Hindawi
Journal of Food Biochemistry
Volume 2024, Article ID 8464817, 11 pages
https://doi.org/10.1155/2024/8464817

Research Article

Capsaicin Enhances Temozolomide-Resistant Glioblastoma Cells' Chemosensitivity and Ferroptosis through FHOD1/IRF2 Downregulation

Ceyhan Hacioglu [1,2]

[1]*Düzce University, Faculty of Pharmacy, Department of Biochemistry, Düzce, Türkiye*
[2]*Düzce University, Faculty of Medicine, Department of Medical Biochemistry, Düzce, Türkiye*

Correspondence should be addressed to Ceyhan Hacioglu; ceyhanhacioglu@duzce.edu.tr

Received 6 January 2024; Revised 28 February 2024; Accepted 6 March 2024; Published 16 March 2024

Academic Editor: Swapan Ray

Resistance of tumor cells to chemotherapy, particularly in the case of glioblastoma (GBM), a common brain tumor, presents a substantial challenge in oncology. In this study, we investigated the potential of capsaicin to overcome drug resistance in temozolomide (TMZ)-resistant U87 and U251 cells by targeting ferroptosis-mediated interferon regulatory factor (IRF)-2 and formin homology-2 domain-containing protein-1 (FHOD1) signal pathways. First, we induced TMZ resistance in these cells by treating them with TMZ for three weeks. Subsequently, we assessed the impacts of capsaicin on various aspects, including cell viability, proliferation, ferroptosis markers, levels of IRF2 and FHOD1, intracellular iron concentrations, and cell migration in these cells. Our results indicate that capsaicin treatment resulted in a significant decrease in both cell viability and proliferation in TMZ-resistant U87-R and U251-R cells. In addition, it effectively suppressed cell migration rates in these cells, targeting TMZ resistance. Furthermore, capsaicin demonstrated its ability to downregulate FHOD1, IRF2, glutathione, and glutathione peroxidase 4 levels in TMZ-resistant cells. This was accompanied by an increase in intracellular iron, total oxidant status, and increased malondialdehyde levels. Significantly, the treatment with capsaicin led to a notable decrease in the expressions of FHOD1 and IRF2 at both the mRNA and protein levels in U87-R and U251-R cells. In summary, our results emphasize the substantial potential of capsaicin in enhancing the sensitivity of TMZ-resistant GBM cells to chemotherapy. This effect is achieved through its modulation of ferroptosis-related pathways, involving the regulation of FHOD1 and IRF2 expressions.

1. Introduction

Glioblastoma (GBM) is the most aggressive brain tumor among malignant brain tumors and has the lowest survival time in patients [1]. In the traditional approach to the treatment of GBM, the prognosis is often poor despite surgery and simultaneous radiation therapy and chemotherapy treatments [2]. While chemotherapy, particularly utilizing the drug temozolomide (TMZ), has shown some advancements by extending survival rates by approximately 2.5 months, its efficacy is constrained due to the emergence of drug resistance [3]. TMZ, an oral alkylating agent, serves as the primary chemotherapy drug used against GBM. Extensive research efforts have been dedicated to unraveling the mechanisms underlying TMZ resistance. Among these, much attention has been directed toward O6-methylguanine-DNA methyltransferase (MGMT) since it directly influences the cytotoxic effects of TMZ [4]. Nevertheless, the role of MGMT alone does not completely explain GBM resistance to TMZ, as over 40% of GBMs with low MGMT levels still demonstrate resistance to the drug [5].

The formin family, which includes formin homology-2 domain-containing protein-1 (FHOD1), plays a role in modulating the capping and bundling of actin to reshape the actin cytoskeleton [6]. Recently, research has highlighted FHOD1's involvement in various processes related to cancer. As an example, in squamous cell carcinoma, increased levels of FHOD1 were associated with the promotion of cancer cell migration and invasion, achieved through the induction of epithelial-mesenchymal transition [7].

Knocking down FHOD1 in the breast cancer cell line, MDA-MB-231 had a notable impact on invasion, migration, and proliferation [8]. In addition, in GBM cells, the down-regulation of FHOD1 led to a significant reduction in cell proliferation and induced cellular sensitivity to ferroptosis [9]. Ferroptosis, a recently identified form of regulated cell death, is known to be initiated by several biological processes [10]. Indeed, exploring FHOD1 and its interconnected signaling networks that contribute to cell death mechanisms holds the potential to establish a solid foundation for developing strategies aimed at sensitizing cancer cells to therapies centered around inducing cell death.

Interferon regulatory factors (IRFs) consist of 9 different members, and among them, IRF2 plays a role in the regulation of a wide variety of cellular metabolic processes [11]. Although some studies have illuminated the association between IRF2 and tumor immunity within gliomas, these investigations have often lacked a comprehensive consideration of additional tumor characteristics [12]. Therefore, there is a pressing need to deepen our understanding of IRF2, particularly in the context of its broader implications within tumor biology.

Capsaicin, a lipophilic protoalkaloid renowned for its characteristic pungency, is primarily found in hot peppers (*Capsicum annuum* L.). It has garnered attention for its utility in alleviating pain associated with neuralgias and neuropathies [13]. Beyond its analgesic properties, capsaicin has also been the subject of numerous studies revealing its potential as an agent with anticancer properties [14]. Notably, our previous research has highlighted capsaicin's role as a potential inducer of ferroptosis, a type of regulated cell death, and demonstrated its capacity to curtail the proliferation of GBM cells [15]. While the roles of FHOD1, IRF2, and capsaicin in tumorigenesis, cancer progression, ferroptosis, and chemoresistance have been increasingly recognized, there remains a notable gap in our understanding regarding their preventive potential in conferring resistance to TMZ chemotherapy in GBM. As of now, no conclusive evidence has emerged to establish whether FHOD1 and IRF2 inhibit TMZ chemoresistance in GBM. In light of this knowledge gap, the current study was undertaken to explore the possibility that capsaicin treatment could regulate the FHOD1/IRF2 signaling pathways. This investigation aimed to ascertain whether these pathways exert an influence on ferroptosis and if capsaicin treatment could potentially prevent TMZ resistance in GBM cells.

2. Materials and Methods

2.1. Cell Lines and Cell Culture. U87 and U251 GBM cells were purchased from the American Type Culture Collection. These cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 2 mM glutamine and 10% fetal bovine serum. Cells were maintained in an incubator with control environmental conditions at 37°C and 5% CO_2.

To induce resistance to TMZ in GBM cell lines, we adapted the techniques described by Zhu et al. [16] and Xu et al. [17]. U251 and U87 cells (at a density of 1×10^5 cells) underwent a 3-week treatment with 200 μM TMZ. Initially, the two cell lines were cultured separately in 12-well plates and allowed to adhere overnight at 37°C. Subsequently, TMZ treatment was administered every 72 hours for 3 weeks, with a fresh medium containing TMZ provided during each cycle. The majority of cells died during TMZ treatment, and a small group of cells survived by resisting TMZ. These surviving cells were isolated from the culture medium and used to establish TMZ-resistant U251 (U251-R) and U87 (U87-R) cell lines. U251-R and U87-R cells were employed in all subsequent experimental analyses. In a prior investigation, we determined that the cytotoxic concentration of capsaicin in U87 and U251 cells, when treated for 48 hours, was 120 μM [18], and this capsaicin concentration was utilized in other analyses in the present study.

