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Research Paper / Araştırma Makalesi

Effect of Hardaliye on FoxM1 Gene Expression Level of HT-29, DU-145, HeLa Cancer Cells and CF-1 (Mouse Embryonic Fibroblast)

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ABSTRACT

Chemotherapeutic agents may influence both cancer cells and healthy cells, therefore alternative therapeutic agents or targets have become a milestone of cancer cure. Forkhead box M1 (FoxM1) is a transcriptional regulator and a novel cancer therapy target as overexpressed in cancer cells, and its inhibitors are considered as potential therapeutic agents to halt cancer progression. Therefore, in this study, the effect of “Hardaliye” on cancer cells and a healthy cell line was primarily investigated by MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) and later on, FoxM1 gene expression levels were determined. Hardaliye, especially when diluted ten and twenty-fold, decreased viability percentage of all cancer cells and did not affect healthy cells. FoxM1 levels of cancer cells drastically decreased especially in HT-29 cells while did not statistically change their levels in the healthy cell line.

Keywords: FoxM1, qRT-PCR, Cancer cell lines, CF-1, Gene expression

Hardaliyenin HT-29, DU-145, HeLa Kanser Hücreleri ve CF-1 (Fare Embriyonik Fibroblast) Hücresi FoxM1 Gen Expresyon Seviyelerine Etkisi

ÖZ

Kemoterapötik ajanlar hem kanser hücrelerini hem de sağlıklı hücreleri etkilemektedir bu nedenle alternatif terapötik ajanlar veya hedefler kanser tedavisinin kilometre taşı olmuştur. Forkhead box M1 (FoxM1) transkripsiyonel regülatördür ve kanser hücrelerinde daha fazla eksprese olması nedeniyle yeni kanser terapi hedefi olmuştur. Fox M1 inhibitörleri, kanser gelişimini engelleyen potansiyel terapötik ajanlardır. Bu nedenle, çalışmada, Hardaliye’nin kanser hücreleri ve sağlıklı hücre hattı üzerine etkisi MTT yöntemiyle öncelikle belirlenmiş ve sonrasında, FoxM1 gen ekspresyon seviyeleri tayin edilmiştir. Hardaliye, özellikle on ve yirmi-kat seyreltildiğinde bütün kanser hücrelerinin yüzde canlılığını düşürmüş ancak sağlıklı hücreleri etkilememiştir. FoxM1 seviyeleri sağlıklı hücre hatlarında istatistiksel olarak değişmezken, kanser hücrelerinde özellikle HT-29 hücrelerinde epeyce düşmüştür.

Anahtar Kelimeler: FoxM1, qRT-PCR, Kanser hücre hatları, CF-1, Gen ekspresyonu

INTRODUCTION

FoxM1 (Forkhead transcription factor) is a member of transcriptional regulators and a key regulator of processes including cell proliferation, G1–S and G2–M cell cycle transition, mitotic spindle integrity, cell differentiation, DNA damage repair, tissue homeostasis, angiogenesis, chemotherapeutic drug response and apoptosis [1-4]. An elevated expression of FoxM1 has been recently reported in the wide majority of tumor cells such as liver, prostate, brain, breast, lung, colon, pancreas, skin, cervix, ovary, mouth, blood and nervous while its expression is low in normal cells [5]. FoxM1 has been also demonstrated that activates genes related to

metastasis [6]. Some studies conducted in carcinogenic mouse models, where FoxM1 gene has been deleted, have shown that cancer cell proliferation have reduced [3].

Reactive oxygen species (ROS) trigger expression of FoxM1 and interestingly, FoxM1 also triggers expression of ROS scavengers such as Superoxide Dismutase (SOD), Catalase [6, 7]. Overexpression of FoxM1 renders cancer cells resistance to ROS-mediated cell death. Thus, tumor cells overexpressing FoxM1 coincides with resistance to apoptosis and chemotherapeutic drugs cisplatin, trastuzumab, and paclitaxel [7]. Thereby, inhibitors of this gene have been considered as potential therapeutic agents to halt cancer progression [1-3] and to increase the sensitivity of cancer cells to ROS-mediated cell death [7].

Hardaliye is a fermented non-alcoholic traditional beverage produced from the black grape, black mustard seeds, and cherry leaves in the Thrace Region of Turkey. Mustard seeds and grapes are slightly cracked; barrels are filled with a layer of cracked black grape, mustard seeds, and a layer of cherry leaves. The mixture is fermented for 10-15 days. The phenolic content of Hardaliye used in this study was detected by LC-MS/MS and elemental content was detected by ICP-MS in our previous study [8]. Its antioxidant capacity has been also shown by other researchers [9]. However, currently, there is no clear evidence about the effect of antioxidant foods on FoxM1 activity as potential anticancer sources. Therefore, determining the effect of Hardaliye, which is an antioxidant beverage, on FoxM1 gene expression of three cancer cell line, namely; HT-29 (colon cancer), DU-145 (prostate cancer), HeLa (cervical cancer) and healthy cell culture CF-1 (mouse embryonic fibroblast) was aimed in this study.

