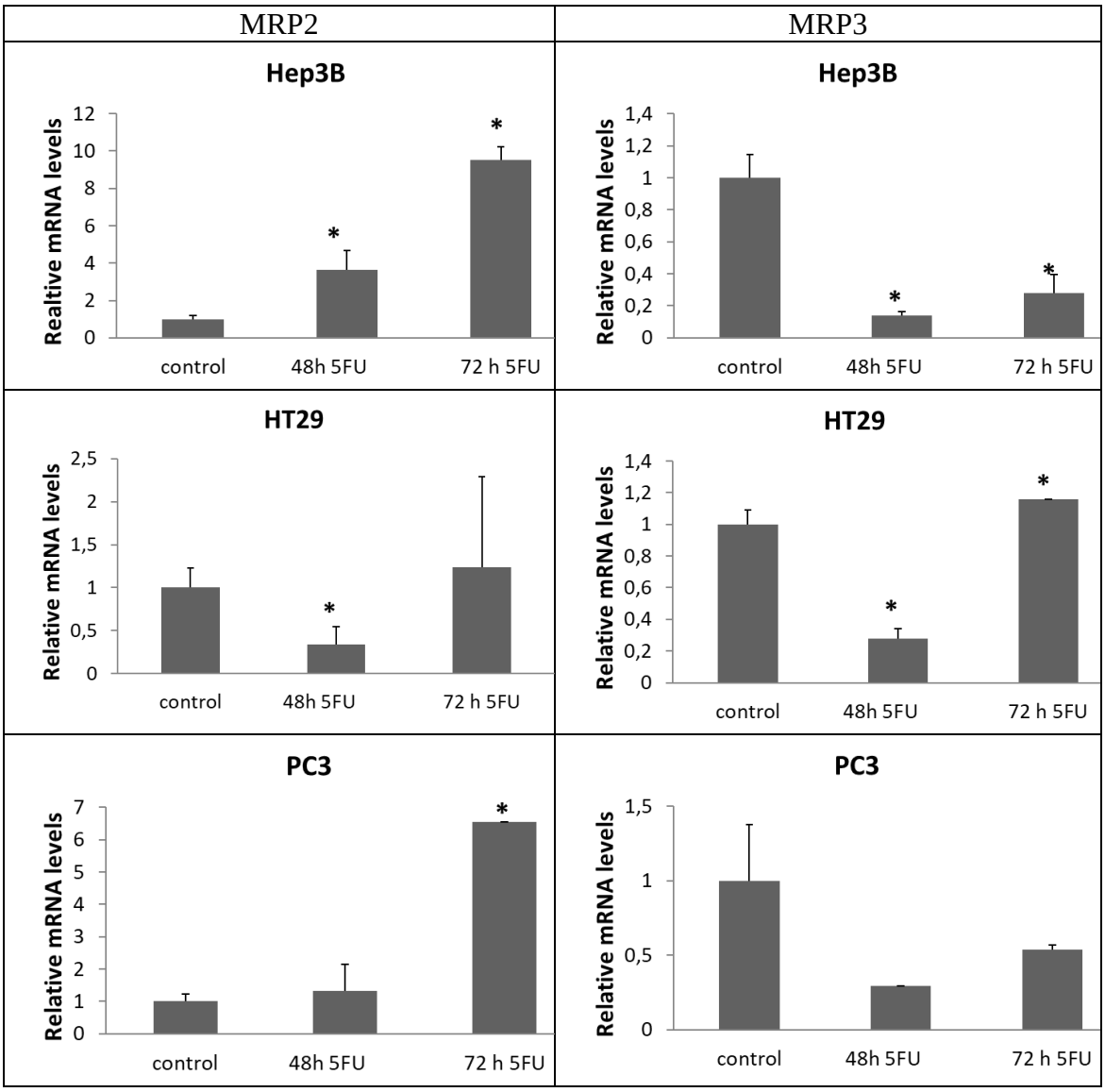

Figure 4. Changes in relative mRNA levels of MRP2 and MRP3 in 5FU treated PC3, Hep3B and HT29 cell lines for 48 and 72h. (*p <0.05).