2.2. Cell Counting Assay for Cell Viability. The viability analysis of cells was evaluated using the Cell Counting Kit-8 (CCK-8, Cat no. E-CK-A362) assay. Cells (1×10^5 per well) were cultured in 96-well plates. After a 24-hour adaptation period, cells were exposed to TMZ at concentrations ranging from 0 to 2000 μM for 48 hours. Following this, the cells were incubated with 10 μl of the CCK-8 solution for 2 hours. Subsequently, a microplate reader (BioTek) was used to measure cell viability at a wavelength of 490 nm.

2.3. Cell Proliferation Assay. Cell proliferation was assessed utilizing the 5-bromo-2′-deoxyuridine (BrdU) cell proliferation assay kit (2750, Sigma-Aldrich), adhering to the prescribed guidelines from the manufacturer. The BrdU incorporation method stands as a widely employed assay for the quantification of *in vitro* cell proliferation. Initially, cells (1×10^5 per well) were incubated in 96-well plates and analyses were performed according to the manufacturer's instructions. Subsequently, the cells were subjected to varying concentrations of capsaicin over a 48-hour period. The absorbance values of the samples were subsequently gauged at a wavelength of 450 nm.

2.4. Cell Cycle Analysis. TMZ-resistant cells were seeded in 12-well plates (1×10^5 per well). Subsequently, these cells underwent a 48-hour exposure to both TMZ and capsaicin. Following the treatment interval, cellular fixation was achieved by using a 4% paraformaldehyde solution, ensuring the preservation of cellular structures and preventing further alterations. The fixed cells underwent staining with propidium iodide (PI) to visualize distinct phases of the cell cycle, encompassing G1 (gap phase 1), S (DNA synthesis phase), G2 (gap phase 2), and M (mitosis phase). The fluorescence intensity emitted by the stained cells was quantified by employing flow cytometry, specifically by utilizing the Muse® flow cytometry system.

2.5. Biochemical Analysis. FHOD1, IRF2, intracellular levels of ferrous (Fe^{2+}), glutathione peroxidase 4 (GPX4), malondialdehyde (MDA), cellular total oxidant status (TOS), and reduced glutathione (GSH) levels were assessed using enzyme-linked immunosorbent assay (ELISA) kits

(MBS9322747, MBS166682, MBS2000338, MAK025, MAK085, Rel Assay Diagnostics, and MBS727656, respectively) by following the manufacturer's instructions. To carry out these analyses, U251-R and U87-R cells were seeded in 96-well plates at a density of 1×10^5 cells and then treated with 120 μM of capsaicin for 48 hours. According to the manufacturer's instructions, the absorbance value of each sample was analyzed with a microplate reader.

2.6. Cell Migration Assay. U251-R and U87-R cells were seeded into a 6-well plate and allowed to grow until they reached a confluence level of over 90%. To commence the scratch assay, the aseptic technique was employed with a sterile pipette tip, creating a straight vertical line along the center of the plate. Following the creation of the scratch, the cells were treated with capsaicin. Images of the scratch area were captured by using an inverted microscope at two distinct time points: immediately after creating the scratch (0 hours) and 24 hours after capsaicin treatment. To quantify the extent of scratch closure, ImageJ software was utilized.

2.7. Quantitative Reverse-Transcription Polymerase Chain Reaction and Western Blotting Assay. TMZ-resistant cells (1×10^5 per well) were cultured in 96-well plates. Following this, the cells underwent treatment with capsaicin for 48 hours. The transcription levels of FHOD1 and IRF2 were assessed using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). To initiate this process, total RNA was extracted from FHOD1 and IRF2 using TRIzol® Reagent (Invitrogen, 12594025). The extracted RNA was then subjected to cDNA synthesis by using the SuperScript™ IV One-Step kit (Invitrogen, 12594025). For the qRT-PCR analysis, the StepOnePlus™ Real-Time PCR System was employed in conjunction with SYBR Green Master Mix. The relative mRNA levels of FHOD1 and IRF2 were determined using the $2^{-\Delta\Delta CT}$ method, with β-actin used as an endogenous control. The qRT-PCR was carried out with the following conditions: an initial preincubation step for 15 min at 60°C, followed by polymerase activation for 10 min at 95°C. This was succeeded by 45 cycles, each consisting of denaturation at 95°C for 10 s, and annealing and extension at 60°C for 40 s. The primer sequences were as follows: FHOD1 forward 5′-CCT CAG CTG ACA CCT CCA GC-3′, FHOD1 reverse 5′-CAG CGC AAC CTG CTT CTC-3′, IRF2 forward 5′-CGA CCG ATC GCT CGG GAC-3′, IRF2 reverse 5′-GCT GCA GAG TGG GCC ATG-3′, β-actin forward 5′-GCC ATG GCC ATC ATG AAG-3′, and β-actin reverse 5′-GTC GTA CGG AGA TGC CCA ACG-3′.

FHOD1 and IRF2 protein levels in U251-R and U87-R cells were assessed using Western blot analysis. This process was executed step by step following the methodology described in our previous study. The antibodies used for analysis were as follows: FHOD1 antibody, diluted to 1 : 1000 (PA5-115233, Invitrogen), and IRF2 antibody, diluted to 0.2 μg/mL (PA5-79515, Invitrogen). To visualize the protein bands, we employed an enhanced chemiluminescence kit (34579, Thermo Scientific). The images were analyzed using ImageJ.

2.8. Statistical Analysis. Statistical analysis was carried out using GraphPad Prism 8 (GraphPad Inc., USA). All experiments were performed with three replicates in three independent experiments. The data were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare three or more groups, followed by Tukey's post hoc test for multiple-group comparisons. Statistical significance was determined when the P value was less than 0.05.

3. Results

3.1. Capsaicin Suppressed Cell Viability and Proliferation in U87-R and U251-R Cells. To investigate the cytotoxic effect of TMZ on viability and proliferation in GBM cells, as shown in Figure 1, we analyzed the response of U87, U251, U251-R, and U87-R cells to TMZ treatment. U87 and U251 cells did not exhibit viability at TMZ concentrations above 1 mM. Following a 48-hour exposure to 200 μM, 400 μM, and 800 μM TMZ, the cell viability of U87 cells exhibited reductions of 28.5%, 70.2%, and 89.6%, respectively, in comparison to the control group. Concurrently, U251 cells demonstrated decreases of 34.1%, 82.7%, and 94.3%, respectively ($P < 0.0001$; Figure 1(a)). Nevertheless, U87-R cells, when subjected to 400 μM, 800 μM, 1 mM, and 2 mM TMZ for 48 hours, exhibited reductions in cell viability by 11.4%, 37.2%, 78.3%, and 90.1%, respectively, compared to the control group ($P < 0.01$ and $P < 0.0001$). Notably, there was no significant variance in the response to the 200 μM TMZ treatment between U87-R and U251-R cells ($P > 0.05$). Furthermore, exposure of U251-R cells to 400 μM, 800 μM, 1 mM, and 2 mM TMZ resulted in reductions of cell viability by 17.5%, 48.3%, 82.6%, and 97.1%, respectively, compared to the control group ($P < 0.0001$). As per the CCK-8 results, the IC_{50} TMZ values for U87, U251, U87-R, and U251-R cells were determined as 337.2 μM, 264.8 μM, 912.7 μM, and 820.8 μM, respectively. These findings collectively indicate the presence of TMZ resistance in U87-R and U251-R cells.