MATERIALS AND METHODS

Cell Lines and Treatment with Hardaliye

Human HT-29, DU-145, HeLa and Mouse embryonic fibroblast cells (CF-1) cell cultures were purchased from ATCC (Manassas, VA, USA). Cells were initially cultured in 75 cm^2 flasks with DMEM (Dulbecco's Modified Eagle's medium) supplemented with 10% FBS and Penicillin/Streptomycin (100 IU/100 µg/mL) (Wisent Inc., Canada) and grown at 37ºC in the presence of 5 % CO_2. Cells were trypsinized at 37°C washed with PBS and were seeded in 6- well plates (~10^6 cell per well). Hardaliye was filtered with a 0.22 µm sterile syringe filter and in 96-well plates (~ 5000-10.000 cell per well) for MTT analysis. Exponentially growing cells were treated with 10 to 160-fold Hardaliye for MTT assay and with 5 and 10-fold (abbreviated as H5 and H10) Hardaliye but control for further analysis. Each experiment was repeated independently at least three times.

MTT Cell Viability Assay

MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) (Biomatik Cambridge, Ontario) is an assay to determine the viability of cells with the conversion of the water-soluble MTT to an insoluble purple formazan by respiring cells. MTT assay was carried out after 24h and 48h of the experiment. 20 µL MTT (5 mg/mL in PBS) was added and incubated at 37°C for 4h. Medium with MTT was carefully removed from all wells and the formazan crystals were dissolved in 200 µL of DMSO and incubated at 37ºC for 5 min. Absorbance was recorded at 492 nm using Multiscan Go microplate reader (Thermo Scientific, USA). The viability % was calculated [10].

RNA Extraction and Reverse Transcription

All cells were trypsinized, centrifuged and 500µl lysis buffer was added to the pellet. Total RNA was isolated according to manufacturer's instruction with PureLink® RNA Mini Kit (Life Technologies, USA). The RNA concentrations were determined by measuring UV absorbance at 260 nm with NanoQ (Pathtech, Australia). RNAs were equalized to 100 ng/µL and reverse transcription was performed using High Capacity cDNA synthesis kit (Life Technologies, USA) on Veriti 96-Well Thermal Cycler system with the following protocol: step 1, 25°C, 10 min; step 2, 37°C, 120 min; step 3, 85°C, 5 min. RNA was stored at -80°C while cDNA was stored at -20°C for subsequent steps.

Detection of Relative Gene Expression Level of FOXM1 by Qrt- PCR (Quantitative Real-Time PCR)

Expression level of FOXM1 gene was detected using following protocol on ABI 7500 Fast qRT-PCR system (Life Technologies, USA): 20 µL reactions containing 0.2 µM each primer (the forward primer was 5'-AACCGCTACTTGACATTGG-3' and the reverse primer was 5'-GCAGTGGCTTCATCTTCC-3'), 10 µL SYBR Select Master Mix (Life Technologies, USA) (2x), 2 µL template cDNA and DNAse/RNAse free water [11]. After initial denaturation 1x 94°C for 3 min; amplifications were performed for 40 cycles 94°C for 45 s; 58°C for 45 s; and 72°C 1 min and 1x 72°C 10 min. 18S mRNA expression was used as housekeeping gene for normalization. Relative fold change of mRNA expression level was determined by the Comparative CT method ($2^{-\Delta\Delta CT}$ method) [12] and presented as mean ± SD. All reactions were set up in triplicates.

Statistical Analysis

Differences between control and treatments were analyzed using a statistical software JMP 12.0 (SAS Institute, Cary, NC, USA) with one-way ANOVA followed by Tukey-Kramer multiple comparison test. For MTT test, control was considered as % 100 and results were compared with control. Differences were considered significant at $p < 0.05$ and specified with asterisk as *** $p< 0.001$, **$p<0.01$, *$p<0.05$.

RESULTS AND DISCUSSION

The hardaliye used in this study mostly contains gallic acid, resveratrol and caffeic acid, namely; 6 ppm, 2