4. Discussion

Cytostatics such as platinum drugs or taxanes are still main approach for several cancer therapies. But the occurence of MDR limits the application of chemotherapy. Cisplatin was one of the first chemotherapeutic agents employed for many human cancers and keeping its popularity as the most widely used anticancer agent. Doublet therapy with platinum drugs, generally cisplatin in combination with either infused 5FU or an oral 5FU, is current standard application in many countries [12, 13]. The aim of the current study was to investigate the cytotoxic effects of cisplatin and 5FU in mainly hepatoma cell model, Hep3B and other cell lines namely human colorectal adenocarcinoma cell line HT29 and human prostate cancer cell line PC3. The expression levels of MRP2 and MRP3 drug transporters were also investigated using quantitative RT-PCR analysis. MRP2 and MRP3genes encode drug transporter proteins and they are mainly expressed in the liver. Koike et al., showed that using anti-sense cDNA of MRP2 in HepG2 cells

resulted sensitization to important series of drugs like cisplatin. The study also showed that using anti-sense cDNA of MRP2 caused significant decrease in MRP2 and increase in cellular glutathione up to 4 fold [14]. Even several studies have already demonstrated the differential expression of drug transporters in HCC patients and hepatoma cell lines [6, 7, 15, 16]. We focused on simultaneous MRP2 and MRP3 expression in mainly hepatoma cell line Hep3B and in comparison, with prostate cell line PC3 and colon cancer cell line HT29. To this end, the cytotoxic activity of the cisplatin and 5FU drugs was tested by MTT assay for 48 and 72 hours. As there are quite much knowledge about the antiproliferative activity of these drugs on different cancer cell lines, MTT assay showed that PC3 is the most sensitive cell line to both cisplatin and 5FU treatment. 5FU treatment did not reduce the proliferation of the Hep3B and HT29 cells for the concentrations used in this study, but the proliferation of PC3 cells altered by 5FU treatment for 48 and 72 h treatment. Correlatively, the study comparing cytotoxic effects of cisplatin, paclitaxel and 5FU in two different hepatoma cell lines, Hep3B and HepG2, showed that while both cell lines were highly susceptible to cisplatin and paclitaxel at the concentrations lower than 100 μg/ml, 5FU was only active at higher concentrations than the other drugs, after 48h of treatment [17]. Separately cisplatin and 5FU-based therapies and/or combinations of cisplatin and 5FU are used frequently for many cancers but the most suitable formula has not been found [12]. Two randomized study including different groups of patients showed there is not survival advantage between the 5FU and cisplatin-based chemotherapy groups [16, 18]. The difference was that cisplatin group found more responsive to chemotherapy, 5FU alone was found less toxic [12, 18, 19].

Drug resistance is generally mediated by decrease in drug uptake and increase in drug efflux [6, 20]. The membrane-located proteins throw out the chemotherapeutic drugs or their metabolites from cells leading to resistance of drugs [5, 21]. Specifically, multidrug resistance related proteins, MRP2 and MRP3, were found to be important transporters in the liver, playing an important role in bile formation and excretion of various toxic substances [7]. Nies et al. analyzed the expression of MRP1, MRP2 and MRP3 in HCC samples and demonstrated that MRP2 and MRP3 mRNA expression was at least 10 fold higher than MRP1. As a result of their study, expression of MRP2 and MRP3, but not MRP1, was contributed to the MDR phenotype of HCC [15]. In this work, we analyzed the mRNA expression levels of drug transporters MRP2 and MRP3 by qRT-PCR from cisplatin and 5FU treated cells (Fig.3 and Fig.4). Cisplatin treatment caused decrease in MRP3 mRNA level compared to control groups in all cell lines however, MRP2 expression was upregulated in Hep3B and PC3 cells. MRP3 mRNA expression was down regulated in Hep3B and PC3 cells after treatment of 5FU for 72h. Yu and colleagues showed that down regulation of MRP3 caused significant increase of 5-fluorouracil or irradiation-induced cell apoptosis and attenuated tumor growth following irradiation in animal models [22]. MRP2, primarily accepts amphipathic cations and neutral compounds as substrates, seems to be involved in the resistance to cisplatin [15]. Another hepatic MRP isoform, MRP3, is localized to the basolateral membrane of hepatocytes and MPR3 also confers resistance to chemotherapeutic agents such as etoposide and methotrexate [15, 23, 24]. MRP3 was also reportedly correlates with resistance to doxorubicin in lung cancer and also found to be associated with resistance to vincristine and methotrexate [24, 25, 26]. As a result, in the current study we determined and compared the antiproliferative activity of cisplatin and 5FU drugs in human hepatoma cell line Hep3B, human prostate cancer cell line PC3, and human colorectal carcinoma cell line HT29. Cisplatin altered the proliferation of all cell lines,