Moreover, in order to illustrate the substantial influence of capsaicin on TMZ resistance in U87-R and U251-R cells, we examined the reaction of these cells to TMZ treatment. As depicted in Figure 1, the inclusion of capsaicin in U87-R and U251-R cells led to a substantial decrease in cell viability following TMZ treatment. U87-R and U251-R cells treated with 120 μM showed no viability at 2 mM TMZ exposure. U87-R cells, subjected to 120 μM capsaicin and exposed to 200 μM, 400 μM, 800 μM, and 1 mM TMZ for 48 hours, manifested a reduction in cell viability by 18.1%, 41.7%, 83.5%, and 92.8%, respectively, compared to the control group ($P < 0.0001$; Figure 1(b)). In addition, the treatment of U251-R cells with 200 μM, 400 μM, 800 μM, and 1 mM TMZ resulted in a decrease in cell viability by 24.6%, 49.2%, 88.5%, and 98.1%, respectively, compared to the control group ($P < 0.0001$). Consistent with the CCK-8 results following the capsaicin treatment, the IC_{50} TMZ values for U87-R and U251-R cells were determined as 522.6 μM and 407.2 μM, respectively.

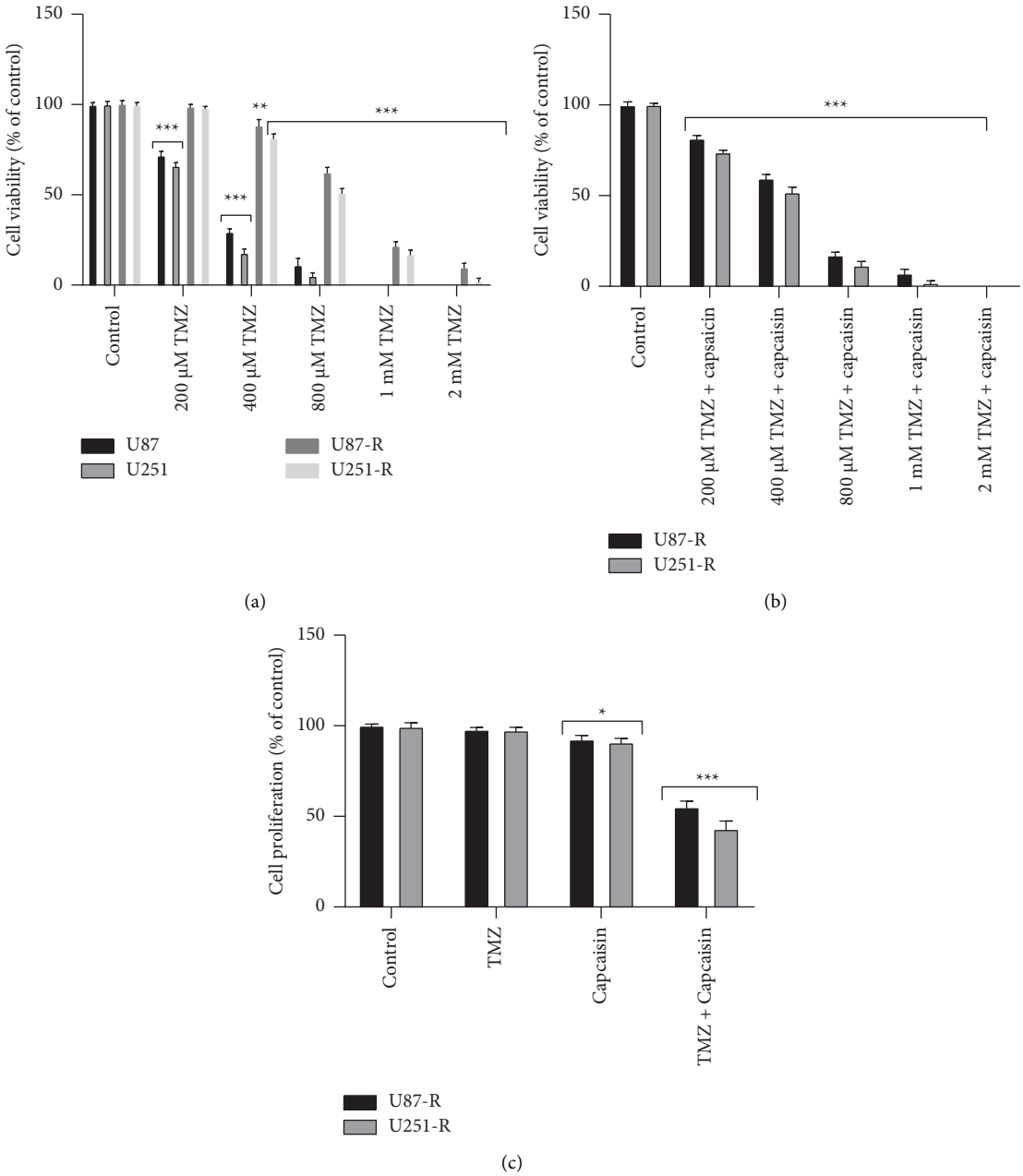


Figure 1: Capsaicin treatment showed antiproliferation effects in TMZ-resistant and TMZ-free GBM cells: (a) CCK-8 results in U87, U251, U87-R, and U251-R cells treated with TMZ, (b) CCK-8 results in U87-R and U251-R cells treated with TMZ and capsaicin, and (c) BrdU incorporation in U87-R and U251-R cells treated with TMZ and capsaicin. $^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ vs. control groups.

In Figure 1(c), to evaluate the impact of capsaicin on TMZ resistance in U87-R and U251-R cells, we examined cell proliferation in both TMZ-treated and untreated cells. An intermediate concentration of 650 μM TMZ was selected for treating U87-R and U251-R cells based on CCK-8 analysis for BrdU analysis and subsequent biochemical analysis. The proliferation of U87-R and U251-R cells treated with TMZ did not exhibit a statistically significant decrease compared to the control groups ($P > 0.05$). However, subsequent to capsaicin treatment, the proliferation of U87-R and U251-R cells decreased by 7.1% and 9.3%, respectively, compared to the control group ($P < 0.05$). Furthermore, the proliferation of U87-R and U251-R cells cotreated with capsaicin and TMZ experienced a reduction of 42.7% and 55.1%, respectively, compared to the control group ($P < 0.0001$). Our findings suggest that capsaicin treatment diminishes cell viability and proliferation by mitigating TMZ resistance in U87-R and U251-R cells.

3.2. Capsaicin Specifically Induced Cell Cycle Arrest in U87-R and U251-R Cells. As seen in Table 1, TMZ treatment did not cause a significant change in the cell cycle in TMZ-resistant cells (U87-R and U251-R). However, capsaicin treatment

caused an increase in the number of U87-R and U251-R cells in the G0/G1 phase. Furthermore, TMZ + capsaicin treatment reduced the number of U87-R and U251-R cells in the G2/M phase. Thus, simultaneous treatment of TMZ and capsaicin regulates the cell cycle of U87-R and U251-R cells, causing an arrest in the G0/G1 phase of these cells.

