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Capsaicin Alters the Expression of Genetic and Epigenetic Molecules In Hepatocellular Carcinoma Cell

Beren Ates[1], Çağrı Öner[2*], Zeynep Akbulut[2], Ertuğrul Çolak[3]

1. Maltepe University, School of Medicine, İstanbul, Turkey.
2. Maltepe University, Medical Faculty, Department of Medical Biology and Genetics, İstanbul, Turkey.
3. Eskişehir Osmangazi University, Medical Faculty, Department of Biostatistics, Eskişehir, Turkey.

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ABSTRACT

Capsaicin is a natural product which is extracted from pepper and has the potential to be used in cancer treatment because of its anti- proliferative effects. The aim of the study was to determine the effect of capsaicin on the hepatocellular carcinoma cell proliferation and the expressions of related genetic markers as Ki-67, PI3K/AKT/mTOR and epigenetic markers as miR-126 and piR-Hep-1. The inhibitory concentration of capsaicin in HepG2 cells was determined. piR-Hep-1 and miR-126 expressions and Ki-67, PI3K, AKT and mTOR gene expressions were examined by RT-PCR. The inhibitory concentration of capsaicin for HepG2 cells was 200 nM and the decreased proliferation was observed at 24th hour. As epigenetic markers, an up regulation of miR-126 and down regulation of piR-Hep-1 expression were determined after treatment. Moreover, Ki-67, PI3K and mTOR gene expressions decreased while AKT gene expression increased after the treatment ($p<0.001$). According to the obtained data, capsaicin has an impact on proliferation both genetically and epigenetically. Furthermore, treatment of capsaicin effects miR-126 and piR-Hep-1 expressions which effect carcinogenesis in different way. Moreover, there are some clues which indicate that these two small non-coding RNA might affect each other and share the same target molecules post-transcriptionally.

Keywords: Capsaicin, miR-126, piR-Hep-1, PI3K/AKT/mTOR signalling

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Introduction

Carcinogenesis refers to the uncontrolled division and proliferation of cells in organs or tissues as a result of genetic mutations and errors in epigenetic arrangements (1). The most frequent type of primary liver cancer is hepatocellular carcinoma (HCC), which is the sixth most frequent malignancy and the second leading cause of cancer mortality (2). HCC pathogenesis is a multistep process that involves the slow

***Corresponding Author:** Çagrı Öner
Address: Maltepe University, Medical Faculty, Department of Medical Biology and Genetics, İstanbul, Turkey.
E-mail: cagri.oner@maltepe.edu.tr

accumulation of genomic, transcriptomic, and epigenetic alterations that control many molecular and cellular activities (3).

Capsaicin comes from the *Capsicum annum* species of the Solanaceae family, which includes a variety of red peppers. Capsaicin is produced in chili peppers through the condensation of phenylpropanoid and fatty acid routes with capsaicin synthase. Capsaicin (8-methyl-N-vanilyl-6-nonenamide) is a fat-soluble homovalinic acid derivative (4). Recent studies have indicated that capsaicin metabolites might disrupt cell signalling pathways, limiting cellular development and increasing carcinogenesis; hence, targeting cellular metabolic processes may be a novel cancer therapy technique (5, 6). Anticancer properties of Capsaicin have been studied in vivo and in vitro in a variety of malignancies, including lung, breast, stomach, and prostate cancers, as well as cholangiocarcinoma (7). It is worth noting that utilizing a static magnetic field (SMF), capsaicin affinity to the TRPV1 receptor may be boosted, improving capsaicin's anti-cancer impact on HepG2 cells via caspase-3 death (8).

It is worth noting that small, non-coding RNAs play an important role in cell development, proliferation, apoptosis, and differentiation (9, 10). The Argonaute (AGO) protein family, which is a prominent component of RNA silencing complexes and is strongly maintained in all organisms, has a regulatory role (11). The deregulation of ncRNAs in cancer, in particular, has been linked to tumour genesis, development, and metastasis (10, 12). The PIWI-interacting RNAs (piRNAs), which belong to the small, non-coding RNA (ncRNAs) category, are important cell biology mediators (10). miR-126 is an evolutionarily conserved gene that is expressed more abundantly in vascularized areas (13). In hepatocellular carcinoma, miR-126 is thought to have a tumour suppressive function. *Wong* et al. (2008) observed that the expression level of miR-126 significantly decreased in patients who were chronic carriers of HBV and HCV, compared to healthy controls (14). In particular, piR-Hep-1 is expressed in hepatocellular carcinoma cells and its expression cannot be observed except in hepatocellular carcinoma (15).

