Original Article

J Babol Univ Med Sci
22; 2020. P: 388-395

The Effect of Hyperthermia on the Gene Expression of MDR1 and MRP4 Drug Efflux in Colorectal Cancer Cells

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J Babol Univ Med Sci; 22; 2020; PP: 388-395

Received: May 20th 2020, Revised: Aug 25th 2020, Accepted: Sep 5th 2020.

ABSTRACT

BACKGROUND AND OBJECTIVE: Hyperthermia has been reported as a new and adjunctive treatment of cancer in inhibiting DNA repair, increasing radiation sensitivity of cancer stem cells, increasing the sensitivity of drug-resistant cancer cells, and inhibiting cancer signaling pathways that cause apoptosis, suppression of cancer stem cell proliferation and disruption in cellular function. The aim of this study was to investigate the effect of hyperthermia on the gene expression pattern of drug resistance and cell survival.

METHODS: In this in vitro study, two cell lines of human colorectal adenocarcinoma HT-29 and SW-48 were cultured. The cells of the hyperthermia and control groups were exposed to 42 or 43 °C and 37 °C for 2 hours, respectively. Then the effect of hyperthermia on cell survival was investigated by MTT method. The expression pattern of MDR1 and MRP4 genes was also measured using qRT-PCR.

FINDINGS: Hyperthermia reduced cell survival, but this reduction was not significant. Hyperthermia decreased MDR1 gene expression in SW-48 cells (p=0.007). Although MDR1 expression in HT-29 cells was significantly reduced at 42 °C, no significant difference was observed between the hyperthermia and control groups. Hyperthermia also had no significant effect on MRP4 gene expression in SW-48 and HT-29 cell lines.

CONCLUSION: The results showed that hyperthermia reduces the gene expression related to drug resistance, but has no significant effect on cell survival.

KEY WORDS: *Drug Resistance Genes, Colorectal Cancer, Gene Expression, Hyperthermia, Tumorigenesis.*

Please cite this article as follows:

Ghorbani Z, Heidari M, Jafarinia M, Rohani M, Akbari A. The Effect of Hyperthermia on the Gene Expression of MDR1 and MRP4 Drug Efflux in Colorectal Cancer Cells. J Babol Univ Med Sci. 2020; 22: 388-95.

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[DOR: 20.1001.1.15614107.1399.22.1.54.6]

[DOI: 10.22088/jbums.22.1.388]

Introduction

Colorectal Cancer (CRC) is one of the leading causes of cancer death worldwide. Therefore, the study of cancer, its treatment and prevention is very important (1-3). About 90% of cases of CRC can be prevented if tested and screened early. However, due to the complexity caused by a large number of genetic changes and disruption of different signaling pathways, poor prognosis and lack of definitive early diagnostic methods, about half of people with CRC are diagnosed in the late stage. This situation emphasizes the importance of prevention and early diagnosis of this disease (4-7).

At present, cancer can be treated or cured by three main methods: surgery, radiotherapy and chemotherapy. Despite advances in treatment, resistance to chemotherapy has been frequently seen in a number of malignant cancers, such as colorectal cancer (8). Most of these treatments do not differentiate between healthy and cancer cells, and this causes harmful side effects in body tissues (9). One of the newest treatments for cancer is hyperthermia, which is based on focusing the high temperature on the tumor so that other parts of the body are protected from its side effects. In hyperthermia, temperature above 42-44 °C is often used for 2-4 hours to kill tumor cells (10). Hyperthermia is used as an external heat source to raise tissue temperature and kill cancer cells or prevent them from growing further (11).

Researchers have shown that high temperatures kill cancer cells and destroy the protein structure of the tumor cell, resulting in a reduction in tumor size. When hyperthermia is used concomitantly or shortly after the start of treatment, it disrupts cell DNA, activates heat shock proteins, and improperly folds proteins, resulting in loss of cell function and cell death (12). Despite recent studies, the exact mechanism of hyperthermia-induced cell death is not well understood. The combination of both heat-induced necrosis and inactivation of enzymatic proteins appears to be factors in DNA damage (13). Cancer cells show varying degrees of sensitivity to high temperatures, depending on the different induction of survival pathways (14).