3.3. Effects of Capsaicin on Biomarkers in U87-R and U251-R Cells. The findings presented in Figures 2(a)–2(g) indicate that capsaicin treatment had a significant impact on various factors in U87-R and U251-R cells. In response to capsaicin treatment, observable changes occurred in FHOD1, IRF2, MDA, GPX4, intracellular iron, TOS, and GSH levels. However, no statistically significant differences were observed in these levels between the control group and cells treated with TMZ ($P > 0.05$). In both U87-R and U251-R cells, capsaicin led to a reduction in FHOD1, IRF2, GSH, and GPX4 levels while causing an enhancement in intracellular iron, TOS, and MDA levels. ELISA analysis results revealed specific percentage changes in these factors. Remarkably, the combined administration of capsaicin and TMZ had a significant impact on various biochemical biomarkers in both TMZ-resistant U87-R and U251-R cells. In U87-R cells, TMZ + capsaicin treatment resulted in a 37.3% reduction in FHOD1, a 29.2% reduction in IRF2, a 40.6% reduction in GSH, and a 35.8% reduction in GPX4 levels ($P < 0.0001$ vs. control). Conversely, this treatment led to a 24.7% increase in intracellular iron, a 42.1% increase in TOS, and a 30.5% increase in MDA levels ($P < 0.0001$). Likewise, TMZ + capsaicin treatment in U251-R cells caused a 40.4% reduction in FHOD1, a 35.3% reduction in IRF2, a 52.3% reduction in GSH, and a 47.2% reduction in GPX4 levels ($P < 0.0001$ vs. control). TMZ + capsaicin also triggered a 29.3% increase in intracellular iron, a 51.4% increase in TOS, and a 38.5% increase in MDA levels ($P < 0.0001$ vs. control).

3.4. Capsaicin Treatment Regulated Wound Healing in U87-R and U251-R Cells. As shown in Figure 3, capsaicin clearly suppressed the migration ability of U87-R and U251-R cells according to the wound healing assay. Initially, in the untreated cells, a substantial portion of the initial wound area was observed to close within a span of 48 hours. This closure amounted to 84.26% in the case of U87-R cells and 77.52% in U251-R cells, indicating a significant wound-healing process ($P < 0.0001$ compared to control cells). However, with capsaicin treatment, a remarkable deceleration in the rate of wound healing was evident in both U87-R and U251-R cells. In particular, following 48 hours of treatment with TMZ + capsaicin, U87-R cells exhibited a 25.83% expansion in the wounded region ($P < 0.0001$ compared to control cells; Figure 3(a)). Similarly, U251-R cells experienced an expansion of 20.74% ($P < 0.0001$ compared to control cells; Figure 3(a)) after the same treatment duration. These outcomes strongly underscore the inhibitory effect of capsaicin treatment on the migratory capacity of both U87-R and U251-R cells.

Table 1: Capsaicin treatment arrested U87-R and U251-R cells in the G0/G1 phase.

Cells	Groups	Cell cycles		
		G1/G0	S	G2/M
U87-R	Control	60.2	11.5	17.3
	TMZ	59.2	15.8	20.1
	Capsaicin	65.7	10.3	12.6
	TMZ + capsaicin	71.9	7.5	9.2
U251-R	Control	54.2	18.3	25.7
	TMZ	55.4	16.8	21.6
	Capsaicin	60.8	14.5	17.2
	TMZ + capsaicin	78.4	4.2	10.8

% cell viability of each cell cycle phase (G0/G1, S, and G2/M).

3.5. Capsaicin-Regulated FHOD1 and IRF2 Expressions and Protein Levels in U87-R and U251-R Cells. The observations presented in Figures 4 and 5 reveal substantial changes in the expression and protein levels of FHOD1 and IRF2 subsequent to capsaicin treatment in both U87-R and U251-R cells. Notably, TMZ treatment in U87-R and U251-R cells did not elicit statistically significant alterations in the mRNA levels of FHOD1 and IRF2. However, as depicted in Figures 4(a) and 4(b), FHOD1 mRNA levels in U87-R and U251-R cells experienced reductions of 34.7% and 41.3%, respectively, under TMZ + capsaicin treatment ($P < 0.0001$). Furthermore, IRF2 mRNA levels in U87-R and U251-R cells treated with TMZ + capsaicin exhibited reductions of 25.8% and 37.2%, respectively ($P < 0.0001$; Figures 4(c) and 4(d)). In alignment with the mRNA outcomes, Western blot analysis indicated that capsaicin treatment led to diminished levels of FHOD1 and IRF2 proteins in both U87-R and U251-R cells. Notably, a more pronounced suppressive effect of capsaicin on these proteins was evident in U251-R cells when compared to capsaicin-treated U87-R cells.

4. Discussion

Drug resistance plays a pivotal role in clinical outcomes, influencing tumor relapse rates and patient survival. In the context of GBM, TMZ resistance is a key contributor to treatment failure. Researchers have vigorously examined the molecular underpinnings of this chemoresistance in GBM, particularly focusing on genes involved in DNA repair mechanisms [19]. However, despite extensive research, the mechanism of resistance developed by GBM cells against TMZ is not clearly understood. The primary objective of this study was to elucidate the roles played by FHOD1 and IRF2 in the regulation of ferroptosis in TMZ-resistant GBM cells, particularly through the application of capsaicin treatment. Notably, previous research has observed significantly elevated FHOD1 and IRF2 levels in both glioma tissues and cell lines [20]. Consequently, we uncovered that the downregulation of FHOD1 and IRF2 through capsaicin treatment in TMZ-resistant GBM cells led to a noteworthy increase in both drug sensitivity and susceptibility to ferroptosis. These findings collectively contribute to elucidating potential avenues for enhancing therapeutic strategies in TMZ-resistant GBM cases.

