

# Temozolomide Increases HSPs in Extracellular Vesicles Released from Glioblastoma Cells

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## Research Article

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# Abstract

Glioblastoma (GBM) is the most malignant and the fastest-progressing type of primary brain tumours. Temozolomide (TMZ) is a chemotherapeutic drug for the treatment of GBM. Extracellular vesicles (EVs) have been recently confirmed to have a substantial role in the GBM, and their contents released from GBM cells have been considered a target for treatment. Here, we assessed the impact of TMZ on heat shock proteins (HSPs) derived from extracellular vesicles (EVs) originated from GBM cell lines (U87-MG and LN229) and the significance of EVs in response to chemotherapy in GBM. We first characterised EVs and detected that U87-MG cells release many EVs compared to LN229 cells. Interestingly, TMZ was found to be led to increases in HSF-1, Hsp90, Hsp70, Hsp60 and Hsp27 expression in GBM cells and their EV contents, which these increases are related to therapeutic resistance. What is more, in studies showing which signalling pathways might be associated with these increases, it was observed that TMZ triggered the expression of RAD51 and MDM2 genes in cells and EV contents. More strikingly, we discover a correlation between EV and parental cells in regard of mRNA and protein level in both cell lines as a result of TMZ treatment. Therefore, this data suggest of EVs in the treatment of GBM may have potential biomarkers that can be used to investigate the treatment response.

## Introduction

Glioblastoma (GBM) is the most malignant and the fastest-progressing primary brain tumour in adults. Despite all the possibilities of treatment involving surgical resection, radiation and chemotherapy, the overall response remains poor due to the presence of acquired resistance and blood-brain barrier [1, 2]. Temozolomide (TMZ), a chemotherapeutic drug, is a crucial therapeutic employed in the treatment of GBM because of its structure, which allows it to pass the blood-brain barrier [3].

Heat shock proteins (HSPs) are evolutionarily conserved proteins. The majority of HSPs (also known as stress proteins) have significant roles in biosynthesis, folding and unfolding, transport, and interaction of other proteins. The stimulation of HSP-encoding genes necessitates the use of specific transcriptional regulators known as "Heat Shock Factors" (HSF). [4–6]. It is known that some HSPs have been found to be elevated in many cancer types, including GBM and their function aid in progression of the disease. High HSP expression is linked to tumour development, metastasis, treatment resistance, and apoptotic suppression. As a result, HSPs are thought to be viable therapeutic targets for GBM [7].

Extracellular vesicles (EVs), including exosomes, micro-vesicles and apoptotic bodies, which are small vesicles secreted by all cell types such as immune cells, neurons, tumour cells into the extracellular space, play a part in many pathological and physiological processes and can be detectable in plenty of biofluids such as saliva, urine, blood, breast milk, amniotic fluid, cerebrospinal fluid [8–10]. Several studies have demonstrated that EVs carry nucleic acids, proteins, mRNA, miRNA, nucleoproteins, and various enzymes, together with some specific markers (CD9, CD63, CD81, ALIX...) playing their biogenesis [11, 12]. They indicate individualities, which enable tracking of their cellular origin and reflect the state of the host cell [13]. EVs originating from the tumour with the help of their content, especially their nucleic acids and

oncogenic signal proteins such as heat shock proteins (HSP), which have been confirmed to play significant roles in EVs and cancers, can be involved in cancer progression, metastasis, invasion, and resistance formation. Therefore, targeting tumor-derived EVs packaged with HSP has become promising approach in cancer therapy since they are emerged as potential biomarkers for cancer diagnosis and prognosis.

The aim of this study is to understand the effects of TMZ on the expression levels of cellular and EV-HSPs, to investigate which signalling pathways contribute to this effect and to reveal the role of EVs in response to chemotherapy in GBM. The two cell lines U87-MG and LN229 were chosen because of their differences in terms of their mutations, aggressiveness, and treatment responses. We firstly investigated the cytotoxic effect of TMZ on U87-MG and LN229 human glioma cell lines and then the expression levels of different stress proteins were evaluated in both cells and their EV's. In the next stage, various genes, which thought to be related to HSPs response, involved in diverse processes such as apoptosis, DNA damage response, cell cycle regulation, stress response, tumour formation and suppression in the cell was analysed by applying TMZ to human GBM cells. It was observed that in U87 -MG cells, the HSP response has been linked to a rise in the RAD51 gene, whereas in LN229 cells, it has been associated with the MDM2 gene. Furthermore, the expression of these genes was found to escalate in their EVs produced from parental cells. All findings obtained from this study emphasise that EVs can be potential biomarkers for investigating treatment response in GBM.