The nucleus core protein known as Ki-67 can control cell growth, particularly in tumour cells. In cell lines, the G2 phase and mitosis of the cell cycle occur when Ki-67 expression is the highest, and this higher expression causes an accumulation of a transcription factor called E2F (16). The PI3K/AKT/mTOR signalling pathway promotes tumour growth, development, invasion, and chemical resistance, all of which lead to enhanced cell proliferation (17, 18). While the PI3K/AKT pathway maintains cell survival during metabolic stress, it also inhibits pro-apoptotic components, resulting in an increase in NF-kB expression, which impacts the cell's survival (19). The essential protein mTOR functions both upstream and downstream of AKT in the pathway (18). One of the most frequently active protein kinases in tumours is AKT. High expression of AKT may promote cell proliferation, growth, and resistance to apoptosis (20). The fact that a conditional PTEN loss in a mouse model, which causes an increase in AKT signalling, may cause metastatic cancer growth provides more evidence for the AKT pathway's carcinogenic potential (21).

We aimed to observe capsaicin effect on proliferation related PI3K/AKT/mTOR signalling. Furthermore, we also wanted to show the correlation between miRNA and piRNA relationship epigenetically.

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Materials and methods

Cell Culture of HepG2 Cells

Hepatocellular carcinoma cell line HepG2 (ATCC, USA) was cultured in our laboratory under standard conditions, in an incubator with 5% CO at 37°C, in tissue and cell culture flasks (Greiner, Austria), 10% Foetal Bovine Serum (FBS; Gibco, USA) and 1% Penicillin/streptomycin (Biowest, USA) containing Phenol red, Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA).

Inhibitory Concentration 50 (IC50) Assay of Capsaicin

$7x10^3$ cells were seeded into each well of 96-well plates (Greiner, Austria) before treatment with Capsaicin (Cayman, Germany). 200 nM, 160 nM, 120 nM and 80 nM doses of Capsaicin were administered to cells at 24th, 48th, and 72nd hours to determine the inhibitory concentration 50 (IC50) of Capsaicin on HepG2 cells. After treatment with Capsaicin, XTT (Biological Industries, Israel) was added to wells and incubated for 2 hours. The absorbance of the groups was then measured at 450nm using a microplate reader (Biotek, Japan).

Total RNA Isolation and Real Time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from 200 nM Capsaicin treated HepG2 cells at the 24th hour according to the manufacturer's instructions of RNA purification kit (Nucleospin RNA, Macherey-Nagel, Germany). Total RNA was isolated and converted to cDNA by using reverse transcription kit (Bioneer, Korea). RT-PCR was performed by Roche Lightcycler96 (Vedbaek, Denmark). Ki-67, hTERT, PI3K, AKT, mTOR gene expressions; and miR-126 and piR-Hep-1expressions were determined after cDNA conversion. Gliseraldehide-3-Phosphate Dehydrogenase (GAPDH) served as the assay's internal control, and the expression of associated genes was normalized by GAPDH expression. The condition of qPCR was set as: at 95℃ for 15 s, at 60℃ for 30 s, and 40 cycles of at 95℃ for 30 s.

Statistical Analyses

The Kolmogorov-Smirnov suitability test was used to ensure that the continuous variables had a normal distribution. One-way variance analysis was used to analyse comparisons between groups of normally distributed data (ANOVA). Tukey HSD test was used for numerous comparisons. The student t-test was used to assess multiple gene expression comparisons. The IBM SPSS Statistics 21.0 software package was used for all analyses. The mean and standard deviation of the obtained data were calculated (sd). Only mean values have been shown in the graphs.

Results

200 nM Capsaicin at the 24th Hour is the Inhibitory Concentration 50 (IC50) data for HepG2 cells

According to the obtained data, 200 nM capsaicin treatment at the 24th hour was effective to decrease proliferation of treated group (231100±7) compared to control group (432776± 4, 04145; $P<0.001$). Other concentrations caused cell death at 24, 48, and 72 hours, but were no more effective than 200 nM at 24 hours in 50 percent of all cases (Figure 1).

200 nM Capsaicin and Ki-67 Gene Expression

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The aberrantly inhibition in Ki-67 expression was determined in 200 nM capsaicin treated HepG2 cells (0,626±0.007) compared to the control group (269,349± 0.007; p<0.001; Figure 2A).