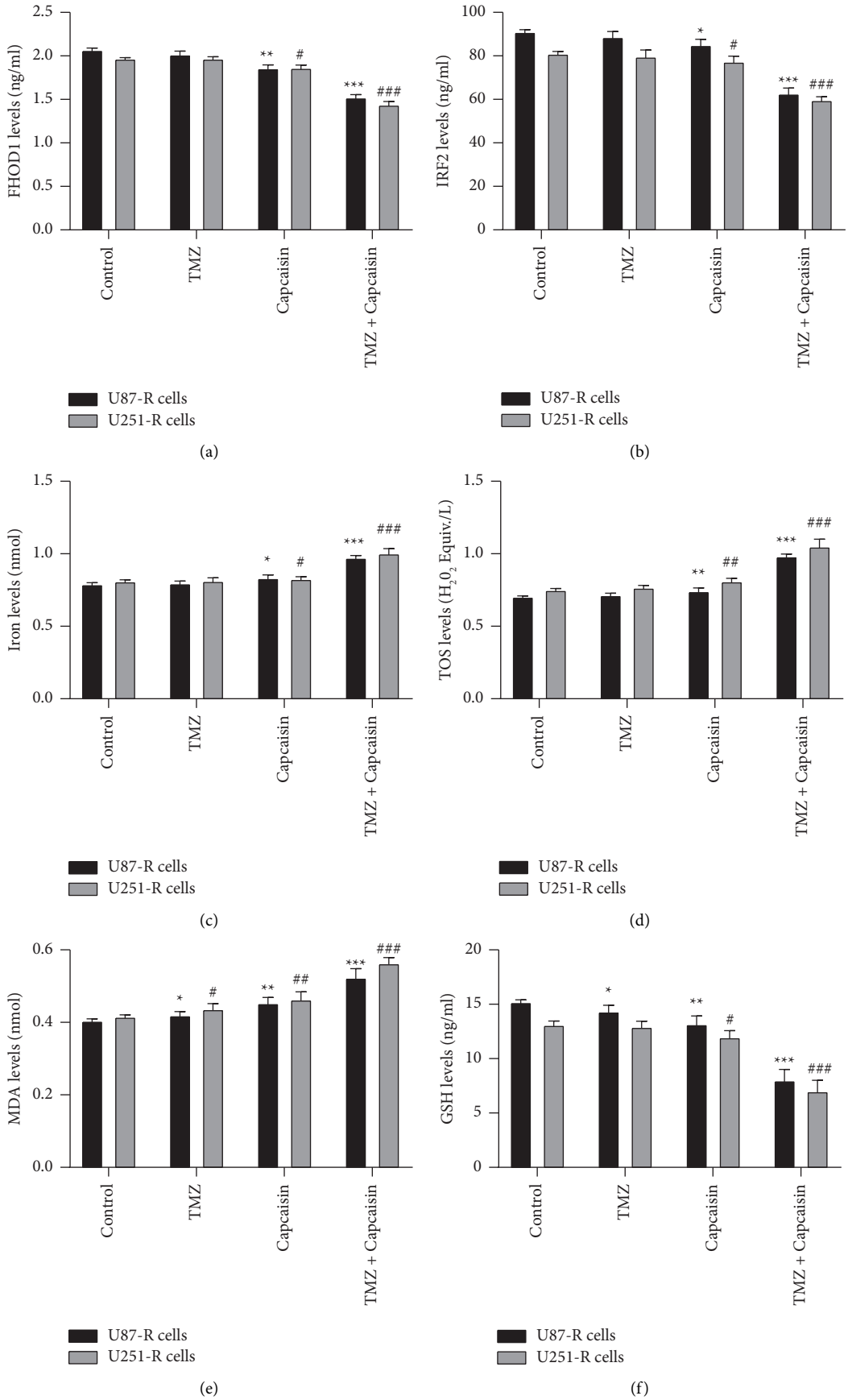
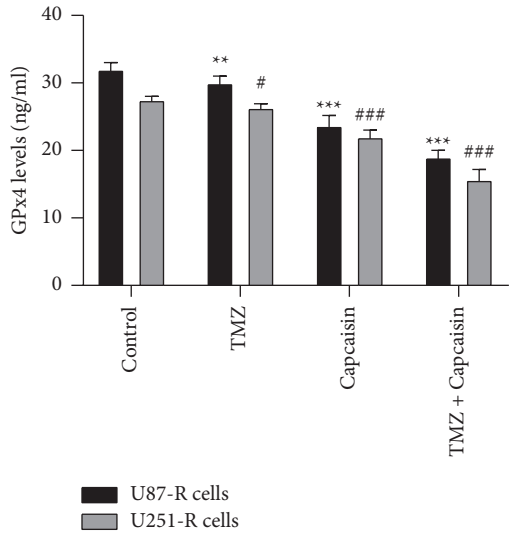
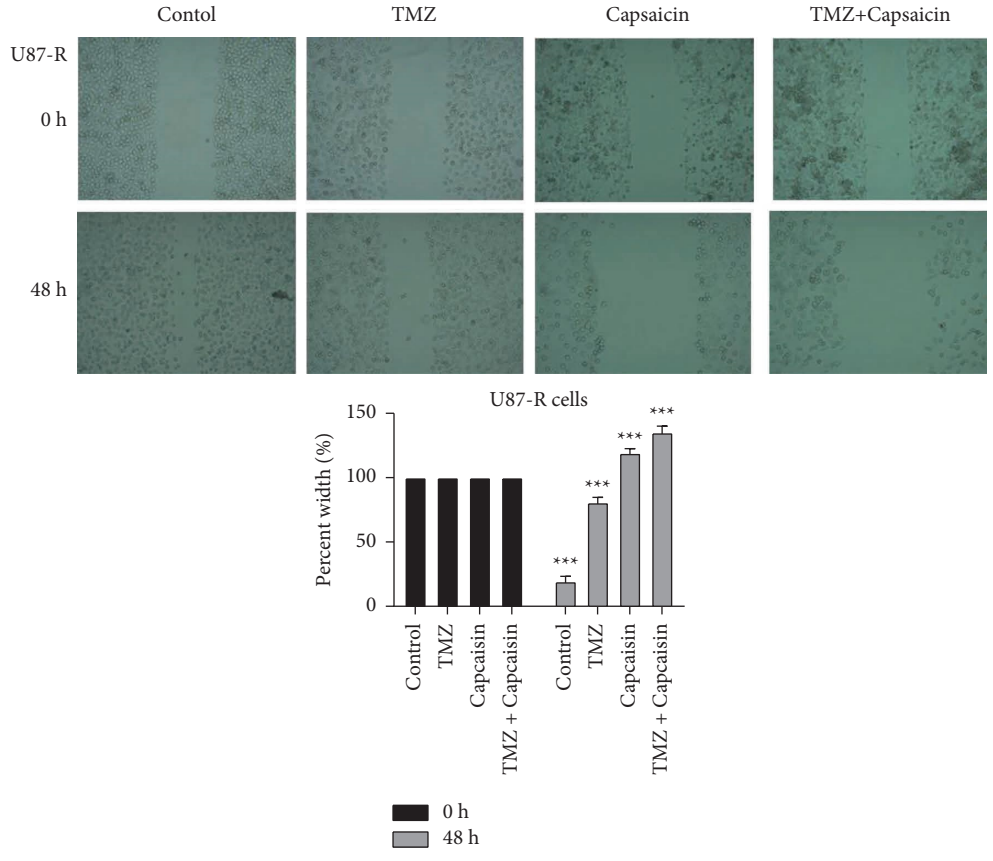


Figure 2: Continued.



(g)

Figure 2: Capsaicin treatment regulated FHOD1, IRF2, intracellular iron, TOS, MDA, GSH, and GPX4 levels in U87-R and U251-R cells: (a) FHOD1 levels in U87-R and U251-R cells, (b) IRF2 levels in U87-R and U251-R cells, (c) iron levels in U87-R and U251-R cells, (d) TOS levels in U87-R and U251-R cells, (e) MDA levels in U87-R and U251-R cells, (f) GSH levels in U87-R and U251-R cells, and (g) GPX4 levels in U87-R and U251-R cells. $^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ vs. the U87-R control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, and $^{\#\#\#}P < 0.0001$ vs. the U251-R control group.



(a)

Figure 3: Continued.