The effectiveness of chemotherapy is one of the most important challenges in cancer treatment. One of the major barriers to long-term recovery and definitive treatment in cancer patients is the multidrug resistance proteins (MRPs) in cancer cells. The main mechanism in MDR is the overexpression of ATP-binding cassette transporters (ABC transporters), which has the ability to increase the outflow of drugs from cancer cells and thus reduce the concentration of drug within the cell (drug efflux). ABC transporter protein regulators increase the effectiveness of anticancer drugs (15). Drug resistance is a well-known phenomenon that was first discussed in the resistance of bacteria to certain antibiotics. However, it has been considered since similar mechanisms have been identified in other diseases, including cancer. Many cancers are initially sensitive to chemotherapy, but over time, through spread and other mechanisms such as DNA mutations and metabolic changes, they inhibit and destroy the drug and create resistance (16).

While diffusion through ABC transporters is a normal physiological process, it is also known as a drug resistance mechanism in cancer cells. These transporters are in a position to pump internal signals and external drugs, thereby affecting cancer progression and drug resistance (17, 18). Drug resistance reduces the effectiveness of anti-cancer therapies in almost all patients with colorectal cancer. Various types of ABC transporters, including MDR1 and MRP4, are up-regulated in colon cancer cells, causing anticancer drugs to erupt from the cancer cells and reduce their therapeutic effects. On the other hand, drug-resistant cancer cells have been shown to be susceptible to anticancer drugs by inhibiting ABC transporters by suppressing their protein expression or by using a modulator at the same time (19).

Considering the importance of drug resistance in the treatment of colorectal cancer and the role of multidrug efflux transporters in chemotherapy resistance, the present study was conducted to evaluate the possible effect of hyperthermia on the expression of genes related to drug resistance factors.

Methods

This case-control laboratory study was conducted under the auspices of Iran University of Medical Sciences with the approved code 96-02-182-31485 from the University Ethics Committee. Cell lines were prepared from Pasteur cell bank and cultured in RPMI 1640 (Gibco, USA) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in the incubator at 37 C° and 5% CO2. The cell culture medium was changed every two days and subjected to hyperthermia when the cells filled 80-90% of the flux. In the hyperthermia groups, the cells were incubated in RPMI with 10% FBS separately in an incubator at 42 and

[DOR: 20.1001.1.15614107.1399.22.1.54.6]
[DOI: 10.22088/jbums.22.1.388]

43 °C for 2 hours. The control group was incubated for 2 hours at 37 °C for each cell line.

MTT (Microculture Tetrazolium Test): The cytotoxic effect of hyperthermia on the mentioned cancer cells was evaluated by MTT (BioIDEA, Iran) and tetrazolium stain in comparison with the control group. For the MTT assay, HT-29 and SW-48 cells were cultured separately in 96-well plate and incubated for 24 to 48 hours. Cell viability was assessed after hyperthermia exposure. After that, the previous culture medium was removed and MTT solution was added to each 10 µl well. It was then incubated at 37 °C for 4 hours. Then 50 µl of DMSO was added to each well. After 10 minutes of incubation at 37 °C, the adsorption rate (630.570 nm) was measured using a microplate reader (BioTek, USA). To increase the accuracy of the test, the test was performed in triplicate for each concentration.

Total RNA extraction: RNA extraction was performed using a kit (Roche, Germany) according to the manufacturer's instructions. Then, NanoDrop instruments (Thermo Fisher Scientifict, USA) were used to evaluate ODs and their concentrations (adsorption ratio of 260.280 of a pure RNA sample is 2±0.1). Moreover, the quality of RNAs was evaluated by 2% agarose gel electrophoresis and finally stored at -80 °C.

cDNA synthesis: cDNA synthesis from appropriate RNA was performed using PrimeScript RT reagent kit (Takara, Japan) containing non-specific Oligo-dT primer, hexamer random, PrimeScript™×5 buffer and PrimeScript Reverse Transcriptase, according to the manufacturer's protocol.

Primer Design: The primers used were designed by GeneRunner software and BLAST by NCBI (Table 1). The primers were synthesized by Pishgam Company. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize.