ppm, and 1 ppm, respectively as well as 156 ppm potassium. Caffeic acid has been shown to have anticancer activity in colon cancer cells [13]. Gallic acid as a chemical agent has been shown to reduce cervical cancer cells and induces apoptosis in hepatocellular carcinoma cells [14, 15]. Also, in a study, gallic acid has been extracted from a herbal medicine plant and its anticancer activity was assessed on numerous cancer cell lines such as breast, cervix, colon, gastric etc. Gallic acid has inhibited cell proliferation of cancer cells [16]. In addition, in vivo studies conducted with gallic acid or resveratrol have shown these compounds to inhibit most of cancer lesions such as osteosarcoma, breast, colorectal, liver, pancreatic, and prostate cancers [17, 18]. The effect of Hardaliye was assessed after 24h and 48h of experiments. According to MTT assay, 10 and 20-fold diluted Hardaliye decreased drastically viability of cancer cells after 48h of treatment. Namely, 10-fold dilution decreased viability of HT-29, DU-145 and HeLa 34±2%, 33±3%, 17±2 % while 20-fold dilution decreased 38±6%, 55±10%, 34±5%, respectively, compared with the control. However, the percentage of healthy cells did not significantly change apart from 20-fold dilution which is 92±5% after 48h (Tables 1-4).

Both H5 (5-fold dilution) and H10 (10-fold dilution) treatment significantly inhibited the gene expression level of FoxM1 in cancer cells while pleasingly did not affect healthy cells after 24h. 5-fold diluted Hardaliye was more effective on HT-29 cells than other cancer cells according to the relative fold changes. FoxM1 relative expression level of HT-29 cells decreased to 0.26 after 24h and 0.1 after 48h compared with the control. However, HeLa and DU-145 cells treated with H10 reached to control after 48h while HeLa cells treated with H5 remained almost at the same level, presumably, due to the fact that a drug resistance effect of the cells revealed at a low level of Hardaliye (Figures 1, 2 and Table 5).

Table 1. The viability (%) analysis of HeLa cells treated with diluted Hardaliye (10, 20, 40, 80, and 160-fold) via MTT assay

			Viability %	SD				Viability %	SD
24h	10 Fold	d***	28.76	1.42	48h	10 Fold	d***	17.35	2.18
	20 Fold	d***	34.22	4.53		20 Fold	c***	34.02	4.93
	40 Fold	c***	71.06	2.15		40 Fold	b***	62.28	7.69
	80 Fold	cb***	72.79	5.32		80 Fold	b***	67.76	7.61
	160 Fold	b*	83.89	8.03		160 Fold	b***	65.63	4.53
	Control	a	100.00	0		Control	a	100.00	0

Data are presented as mean±SD, n=3. Different letters indicate statistically differences. ***p< 0.001, **p<0.01, *p<0.05.

Table 2. The viability (%) analysis of DU145 cells treated with diluted Hardaliye (10, 20, 40, 80, and 160-fold) via MTT assay.

			Viability %	SD				Viability %	SD
24h	10 fold	d***	43.93	2.46	48h	10 fold	c***	32.80	3.43
	20 fold	dc***	53.07	7.92		20 fold	b***	55.13	10.10
	40 fold	c***	59.60	1.92		40 fold	a	89.03	4.29
	80 fold	b***	76.39	0.65		80 fold	a	89.69	7.57
	160 fold	a	90.14	3.54		160 fold	a	88.29	8.95
	Control	a	100.00	0		Control	a	100.00	0

Different letters indicate statistically differences. Data are presented as mean±SD, n = 3. *** p< 0.001, **p<0.01, *p<0.05.

Table 3. The viability (%) analysis of HT-29 cells treated with diluted Hardaliye (10, 20, 40, 80, and 160-fold) via MTT assay.

			Viability %	SD				Viability %	SD
24h	10 fold	c***	58.73	3.45	48h	10 fold	d***	34.49	2.56
	20 fold	c***	59.58	4.00		20 fold	dc***	38.87	5.94
	40 fold	b***	79.70	5.15		40 fold	cb***	60.46	3.56
	80 fold	b**	84.28	5.28		80 fold	b*	73.45	6.29
	160 fold	b*	88.79	3.27		160 fold	a	100.07	16.63
	Control	a	100.00	0		Control	a	100.00	0

Different letters indicate statistically differences. Data are presented as mean±SD, n = 3. *** p< 0.001, **p<0.01, *p<0.05.

Table 4. The viability (%) analysis of CF-1 cells treated with diluted Hardaliye (10, 20, 40, 80, and 160-fold) via MTT assay.

			Viability %	SD				Viability %	SD
24h	10 fold	a	109.45	15.57	48h	10 fold	a	103.06	4.69
	20 fold	a	104.26	17.14		20 fold	b	92.16	5.63
	40 fold	a	102.73	15.16		40 fold	a	113.86	1.69
	80 fold	a	96.09	6.86		80 fold	a	109.52	7.05
	160 fold	a	86.64	6.88		160 fold	ab	100.54	8.93
	Control	a	100.00	0		Control	ab	100.00	0

Data are presented as mean±SD, n= 3. Different letters indicate statistically differences, *** p< 0.001, **p<0.01, *p<0.05.