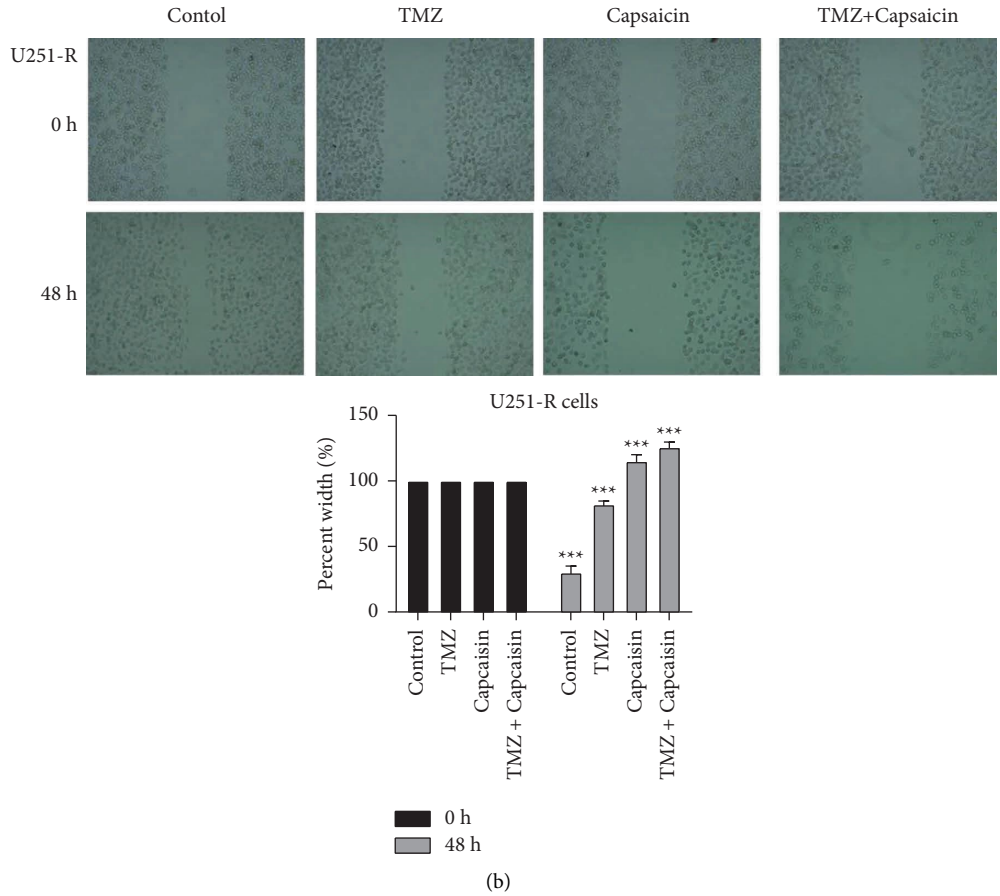


Figure 3: Capsaicin treatment inhibited cell migration rate in U87-R and U251-R cells: (a) microscope images of cell migration rates of capsaicin treatment for 48 hours in U87-R cells and (b) microscope images of cell migration rates of capsaicin treatment for 48 hours in U251-R cells. $^{***}P < 0.0001$ vs. control groups.

The phenomenon of continuous cell proliferation is a hallmark associated with resistance to TMZ and is a key factor contributing to tumor recurrence [21]. In this study, we observed that U87 and U251 cells, which did not possess TMZ resistance, exhibited a higher susceptibility to TMZ treatment than the TMZ-resistant U87-R and U251-R cells. Importantly, even after TMZ treatment, the resistant U87-R and U251-R cells displayed rapid regrowth in the cell migration assay. Furthermore, the viability and proliferation of TMZ-resistant U87-R and U251-R cells were found to be sustained even in the presence of higher concentrations of TMZ, contrasting with the response of U87 and U251 cells. This suggests that the resistant cells have developed mechanisms to withstand the cytotoxic effects of TMZ. This resistance to the effects of TMZ implies a significant potential for tumor recurrence and dissemination of these resistant cells.

Ferroptosis is a form of programmed cell death characterized by the accumulation of cellular iron and reactive oxygen species (ROS) [10]. In a previous study, it was observed that the suppression of FHOD1 significantly increased elastin-induced ferroptosis in T98G and U251 GBM cells [9]. These outcomes strongly suggest that FHOD1-linked signaling pathways potentially hold a critical role in governing resistance to ferroptosis. Furthermore, by employing a knockdown strategy, this study offered pioneering evidence of the indispensability of FHOD1 expression for the efficient migration of U87, U138, T86, and UTGB7 GBM cells [22]. This aligns with findings at the cellular level, where FHOD1 knockdown in basal-like breast cancer cell lines resulted in larger cell areas and compromised abilities to migrate, invade, and proliferate [8]. Besides, FHOD1 expression and functional relevance have been observed in other cancer types such as oral squamous cell carcinoma and melanoma [7, 23]. These studies have indicated that FHOD1 contributes to cellular migration and invasion *in vitro*, suggesting a broader role for FHOD1 in promoting these processes across multiple cancer types. These findings harmonize with our data that identified FHOD1 as a pivotal contributor to facilitating cellular migration. Furthermore, our results showed that when capsaicin was administered to TMZ-resistant U87-R and U251-R cells, it led to the reduction of FHOD1, GSH, and GPX4 levels. This reduction, in turn, triggered an accumulation of intracellular iron within these cells. Consequently, this increase in intracellular iron, in combination with the reduction of FHOD1, GSH, and GPX4 levels, led to the heightened oxidative stress and a surge in lipid peroxidation.

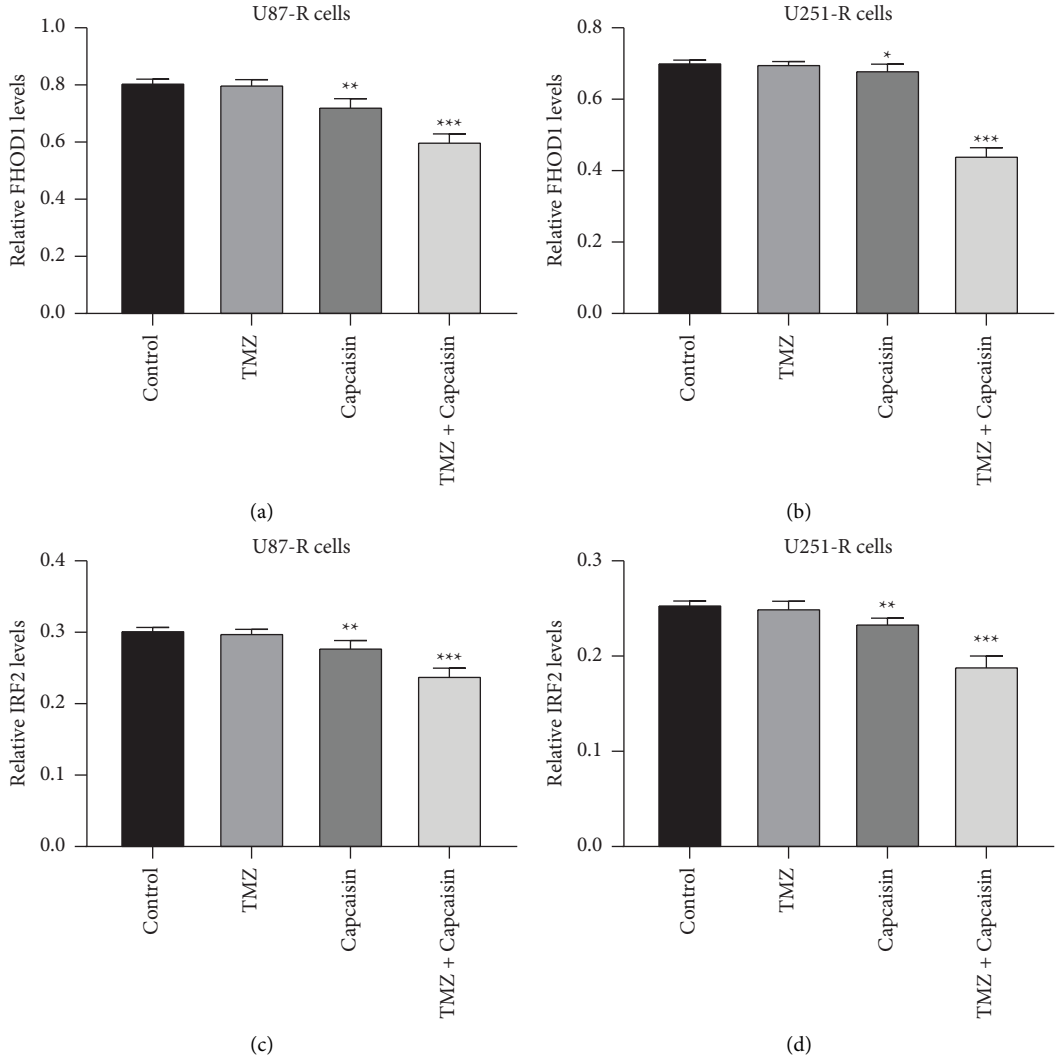


Figure 4: Effects of capsaicin treatment on expression levels of FHOD1 and IRF2 in U87-R and U251-R cells: (a) FHOD1 mRNA levels in U87-R cells, (b) FHOD1 mRNA levels in U251-R cells, (c) IRF2 mRNA levels in U87-R cells, and (d) IRF2 mRNA levels in U251-R cells. $^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ vs. control groups.