200 nM Capsaicin and PI3K/AKT/mTOR Signalling Pathway Gene Expressions

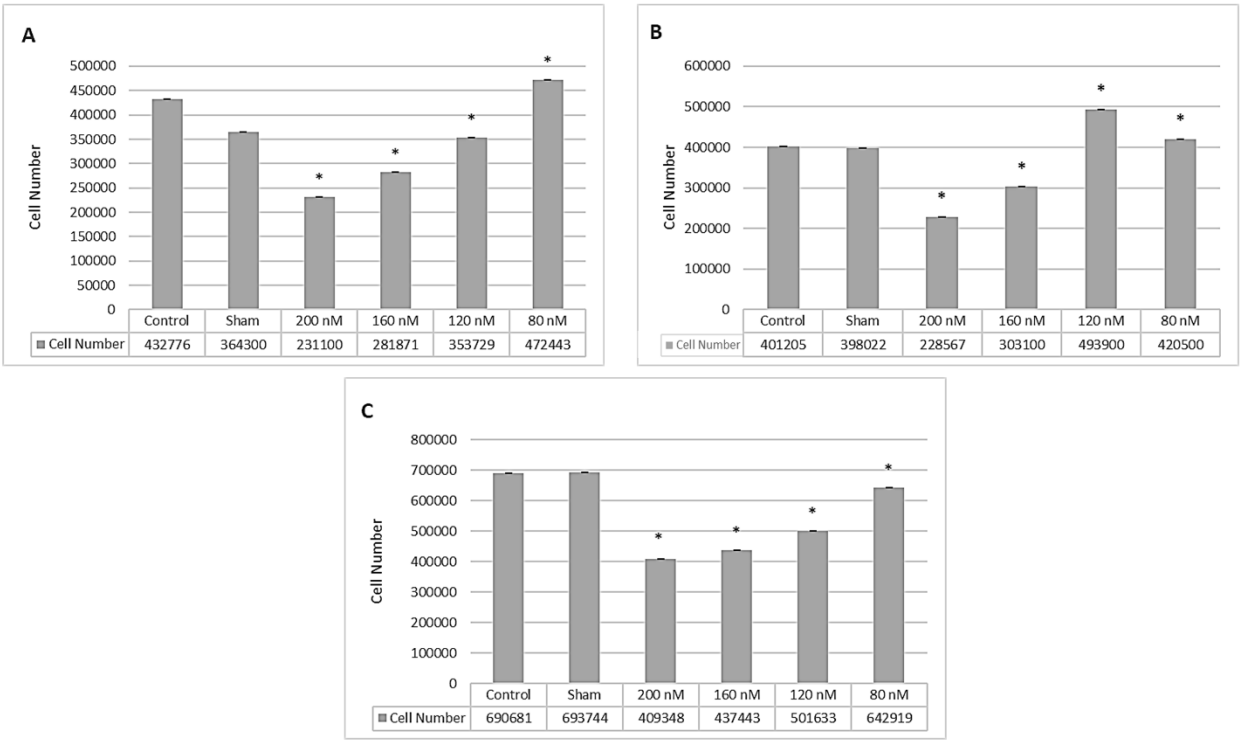

Fig. 1. IC50 values were measured after 24, 48, and 72 hours of treatment with different doses of Capsaicin in HePG2 hepatocellular carcinoma cells. A. The effect of Capsaicin on HePG2 proliferation after 24 hours B. The effect of Capsaicin on HePG2 proliferation after 48 hours C. The effect of Capsaicin on HePG2 proliferation after 72 hours (P<0.001).