but it was found to be the most effective against the prostate cancer cells. In addition, focusing on the MRP2 and MRP3 mRNA expression levels of cisplatin and 5FU treated cells, we obtained that while mRNA expression of MRP2 was generally upregulated in all cell lines, MRP3 expression was found to be decreased after cisplatin and 5FU application for 72h. The results obtained from our study related to drug resistance are limited and the definitive implication of the genes should be rather analyzed due to only mRNA expression is included in our study.

Acknowledgments

This work was partially supported by Scientific Research Council of Balikesir University (Project No.2003/19). The authors are also very grateful to Prof. Dr. Feray KÖÇKAR for her invaluable help.

References

[1] Wakamatsu, T., Nakahashı, Y., Hachımıne, D., Sekı, T., Okazak, K., The combination of glycyrrhizin and lamivudine can reverse the cisplatin resistance in hepatocellular carcinoma cells through inhibition of multidrug resistance-associated proteins, **Internatıonal Journal of Oncology,** 31, 1465-1472, (2007).

[2] Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M., Pastan I., Expression of a multidrug-resistance gene in human tumors and tissues. **Medical Sciences**, 84, 265-269, (1987).

[3] Ma, S., Hu, Y., Wang, F., Huang, Z., Chen, Y., Wang, X., Fu, L., Lapatinib Antagonizes Multidrug Resistance–Associated Protein 1–Mediated Multidrug Resistance by Inhibiting Its Transport Function, **Molecular Medicine,** 20, 390-399, (2014).

[4] Kawase, A., Norıkane, S., Okada, A., Adachı, M., Kato, Y., Iwakı, M., Distinct Alterations in ATP-Binding Cassette Transporter Expression in Liver, Kidney, Small Intestine, and Brain in Adjuvant-Induced Arthritic Rats, **Pharmacokinetics Pharmacodynamics Drug Transport and Metabolism,** 103,2556–2564, (2014).

[5] Warta, R., Theile, D., Mogler ,C., Herpel, E., Grabe, N., Association of Drug Transporter Expression with Mortality and Progression-Free Survival in Stage IV Head and Neck Squamous Cell Carcinoma, **PLOS ONE**, 9, 10, e108908, (2014).

[6] Namisaki, T., Schaeffeler, E., Fukui, H., Yoshiji, H., Nakajima, Y., Fritz, P., Schwab, M., Nies, A.T., Differential Expression of Drug Uptake and Efflux Transporters in Japanese Patients with Hepatocellular Carcinoma, **Drug Metabolism and Disposition**, 42, 2033–2040, (2014).

[7] Zollner, G., Wagner, M., Fickert, P., Silbert, D., Fuchsbichler , A., Zatloukal, K., Denk, H., Trauner, M., Hepatobiliary transporter expression in human hepatocellular carcinoma, **Liver International**, 25, 367–379, (2005).

[8] Prashanth, R., Tagore, S., Pradhyumna, M., Jeffrey, P,R., Update in global trends and aetiology of hepatocellular carcinoma, **Contemporary Oncology ,** 22, 3, 141–150, (2018).

[9] Mosmann, T.R., Coffman ,R.L., Two types of mouse helper T cell clone:implications for immune regulation, **Immunology Today,** 8, 7-8, 223-227, (1987).

[10] Tepsiri, N., Chaturat, L., Sripa, B., Namwat, W., Wongkham, S., Bhudhisawasdi, V., Tassaneeyakul, W., Drug sensitivity and drug resistance profiles of human intrahepatic cholangiocarcinoma cell lines, **World Journal of Gastroenterology,** 11, 18, 2748-2753, (2005).

[11] Livak, K.J., Schmittgen, T.D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(- DeltaDeltaC(T)) method, **Methods,** 25, 402–408, (2001).

[12] Kang, B.W., Kim, J.G., Kwon, O.K., Chung, H.Y., Yu, W., Non-platinum-based chemotherapy for treatment of advanced gastric cancer: 5-fluorouracil, taxanes, and irinotecan, **World Journal of Gastroenterology**, 14, 20, 5396-5402, (2014).