Members of the IRF family have emerged as important regulators of inflammatory and immune microenvironment signaling pathways, exerting crucial roles in the development and progression of cancer [24]. Dysregulation of the IRF family members has been documented in diverse malignancies [25]. However, the comprehensive role of IRFs in glioma remains relatively understudied. In this study, we initiated an investigation into the expression profile, prognostic implications, and biological functions of individual IRF2 within the context of TMZ-resistant U87-R and U251-R cells. As previously mentioned, the levels of IRF family members were noted to be elevated in GBM cells compared to normal cells [20]. Furthermore, the expression levels of different IRFs elevate with tumor progression, yet patients exhibiting lower IRF expression experience significantly prolonged survival [12]. For instance, Liang et al. demonstrated that IRF1 knockdown in a glioma animal model led to an increased apoptosis and enhanced the effectiveness of anti-VEGF treatment, highlighting elevated IRF1 expression in glioma cell lines [26]. Similarly, Jin et al. reported overexpression of IRF7 in glioma cells and linked these elevated levels to decreased patient survival [27]. Moreover, patients with high IRF2 expression in gliomas exhibited poor overall survival [20].

This research effectively employed extensive clinical data to identify heightened IRF2 expression as a noteworthy trait in gliomas, indicating IRF2's potential as a biomarker for glioma. Numerous studies have uncovered IRF2's connections with tumorigenesis and tumor progression [28]. In liver cancer cells, reducing IRF2 has been associated with decreased invasiveness [29]. Conversely, in pancreatic cancer, IRF2 has been linked to the depth of tumor invasion and the promotion of tumor cell growth [30]. It is crucial to acknowledge, however, that the role of IRF2 may vary in different tumor contexts.

In summary, our study provides strong evidence underlining the critical roles of FHOD1 and IRF2 in conferring resistance to TMZ in glioma cells. Our findings

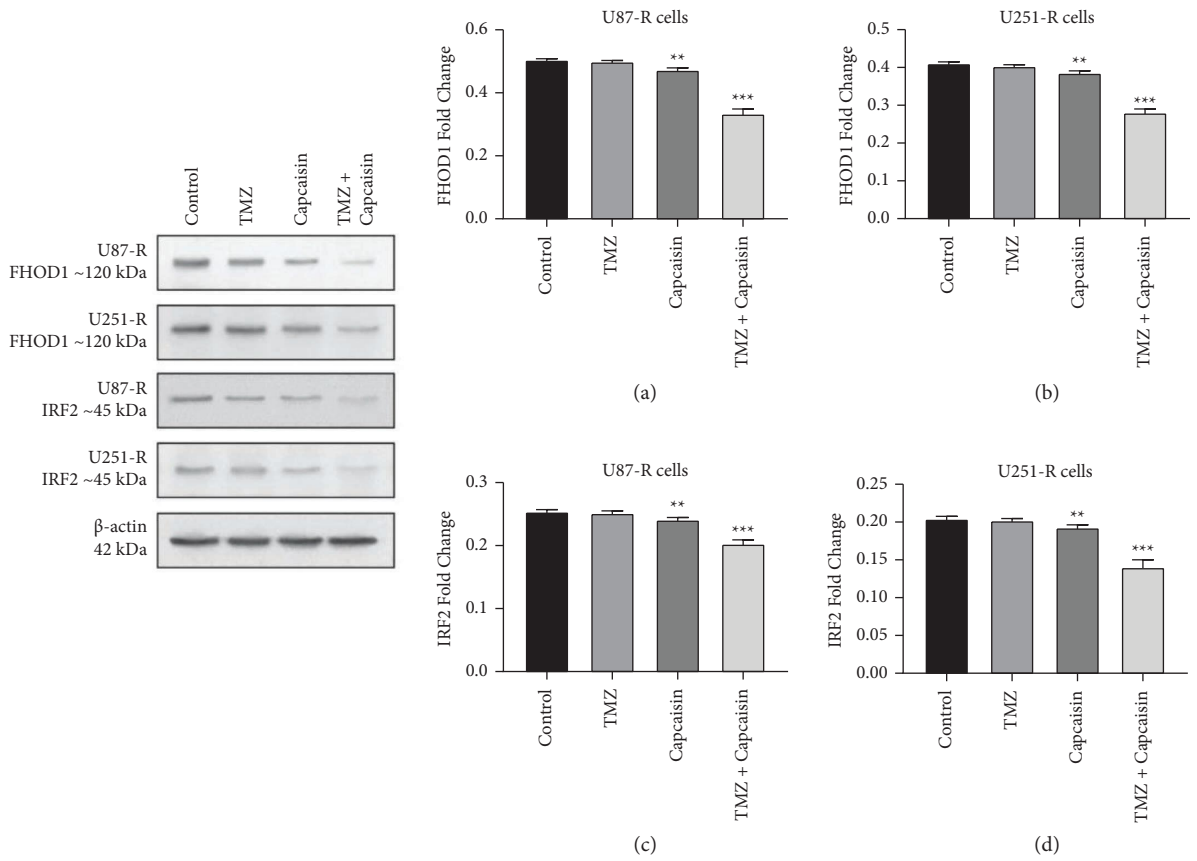


Figure 5: Capsaicin treatment reduced FHOD1 and IRF2 protein levels in U87-R and U251-R cells: (a) FHOD1 protein levels in U87-R cells, (b) FHOD1 protein levels in U251-R cells, (c) IRF2 protein levels in U87-R cells, and (d) IRF2 protein levels in U251-R cells. $^{**}P < 0.001$ and $^{***}P < 0.0001$ vs. control groups.

demonstrate that capsaicin treatment sensitizes glioma cells to TMZ and ferroptosis, leading to a concurrent reduction in FHOD1 and IRF2 levels. However, an important limitation of our study is that silecing and overexpressing experiments of the targeted FHOD1 and IRF2 genes could not be performed. These results highlight FHOD1 and IRF2 as promising targets for the development of chemotherapeutic drugs and suggest their potential as novel diagnostic and predictive biomarkers. Our study underscores the importance of unraveling the mechanisms behind TMZ resistance, given its substantial impact on tumor behavior and the effectiveness of treatment strategies.

Data Availability

All data generated during this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The author declares that there are no conflicts of interest.

Acknowledgments

This study was supported by Tubitak Ulakbim under Grant no. TUB1.

References

[1] Q. T. Ostrom, L. Bauchet, F. G. Davis et al., "The epidemiology of glioma in adults: a state of the science review," *Neuro-Oncology*, vol. 16, no. 7, pp. 896–913, 2014.

[2] V. A. Venur, D. M. Peereboom, and M. S. Ahluwalia, "Current medical treatment of glioblastoma," *Cancer Treatment and Research*, vol. 163, pp. 103–115, 2015.