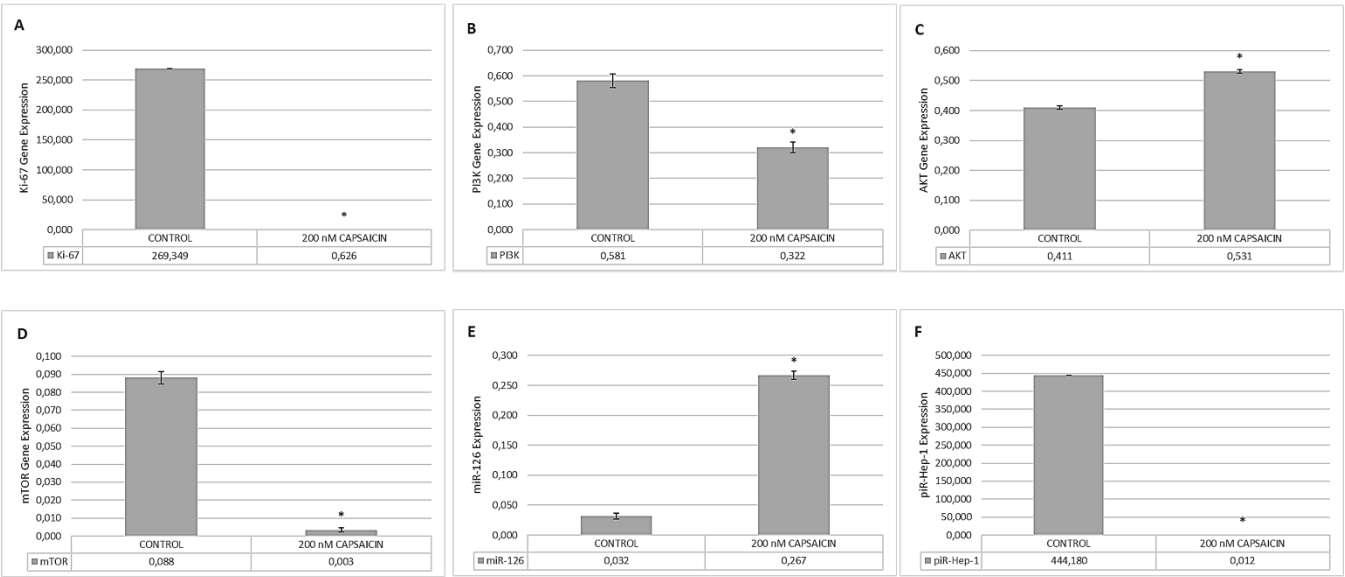

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Fig. 2. The Gene expressions of 200 nM Capsaicin treated HepG2 cells compared to untreated HepG2 hepatocellular carcinoma cells. A. Ki-67 Gene expression B. PI3K Gene expression C. AKT Gene Expression D. mTOR Gene Expression E. miR-126 Expression F. piR-Hep-1 Expression.

The PI3K gene expression decreased in capsaicin-treated HepG2 cells (0,322± 0,021) compared to the control group (0,581± 0,026; p<0.001). AKT gene expressions up regulated in the treated group (0,531±0.006) compared to the control group (0,411±0.006; p<0.001). mTOR gene expressions in 200 nM capsaicin treated group (0,003±0,0012) decreased compared to the control group (0,088±0,0035; p<0.001; Figure 2B-2D).

200 nM Capsaicin and miR-126 and piR-Hep-1 Expressions

The aberrantly up regulation in miR-126 expression was determined in capsaicin treated group (0,267± 0.007) compared to the control group (0,032±0.005; p<0.001; Figure 2E). On the other hand, piR-Hep-1 expression significantly decreased in 200 nM capsaicin treated group (0,012±0.005) compared to the control group (444,180±0.047; p<0.001; Figure 2F).

Discussion

Capsaicin is an important compound that has an impact on various cell mechanisms. Capsaicin has shown clear effectiveness in suppressing cancer cell proliferation and lowering Ki-67 expression in breast cancer in recent studies (22, 23). Capsaicin can increase the activity of the signal transducer and activator of transcription 3 (p-STAT3), promoting autophagy in HepG2 cells via causing ROS production. Capsaicin promotes apoptosis when autophagy is suppressed in assays on the same HCC line (24). Capsaicin inhibits autophagy via inhibiting the Akt/mTOR signalling system, whereas direct suppression of the mTOR pathway induces autophagy on the NPC-TW01 nasopharyngeal cancer cell line; in short, capsaicin can control autophagy by inhibiting the Akt/mTOR signalling pathway (19). *Bort* et al. (2019) determined that capsaicin reduced baseline neutral lipid levels in HepG2 cells. Capsaicin also activated the AMP-activated kinase (AMPK) and inhibited the AKT/mTOR pathway, both of which are important regulators of hepatic lipogenesis. Moreover, capsaicin inhibited autophagy and up regulated PPARγ co-activator 1α (PGC-1 α) protein levels (25). In the apoptotic pathway, capsaicin also has an important role in decreasing proliferation and viability. Capsaicin reduced the cytoplasmic level of anti-apoptotic Bcl-2 to pro-apoptotic Bax and promoted caspase-3 activity in HepG2 cells (26). The effect of capsaicin on reducing cell viability and the Akt/mTOR pathway and its impact in inducing reactive oxygen species (ROS) in HepG2 cells is well known (5). Through the SIRT1/NOX4 signalling pathway, capsaicin may induce oxidative, apoptotic, and DNA damage in HepG2 cells (27). In another study, capsaicin was founded as a hepatocarcinogenesis inhibitor by reducing SIRT1/SOX2 signalling and suggested to have the potential to be a good treatment option for liver cancer (28). According to our data, 200 nM capsaicin administered at the 24th, 48^{th,} and 72nd hour decreased cell proliferation; Figure 1 shows that a 50% inhibitory effect was observed at the 24th hour. These data were also supported by the gene expression of Ki-67, PI3K, and mTOR (Figures 2A, 2B, and 2D), while AKT gene expression was up regulated after capsaicin treatment. The main reason for this up regulation is AKT's multi-signalling property. AKT is triggered by various signalling molecules and epigenetic regulators. However, the signalling pathway of PI3K continues with AKT, so AKT might be