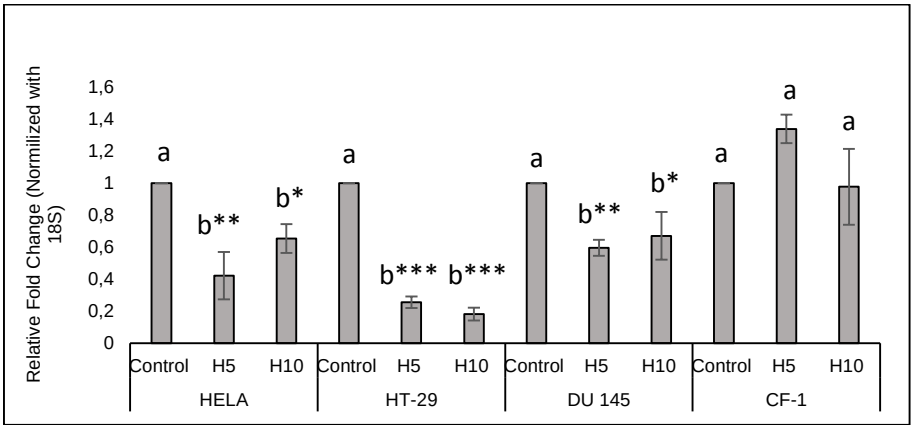

Figure 1: Effect of the Hardaliye on FoxM1 gene expression level after 24h of treatment with H5 and H10. Different letters indicate statistically differences *** p< 0.001, **p<0.01, *p<0.05.

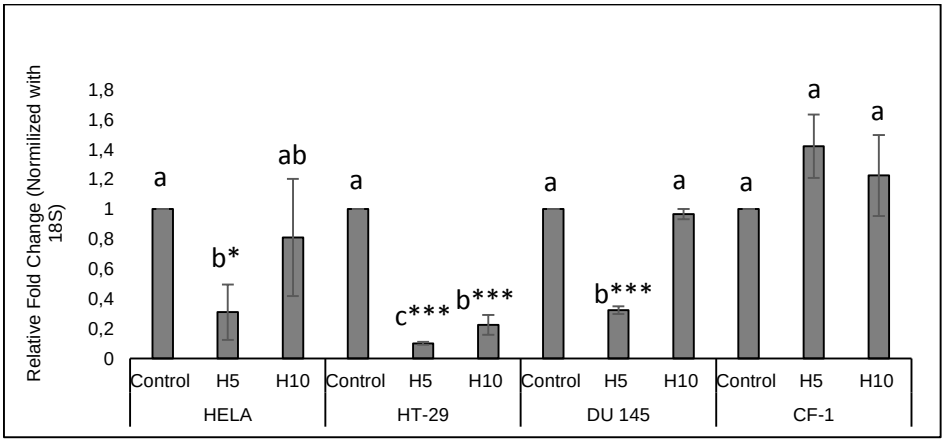

Figure 2: Effect of the Hardaliye on FoxM1 gene expression level after 48h of treatment with H5 and H10. Different letters indicate statistically differences *** p< 0.001, **p<0.01, *p<0.05.

Table 5. Effect of the 5 and 10-fold diluted Hardaliye on relative gene expression level of FoxM1 after 24h and 48h of treatment

	Relative Fold Change (Normalized with 18S)							
		24h				48h		
	Control	a	1	SD	Control	a	1	SD
HELA	H5	b **	0.42	0.15	H5	b*	0.31	0.19
	H10	b*	0.65	0.09	H10	ab	0.81	0.39
HT-29	H5	b***	0.26	0.04	H5	c***	0.10	0.01
	H10	b***	0.18	0.04	H10	b***	0.22	0.07
DU-145	H5	b**	0.60	0.05	H5	b***	0.32	0.03
	H10	b*	0.67	0.15	H10	a	0.97	0.03
CF-1	H5	a	1.34	0.09	H5	a	1.42	0.21
	H10	a	0.98	0.24	H10	a	1.22	0.27

Different letters indicate statistically differences *** p< 0.001, **p<0.01, *p<0.05.

MTT and FoxM1 gene expression results showed that Hardaliye was apparently seen to be more effective on HT-29 cancer cell line. Both methods supported that healthy cells were not affected by Hardaliye treatments. Even though there is no so much study on antioxidants and FoxM1 relation, FoxM1 was shown to be an ROS regulator and antioxidants inhibited FoxM1 expression as also shown in this study while oxidative stress increased [5, 7, 19, 20]. Moreover, the effect of an antioxidant beverage on FoxM1 activity was the first time evaluated with this study.

CONCLUSION

FoxM1 is a transcription factor which is recently a popular target for therapeutic opportunities. Its inhibitors are considered as a potential drug development since it induces apoptosis in human tumor cell lines. Even though detailed studies required to put forward the anti-cancer effect of Hardaliye. This study has become a preliminary study to show up the anti-cancer effect of the Hardaliye via suppressing the FoxM1 expression.

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