## **Materials And Methods**

### **Experimental reagents and antibodies**

Temozolomide was purchased from Thermo Fisher Scientific and dissolved in dimethyl sulfoxide (DMSO). Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12) was obtained from Sigma. Exosome-depleted Fetal Bovine Serum and other standard tissue culture reagents were supplied from Gibco. Amicon® Ultra-15 Centrifugal Filter Units was obtained from Merck - Millipore. ExoEasy Maxi Exosome Isolation Kit and exoRNeasy Serum / Plasma Maxi Kit were purchased from Qiagen. PS Capture™ Exosome ELISA Kit was from obtained Fujifilm. RNA isolation kit was supplied from Macherey–Nagel. cDNA Reverse transcription kit was purchased from Bio Rad. 1X RIPA Buffer was obtained from Santa Cruz Biotechnology 10X RIPA Buffer was obtained from Thermo Fisher Scientific. Pierce™ BCA Protein Assay Kit, PVDF membrane and SuperSignal West Pico PLUS Chemiluminescent Substrate were purchased from Thermo Fisher Scientific. All the other chemicals were obtained from Sigma. The antibodies used in the study are given in Table 1.

Table 1  
Primer and Seconder Antibodies used for Western blotting

Antikor	Host	Company	Catalog Number	Dilution
Anti-Hsp27	Rabbit	Novus	NB120-5579	1:1000
Anti-Hsp60	Rabbit	Novus	NBP1-77397	1:2000
Anti-Hsp70	Rabbit	Novus	NBP2-16896	1:1000
Anti-Hsp90	Rat	Novus	NBP1-97506	1:1000
Anti-HSF1	Rabbit	Novus	NBP1-97475	1:1000
Anti-Alix (1A12)	Mouse	Santa Cruz	sc-53540	1:200
Anti-TSG101	Mouse	Novus	NB200-112	1:500
Anti-RAD51	Rabbit	Novus	NB100-14	1:500
Anti-MDM2	Mouse	Novus	NB100-2736	1:300
Anti-GAPDH	Mouse	Invitrogen	MA5-15738	1:2500
Anti-Mouse IgG	Goat	Boster	BA1050	1:2500
Anti-Rabbit IgG	Goat	Boster	BA1054	1:2500

## Cell and culture conditions

The human glioma cell lines U87-MG and LN229 were obtained from TÜBİTAK Marmara Research Center, Genetic Engineering and Biotechnology Institute. The cell lines were cultured in DMEM/F12, containing 5% fetal bovine serum, and supplemented with 1% antibiotic-antimycotics solution (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B) in 5% CO<sub>2</sub> at 37°C. Cells were subcultured every three days.

## Cell viability assay

The cytotoxic effect of TMZ on U87-MG and LN229 human glioma cell lines were determined by MTT method, following the procedure recommended by Mosmann (1983) [14]. U87-MG and LN229 cell lines were seeded in 96-well cell culture dishes with  $5 \times 10^3$  and  $1 \times 10^4$  cells, respectively. After the 24 hours incubation, the cells were treated with various doses (50-1600 µM) of TMZ for 24, 48 and 72 h. Then, 100 µL of medium containing MTT with a final concentration of 0.5 mg / mL was added to each well, incubated for 4 h. In the final stage, 150 µL of DMSO was added to dissolve the formazan crystals. The absorbance's of the samples were measured using a microplate reader (Biotek, ELx800) at 562 nm wavelength. Experiments were carried out in at least five replicates.

## Extracellular Vesicle Isolation

The cell lines were cultured in DMEM/F12 medium with 5% FBS through 24 hours until 70–80% confluence. Then, cultured cells were washed with PBS and were incubated with different concentrations of TMZ (100  $\mu$ M and 200  $\mu$ M were selected based on cell viability analysis results) containing 5% exosome-depleted FBS for 72 h. Each collected fraction of media was centrifuged 3000 $\times$ g for 5 minutes to remove cell debris. Then, the supernatant was centrifuged at 5000 $\times$ g for 15 minutes at 4°C using a sterile 50 kDa membrane filter (Amicon ultra-15 apparatus 50k Merck) to concentrate. EV isolation from the concentrated medium was performed using the exoEasy Maxi Exosome Isolation Kit according to the manufacturer's instructions.