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affected by another molecule/pathway. A previous study determined that the synergistic effect of sorafenib and capsaicin was more effective in inhibiting phosphorylated AKT expression than the effect of capsaicin alone (26). This result supports the proposition that capsaicin treatment alone may not significantly reduce AKT expression in hepatocellular carcinoma.

Specific and effective biomarkers are required for early cancer detection and the treatment of metastatic and chemo resistant cancers (29). piR-Hep-1 is thought to be a prognostic marker of liver cancer. Various studies have indicated that the overexpression of piR-Hep-1 causes increased migration and invasion of liver cancer cells (15, 30). In our study, capsaicin treatment resulted in a decrease in piR-Hep-1 expression, which was supported by a decrease in Ki-67 gene expression and a decrease in HepG2 cell proliferation. Furthermore, piR-Hep-1 was shown to be substantially expressed in HCC compared to the surrounding non-tumour tissues, and the silencing of piR-Hep-1 affected cell survival, motility, and invasiveness by inactivating PI3K/AKT signalling, implying an oncogenic role for piR-Hep-1–PIWIL2 complexes in HCC (30). The inhibition of proliferation because of capsaicin treatment caused a decrease in PI3K, mTOR, and piR-Hep-1 expression and vice versa.

miR-126 has been proposed as a tumour suppressor in the development of HCC (31) by targeting the Sox2 gene. Down regulation of miR-126 has been linked to HCC metastasis and has been proposed as a factor in the poor prognosis of HCC patients (32). Components of the mTOR pathway are among the targets of miR-126 in cancer cells, and studies have shown that it is also effective in the translation of PI3K and AKT proteins, which are located upstream of the pathway (33). Overall, miR-126 has been discovered as a possible small non-coding RNA involved in angiogenesis, vascular integrity, inflammation, and proliferation inhibition (31, 33, 34). The general inhibitory mechanisms of piRNAs are methylation and histone modifications. Capsaicin treatment increased the expression of miR-126 in HepG2 cells. The down regulation of PI3K and mTOR by capsaicin therapy coincides with upregulation of miR-126. In cancer cells, Peng *et al.* (2016) postulated that piRNAs and miRNAs would have equivalent roles and targets (35). According to the obtained data, capsaicin treatment caused to increase the epigenetic tumour suppressor miR-126 expression. Furthermore, we can observe the correlation between miR-126 and its known target signalling molecules PI3K and mTOR. Moreover, we suggest that there might be a possible relation between miR-126 and piR-Hep-1 because they share the target molecules.

There are restrictions on the present research. Even while natural compounds in cancer treatment research are becoming more and more popular, cell line studies cannot show the clinical chart clearly. Furthermore, we were only able to see alterations in HepG2 hepatocellular carcinoma cell proliferation. Other hepatocellular carcinoma cell lines, tissues, and blood samples should all exhibit capsaicin effects.

Even if the use of natural compounds in direct cancer treatment is not effective, it is known to be effective in increasing the effect of drugs or supplementary food beside cancer treatment. For this reason, studies focusing on the effects of natural components on cancer cells increase day by day and gaining importance. As a result of this study, it suggests that capsaicin can be used as a natural ingredient supplement in the treatment of HCC, since it reduces the proliferation of cells in terms of both metabolic, genetic, and epigenetic markers.

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