[13] Price, T.J., Shapiro, J.D., Segelov, E., Karapetis, C.S., Pavlakis, N., Van Cutsem, E., Shah, M.A., Kang, Y.K., Tebbutt, N.C., Management of advanced gastric cancer, **Expert Review of Gastroenterology and Hepatology,** 6, 199-208, (2012).

[14] Koike, K., Kawabe, T., Tanaka, T., Toh, S., Uchiumi, T., Wada, M., Akiyama, S.I., Ono ,M., Kuwano, M. A., Canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells, **Cancer Research,** 15, 57, 5475-5479, (1997).

[15] Nies, A.T., König, J., Pfannschmidt, M., Klar, E., Hofmann, W.J. Keppler, D., Expression of the multidrug resistance proteins MRP2 and MRP3 in human hepatocellular carcinoma, **International Journalf of Cancer,** 94, 492–499, (2001).

[16] Sun, Z., Zhao, Z., Li, G., Dong, S., Huang, Z., Ye, L., Liang, H., Qu, J., Ai, X., Zhang, W., et al., Relevance of two genes in the multidrug resistance of hepatocellular carcinoma: in vivo and clinical studies, **Tumori,** 96, 90–96, (2010).

[17] Brenes ,O., Arce, F., Gätjens-Boniche, O., Díaz, C., Characterization of cell death events induced by anti-neoplastic drugs cisplatin, paclitaxel and 5-fluorouracil on human hepatoma cell lines: Possible mechanisms of cell resistance, **Biomedicine and Pharmacotherapy**, 61, 6, 347-355, (2007).

[18] Boku, N., Yamamoto, S., Fukuda, H., Shirao, K., Doi, T., Sawaki, A., Koizumi, W., Saito, H., Yamaguchi, K., Takiuchi, H., Nasu, J., Ohtsu, A., Fluorouracil versus combination of irinotecan plus cisplatin versus S-1 in metastatic gastric cancer: a randomised phase 3 study**, The Lancet Oncology,** 10, 1063-1069, (2009).

[19] Ohtsu, A., Shimada, Y., Shirao, K., Boku, N., Hyodo, I., Saito, H., Yamamichi, N., Miyata, Y., Ikeda, N., Yamamoto, S, Fukuda, H., Yoshida, S., Randomized phase III trial of fluorouracil alone versus fluorouracil plus cisplatin versus uracil and tegafur plus mitomycinin patients with unresectable, advanced gastric cancer: The Japan Clinical Oncology Group Study, **Journal of Clinical Oncology**, 21, 54-59, (2003).

[20] Gillet, J.P., and Gottesman, M.M., **Mechanisms of multidrug resistance in cancer** in Methods in Molecular Biology (Zhou J ed), 47–76, Springer, New York, (2010).

[21] Gottesman, M.M., Fojo, T., Bates, S.E., Multidrug resistance in cancer: role of ATP-dependent transporters, **Nature Reviews Cancer**, 2, 48–58, (2002).

[22] Yu, Z., Zhang, C., Wang, H., Xing, J., Gong, H., Yu, E., Zhang, W., Zhang, X., Cao, G., Fu, C., Multidrug resistance-associated protein 3 confers resistance to chemoradiotherapy for rectal cancer by regulating reactive oxygen species and caspase-3-dependent apoptoticpathway, **Cancer Letters,** 353, 2, 182-193, (2014).

[23] König, J., Rost, D., Cui, Y., Keppler, D., Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane, **Hepatology**, 29, 1156-1163, (1999).

[24] Kool, M., van der Linden, M., de Haas, M., Scheffer, G.L., de Vree, J.M., Smith, A.J., Jansen G., Peters, G.J., Ponne, N., Scheper, R.J., Elferink, R.P., Baas, F., Borst P., MRP3, an organic anion transporter able to transport anti-cancer drugs, **Proceedings of the National Academy of Sciences of the United States of America** , 96, 6914–6919, (1999).

[25] Young, L.C., Campling, B.G., Cole, S.P., Deeley, R.G., Gerlach, J.H., Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: Correlation of protein levels with drug response and messenger RNA levels, **Clinical Cancer Research,** 7, 1798–1804, (2001).

[26] Liu, Y.H., Di, Y.M., Zhou, Z.W., Mo, S.L., Zhou, S.F., Multidrug resistance associated proteins and implications in drug development, **Clinical and Experimantal Pharmacology and Physiology,** 37, 115–120, (2009).