Quantitative real-time PCR reaction: To quantify the expression of the desired genes, q-RT PCR reaction was performed using SYBR® Premix Ex Taq ™ II kit (Takara, Japan) and ABI 7500 device (Applied Biosystems; Thermo Fisher Scientific). In each microtube, 10 µl of SYBR Premix Ex Taq II, 0.4 µl of ROX stain, 2 µl of template cDNA, 0.4 µl of each primer and 6.8 µl of sterile distilled water were poured. The q-RT PCR reaction was performed under certain temperature conditions including the following steps: the initial denaturation step (95 °C for 30 seconds) and 40 cycles including 95 °C for 5 seconds and 60 °C for 30 seconds, respectively. All experiments were performed in triplicate. To evaluate the effect of hyperthermia on cell deformation, cell phenotype was evaluated using a reverse microscope. In order to evaluate the expression of genes, first the CTs obtained for each gene were calculated by the formula $2^{-\Delta\Delta CT}$. Then, the mean of the results obtained by SPSS statistical software was calculated in each group. Kruskall-Wallis test was used to evaluate the difference in expression of the desired genes in normal and treatment conditions and $p<0.05$ was considered significant.

Table 1. Sequence of primers and product size of genes

Gene Name	Primer	Sequence from 5' to 3' (5'-> 3')	Product Size
MDR-1	Forward	5´-GGGAGCTTAACACCCGACTTA-3´	154 bp
	Reverse	5´-GCCAAAATCACAAGGGTTAGCTT-3´	
MRP-4	Forward	5´-AGCTGAGAATGACGCACAGAA-3´	125 bp
	Reverse	5´-ATATGGGCTGGATTACTTTGGC-3´	
GAPDH	Forward	5´-CACCAGGGCTGCTTTTAAC-3´	190 bp
	Reverse	5´-GCCAAAATCACAAGGGTTAGCTT-3´	

Results

The results showed that hyperthermia had no effect on cell morphology.

MTT test: Cell viability under the influence of hyperthermia was assessed using the MTT method. Each cell line shows a different pattern in response to hyperthermia. SW-48 cells are more sensitive than HT-29 cell line and the percentage of cell survival decreases further when the temperature rises from 37 °C to 43 °C. However, this decrease in the survival percentage of cell lines treated with increasing temperature was not statistically significant (HT-29: $P_{HT} = 0.452$ and SW-48: $P_{SW}= 0.471$) (Figure 1).

Quantitative Real-Time PCR: In order to investigate possible molecular changes that may have occurred during hyperthermia exposure, the expression pattern of drug resistance-related genes was evaluated using q-RT

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[DOI: 10.22088/jbums.22.1.388]

PCR. Relative changes in MDR1 expression in SW-48 cells due to hyperthermia (42 and 43 °C) compared to the control group (37 °C) decreased gradually. This decrease in expression showed a significant relationship with increasing temperature 43 °C (P_{SW}= 0.007). However, in HT-29 cell line, a different expression pattern was observed. After decreasing MDR1 expression at 42 °C, increased expression was observed at 43 °C. Despite the decrease in temperature at 42 °C, no significant decrease in expression of this gene was observed in HT29 cell line with increasing temperature (P_{HT}= 0.180) (Figure 2).

MRP4 gene expression was increased in HT-29 cell line under the influence of 42 °C. On the other hand, the expression of this gene decreased at 43 °C compared to 42 °C and increased compared to 37 °C. However, these changes in expression were not statistically significant (P_{HT}= 0.288). In SW-48 cell line, with increasing temperature, the expression of this gene increased but no significant change was observed (P_{SW}= 0.733) (Figure 3). The results of this study showed that genes associated with drug efflux show different behavior in colorectal cancer cell lines under different temperature treatments.