## Characterization studies of EVs

Qualitative and quantitative analyses of isolated EVs were performed using the PS Capture™ Exosome ELISA Kit according to the manufacturer's instructions, and this method is based on the presence of CD63, which is considered a common EV marker. Nanoparticle tracking analysis (NTA) was also executed in characterisation studies of EVs. NTA was carried out using the Nanosight NS300 NTA with a blue laser system (NanoSight, Malvern Panalytical) on isolated EVs diluted 100–1000 fold with dH<sub>2</sub>O for analysis. A 50 s video recorded all events for further analysis by NTA software.

## Western blot analysis

The impact of TMZ on cellular protein and EV-protein was investigated in this study. Cellular proteins were extracted in 1X RIPA Buffer while 10X RIPA Buffer was used for EV proteins and then centrifuged at 13000 $\times$ g for 30 min at 4°C. Methanol / chloroform protein precipitation method described in Wessel and Flügge (1984) was used to concentrate EV proteins [15]. The protein levels of RAD51 (1:500), MDM2 (1:300), HSF1 (1:1000), Hsp90 (1:1000), Hsp70 (1:1000), Hsp60(1:2000), and Hsp27 (1:1000) to investigate the effect of TMZ application on both cellular protein and EV-protein and the protein levels of ALIX (1:200) and TSG101 (1:500) for characterization analysis of EVs were analysed by Western blotting using the standard procedure as previously described [7]. At least three independent assays were carried out.

## Reverse transcription quantitative PCR analysis

The effect of TMZ application on both cellular RNA and EV-RNA was examined. Total cellular RNA was isolated using the NucleoSpin RNA II kit according to the manufacturer's instructions and RNA concentrations were measured with aid of a Nanodrop spectrophotometer (NanoDrop™ 1000 Spectrophotometer, Celbio). RNA isolation from the obtained EVs will be done using the exoRNeasy Serum / Plasma Maxi Kit according to the manufacturer's instructions. EV-RNA quality and quantification were done using the Agilent RNA 6000 Pico Assay kit following the manufacturer's protocols. Both cellular RNA and EV-RNA were converted into cDNA using iScript cDNA synthesis kit following the manufacturer's instructions. The effect of TMZ on U87-MG and LN229 cells was evaluated by examining 86 genes (Table 2) involved in different processes, such as apoptosis, DNA damage response, cell cycle regulation, stress response, tumor formation and suppression. Relative quantification technique was used

in PCR analysis of cellular RNAs, and each sample was normalized according to three different internal controls (HPRT-YWAZ-GAPDH). The effects of TMZ on EVs were evaluated using 2 genes (RAD51 and MDM2) whose expression significantly changed in the cell due to TMZ administration. The absolute quantification technique was used in the analysis of EV target genes, and in this context, standard plots were created with the PCR products of the target genes. Each sample was analysed in duplicate, and experiments were carried out in at least 3 replicates. The following primers were used:

Table 2  
The genes examined within the scope of the study and their functions

Functions of Genes	Genes
<i>Apoptosis</i>	BCL2L10, Bcl-xL (BCL2L1), BFA1, BIRC2, BIRC3, BIRC4 /XIAP, BIRC5, BIRC6, BIRC8, BNIP1, BNIP2, APAF1, BMF, CASP1, CASP2, CASP3, CASP4, CASP5, CASP6, CASP7, CASP8, CASP9, CASP10, CASP14, DCR-1, DR4, DR5, FADD, FAS, HRK, MCL1, NFKB1/p50, Noxa (PMAIP1), Puma, RIPK2, TNFRSF11B
<i>DNA Damage Response</i>	ATM, ATR, ERCC1, ERCC3, GADD45A, LIG4, MUYH, OGG1, RAD51, RAD52, XPA, XRCC5
<i>Cell Cycle Regulation</i>	AURKA, CDC2 (CDK1), CDC20, CDC25A, CDK2, CDK4, SIRT 2
<i>Stress Response</i>	CAT, GPX1, NOS2, NOX1, NOX4, PRDX 1
<i>Tumor Formation and Suppression</i>	AKT2, BMI1, BNIP3L, MDM2, p53

Gene specific primer sequences for RAD51 transcript were as follow:

Forward: 5'TTGAAGCAAATGCAGATACTTCAG3'

Reverse: 5'GAGCAGTGTGGCATAAATGCC3'