[3] R. Stupp, W. P. Mason, M. J. van den Bent et al., "Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma," *New England Journal of Medicine*, vol. 352, no. 10, pp. 987–996, 2005.

[4] P. Daniel, S. Sabri, A. Chaddad et al., "Temozolomide induced hypermutation in glioma: evolutionary mechanisms and therapeutic opportunities," *Frontiers Oncology*, vol. 9, p. 41, 2019.

[5] S. Y. Lee, "Temozolomide resistance in glioblastoma multiforme," *Genes and Diseases*, vol. 3, no. 3, pp. 198–210, 2016.

[6] N. Schulze, M. Graessl, A. Blancke Soares, M. Geyer, L. Dehmelt, and P. Nalbant, "FHOD1 regulates stress fiber organization by controlling the dynamics of transverse arcs and dorsal fibers," *Journal of Cell Science*, vol. 127, no. Pt 7, pp. 1379–1393, 2014.

[7] M. Gardberg, K. Kaipio, L. Lehtinen et al., "FHOD1, a formin upregulated in epithelial-mesenchymal transition, participates in cancer cell migration and invasion," *Public Library of Science One*, vol. 8, no. 9, Article ID e74923, 2013.

[8] V. D. Heuser, N. Mansuri, J. Mogg et al., "Formin proteins FHOD1 and INF2 in triple-negative breast cancer: association

with basal markers and functional activities," *Breast Cancer: Basic and Clinical Research*, vol. 12, Article ID 117822341879224, 2018.

[9] F. Zhang, L. Wu, S. Feng et al., "FHOD1 is upregulated in glioma cells and attenuates ferroptosis of glioma cells by targeting HSPB1 signaling," *Central Nervous System Neuroscience and Therapeutics*, vol. 29, no. 11, pp. 3351–3363, 2023.

[10] X. Jiang, B. R. Stockwell, and M. Conrad, "Ferroptosis: mechanisms, biology and role in disease," *Nature Reviews Molecular Cell Biology*, vol. 22, no. 4, pp. 266–282, 2021.

[11] H. Yanai, H. Negishi, and T. Taniguchi, "The IRF family of transcription factors: inception, impact and implications in oncogenesis," *OncoImmunology*, vol. 1, no. 8, pp. 1376–1386, 2012.

[12] J. Lei, M. H. Zhou, F. C. Zhang, K. Wu, S. W. Liu, and H. Q. Niu, "Interferon regulatory factor transcript levels correlate with clinical outcomes in human glioma," *Aging (Albany NY)*, vol. 13, no. 8, pp. 12086–12098, 2021.

[13] M. A. Ilie, C. Caruntu, M. Tampa et al., "Capsaicin: physicochemical properties, cutaneous reactions and potential applications in painful and inflammatory conditions," *Experimental and Therapeutic Medicine*, vol. 18, no. 2, pp. 916–925, 2019.

[14] S. Zhang, D. Wang, J. Huang, Y. Hu, and Y. Xu, "Application of capsaicin as a potential new therapeutic drug in human cancers," *Journal of Clinical Pharmacy and Therapeutics*, vol. 45, no. 1, pp. 16–28, 2020.

[15] C. Hacioglu and F. Kar, "Capsaicin induces redox imbalance and ferroptosis through ACSL4/GPx4 signaling pathways in U87-MG and U251 glioblastoma cells," *Metabolic Brain Disease*, vol. 38, no. 2, pp. 393–408, 2023.

[16] Y. Zhu, Z. Chen, S. N. Kim et al., "Characterization of temozolomide resistance using a novel acquired resistance model in glioblastoma cell lines," *Cancers*, vol. 14, no. 9, p. 2211, 2022.

[17] K. Xu, Z. Zhang, H. Pei, H. Wang, L. Li, and Q. Xia, "FoxO3a induces temozolomide resistance in glioblastoma cells via the regulation of β-catenin nuclear accumulation," *Oncology Reports*, vol. 37, no. 4, pp. 2391–2397, 2017.

[18] C. Hacioglu, "Capsaicin inhibits cell proliferation by enhancing oxidative stress and apoptosis through SIRT1/NOX4 signaling pathways in HepG2 and HL-7702 cells," *Journal of Biochemical and Molecular Toxicology*, vol. 36, no. 3, Article ID e22974, 2022.

[19] S. Yip, J. Miao, D. P. Cahill et al., "MSH6 mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance," *Clinical Cancer Research*, vol. 15, no. 14, pp. 4622–4629, 2009.

[20] S. Tong, L. Ye, Y. Xu et al., "IRF2-ferroptosis related gene is associated with prognosis and EMT in gliomas," *Translational Oncology*, vol. 26, Article ID 101544, 2022.

[21] M. S. Tomar, A. Kumar, C. Srivastava, and A. Shrivastava, "Elucidating the mechanisms of Temozolomide resistance in gliomas and the strategies to overcome the resistance," *Biochimica et Biophysica Acta, Reviews on Cancer*, vol. 1876, no. 2, Article ID 188616, 2021.

[22] V. D. Heuser, A. Kiviniemi, L. Lehtinen et al., "Multiple formin proteins participate in glioblastoma migration," *Bone Marrow Concentrate Cancer*, vol. 20, no. 1, p. 710, 2020.

[23] M. Peippo, M. Gardberg, T. Lamminen, K. Kaipio, O. Carpén, and V. D. Heuser, "FHOD1 formin is upregulated in melanomas and modifies proliferation and tumor growth," *Experimental Cell Research*, vol. 350, no. 1, pp. 267–278, 2017.

[24] E. Fratta, L. Sigalotti, A. Covre, G. Parisi, S. Coral, and M. Maio, "Epigenetics of melanoma: implications for immune-based therapies," *Immunotherapy*, vol. 5, no. 10, pp. 1103–1116, 2013.

[25] Q. Gao, H. Zhu, L. Dong et al., "Integrated proteogenomic characterization of HBV-related hepatocellular carcinoma," *Cell*, vol. 179, no. 2, pp. 561–577.e22, 2019.

[26] J. Liang, Y. Piao, V. Henry, N. Tiao, and J. F. de Groot, "Interferon-regulatory factor-1 (IRF1) regulates bevacizumab induced autophagy," *Oncotarget*, vol. 6, no. 31, pp. 31479–31492, 2015.

[27] X. Jin, S. H. Kim, H. M. Jeon et al., "Interferon regulatory factor 7 regulates glioma stem cells via interleukin-6 and Notch signalling," *Brain*, vol. 135, no. 4, pp. 1055–1069, 2012.

[28] Y. Guo, J. Xu, Q. Du, Y. Yan, and D. A. Geller, "IRF2 regulates cellular survival and Lenvatinib-sensitivity of hepatocellular carcinoma (HCC) through regulating β-catenin," *Translational Oncology*, vol. 14, no. 6, Article ID 101059, 2021.

[29] Y. Yi, H. Wu, Q. Gao et al., "Interferon regulatory factor (IRF)-1 and IRF-2 are associated with prognosis and tumor invasion in HCC," *Annals of Surgical Oncology*, vol. 20, no. 1, pp. 267–276, 2013.

[30] T. Sakai, H. Mashima, Y. Yamada et al., "The roles of interferon regulatory factors 1 and 2 in the progression of human pancreatic cancer," *Pancreas*, vol. 43, no. 6, pp. 909–916, 2014.