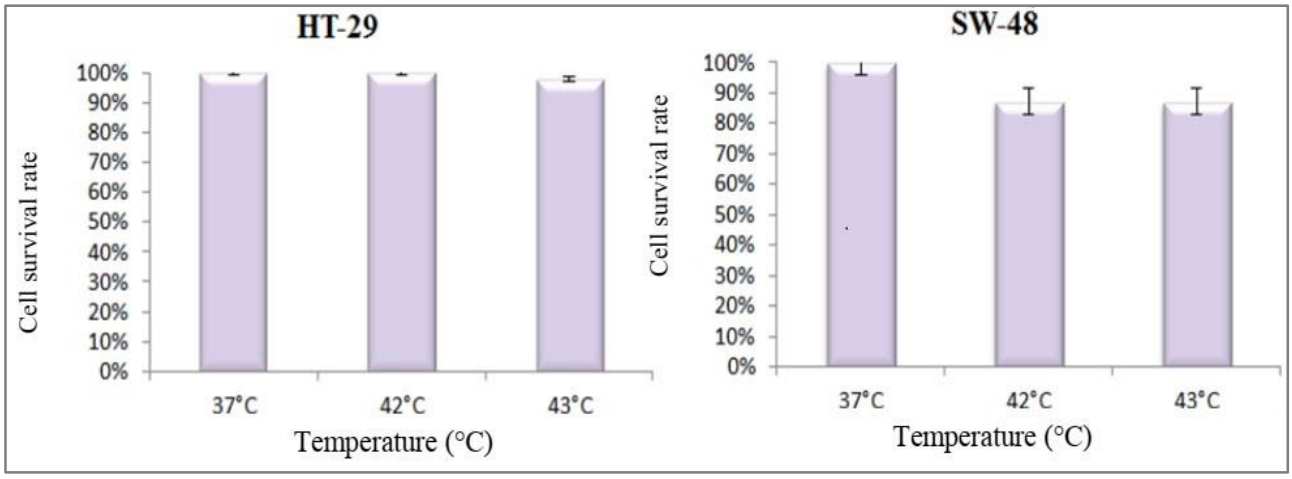

Figure 1. Effect of hyperthermia on cancer cell survival

Increase in temperature had no significant effect on cell survival. All data were compared with the control group (HT-29: P_{HT} = 0.452 and SW-48: P_{SW} = 0.471).

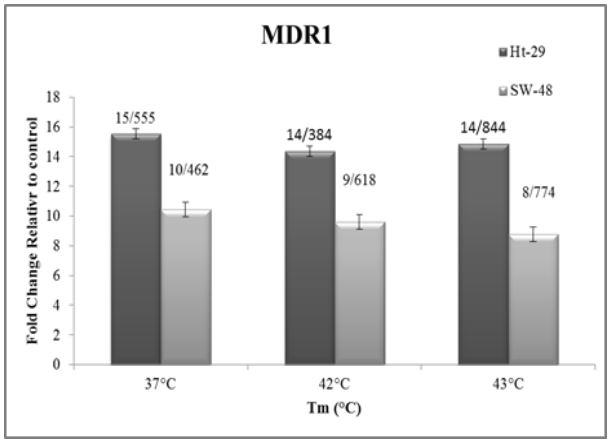

Figure 2. Effect of hyperthermia on MDR1 gene expression pattern in HT-29 and SW-48 cell lines. In HT-29 cells, despite the decrease in MDR1 gene expression at 42 °C, there was no statistically significant difference in MDR1 gene expression between the hyperthermia group and the HT-29 cell line control (P_{HT}= 0.180). In SW-48 cell line, with increasing temperature, the expression level decreased significantly (P_{SW}= 0.007). All data were compared with the control group (37 °C).

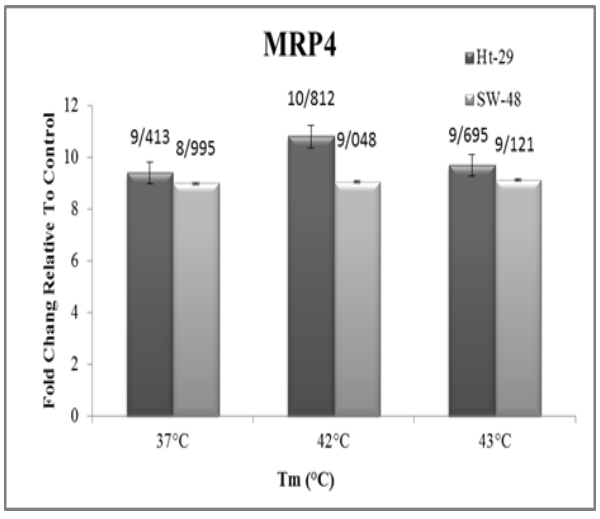

Figure 3. Effect of hyperthermia on MRP4 gene expression pattern in HT-29 and SW-48 cell lines. Increasing temperature had no significant effect on MRP4 gene expression pattern (P_{SW} = 0.733 and P_{HT} = 0.288). All data were compared with the control group (37 °C).

[DOR: 20.1001.1.15614107.1399.22.1.54.6]
[DOI: 10.22088/jbums.22.1.388]

Discussion

In this study, it was found that hyperthermia reduces the expression of genes related to drug resistance. However, in the study of the expression pattern of drug resistance genes in different cell lines treated with hyperthermia, multiple behaviors were observed. Drug resistance genes have received more attention since their association with human tumors after exposure to chemotherapy in cancer was identified. A study by To et al. on lung cancer showed no change in mRNA expression and upregulation of the MDR1 protein in response to hyperthermia in the presence of chemotherapy drugs in cancer cells. Therefore, it seems logical that hyperthermia may limit the activity of anticancer drugs by increasing the expression of extracellular transporters. On the other hand, it has been shown that the activity of these transporters can be modulated by using ATPase inhibitors (20).

Norris et al. showed an association between increased MRP4 and tumor prognosis in patients with primary neuroblastoma. Thus, high MRP4 expression is associated with enhanced MYCN oncogene and severe clinical outcomes. Therefore, MRP4 was introduced as a useful prognostic marker for neuroblastoma (21). Zhang et al. found that MRP4 gene expression is high in cancer cells and insignificant in normal cells. Using RNA interference to reduce the expression of MRP4 in drug-resistant gastric cancer cells, they increased apoptosis and stopped the G1 cell cycle. As a result, they observed an increase in the sensitivity of gastric cancer cells to chemotherapy drugs (22).

The study by Stein et al. also showed that a combination of hyperthermia (40 °C and 43 °C in 15, 30, 60, and 120 min) and drug therapy effectively reduced the survival of HCT15 and HCT116 cells in colorectal cancer. However, an increase in MDR1 and MRP1 expression was observed at the mRNA level in both cell lines (23). The results of the study by Cao et al. showed an increase in the effect of MRP1 on CRC cell resistance to apoptosis. Therefore, inhibition of MRP1 was a new strategy to overcome drug resistance in CRC (24).

In the last decade, the use of hyperthermia to increase the effectiveness of other treatments has received much attention (25). That's because this method has little invasive function and does not affect normal cells and affects countless biological processes in cells including proliferation, migration, invasion and apoptosis (26, 27). Previous studies have shown that hyperthermia affects the survival and expression of genes in cancer cells.

The results of this study showed that hyperthermia reduces MDR1 gene expression in SW-48 cells but has no significant effect on MRP4 gene expression in SW-48 and HT-29 cell lines. Our results also showed that hyperthermia reduced the viability of SW-48 cells but this effect was not significant. As mentioned, hyperthermia can affect a wide range of cellular processes including cellular metabolism, protein synthesis, nucleic acid synthesis and DNA / RNA polymerization (28) and activate heat shock proteins (HSPs) (29).

Kievit et al. showed positive effect of hyperthermia on drug release from nanoparticles in treatment-resistant cancers. In this way, in response to an external stimulus (hyperthermia), the nanoparticles release the encapsulated drug and kill the cancer cells (30).

Therefore, one of the reasons that we did not observe a significant effect of hyperthermia on cell survival in this study could be the use of normal hyperthermia, low temperature or short-term hyperthermia exposure. In order to complete the previous studies and the important role of MDR1 and MRP4 in drug resistance and the treatment process of colorectal cancer, we evaluated the effect of hyperthermia on the expression pattern of drug resistance genes.

The present study showed that hyperthermia, one of the important variables in treatment, is the duration of cell heating. The study found that the duration of 2 hours for the use of hyperthermia alone was short to kill colorectal cancer cells. Increasing the heating time or using combination therapies can be effective.

The results of this study showed that MDR1 gene expression is reduced under the influence of hyperthermia and can cause hypersensitivity and cancer cell death along with other therapies. On the other hand, increasing the temperature and heating time can affect the MRP4 gene and other genes in the ABC family as therapeutic targets. However, the important molecular mechanisms of hyperthermia in therapeutic resistance

[DOR: 20.1001.1.15614107.1399.22.1.54.6]
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must be thoroughly investigated. Our study showed that hyperthermia had no significant effect on cell survival but reduced the expression of genes associated with drug resistance.

Acknowledgment

We would like to thank the Vice Chancellor for Research of Iran University of Medical Sciences and all those who helped us in this research.

[DOR: 20.1001.1.15614107.1399.22.1.54.6]
[DOI: 10.22088/jbums.22.1.388]

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[DOR: 20.1001.1.15614107.1399.22.1.54.6]
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