Gene specific primer sequences for RAD51 transcript (TaqMan Probe) were as follow:

RAD 51 forward: 5'GGCAGTGATGTCCTGGATAATG3'

RAD 51 reverse: 5'CGGTGGCACTGTCTACAATAAG3'

RAD 51 probe: 5'FAM TTCAACACAGACCACCAGACCCAG3' TAMRA

Gene specific primer sequences for MDM2 transcript were as follow:

Forward: 5'GAAATTCCTTAGCTGACTATTGG3'

Reverse: 5'GAGAATTGGCTTCCTGAAGATAA3'

Gene specific primer sequences for GAPDH as house-keeping gene transcript were as follow:

Forward: 5'TGCACCACCAACTGCTTAGC3'

Reverse: 5'GGCATGGACTGTGGTCATGAG3'

Gene specific primer sequences for HPRT1 as house-keeping gene transcript were as follow:

Forward: 5'TGACACTGGCAAACAATGCA3'

Reverse: 5'GGTCCTTTTCACCAGCAAGCT3'

Gene specific primer sequences for YWAZ as house-keeping gene transcript were as follow:

Forward: 5'ACTTTTGGTACATTGTGGCTTCAA3'

Reverse: 5'CCGCCAGGACAAACCAGTAT3'

## Statistical analysis

All experiments were performed randomly at least in three independent repetitions. The program "GraphPad Prism 7.0" was used for statistical analyses (calculation of arithmetic mean, determination of standard deviation value and graph drawing). Multiple comparisons were determined using the one-way analysis of variance ANOVA ("one-way ANOVA") test, and then Dunnett's test was used. Values at  $P < 0.05$  were accepted as statistical significance.

## Results

### TMZ treatment blocks proliferation of human glioblastoma cancer cells in a dose- and time-dependent manner

MTT analysis results showed that administration of TMZ to U87-MG and LN229 cells caused cell death in a dose and time-dependent manner (Fig. 1).  $IC_{50}$  values were shown in Table 3. It showed that 24-hour TMZ administration was insufficient to find the  $IC_{50}$  value in both cells. The  $IC_{50}$  values for U87-MG at 48 and 72 hours were 776.24  $\mu$ M and 398.10  $\mu$ M, respectively, while for LN229 they were 1584.89  $\mu$ M and 758.57  $\mu$ M. While TMZ applied at low doses ( $\leq 50$   $\mu$ M) in both cell lines did not make a significant difference in cell viability, it was observed that TMZ applied at high doses ( $\geq 800$   $\mu$ M) inhibited cell viability over time. To evaluate the effects of TMZ, two doses (100 and 200  $\mu$ M) were chosen to depend on dose-response curves as test concentrations, and we restricted our study to 72 h at which the lowest  $IC_{50}$  was obtained. In the light of the data obtained U87-MG cells were shown to be more sensitive than LN229 cells against TMZ treatments. While a statistically significant difference was determined for all

doses administering the U87-MG cell lines, a notable difference was not detected in LN229. In U87-MG cells, 100  $\mu\text{M}$  and 200  $\mu\text{M}$  doses of TMZ reduced cell viability by 12.1% and 30%, respectively.

Table 3  
Time-dependent  $\text{IC}_{50}$  values of temozolomide in human glioma cancer cells

	U87-MG	LN229
24 h	-	-
48 h	776.24 $\mu\text{M}$	1584.89 $\mu\text{M}$
72 h	398.10 $\mu\text{M}$	758.57 $\mu\text{M}$

## Characterization studies of EVs

Western blot analysis confirmed that EV protein markers (TSG101 and ALIX) were presented in both cell lines even if after TMZ applications (Fig. 2a). In addition to immunological analysis, PS Capture™ Exosome ELISA Kit was also used for the presence of EVs. The amount of CD63 in the EVs secreted by both control and TMZ administration group of U87-MG and LN229 cell lines was presented in Fig. 2b. The amount of CD63 in the EVs released by the U87-MG control group, 100  $\mu\text{M}$  TMZ administration group and 200  $\mu\text{M}$  TMZ administration group were  $799.2 \pm 73.24$  ng/mL,  $729.7 \pm 81.74$  ng/mL and  $866.9 \pm 42.92$  ng/mL, respectively, while the amount of CD63 in the EVs released by the LN229 control group, 100  $\mu\text{M}$  TMZ administration group and 200  $\mu\text{M}$  TMZ administration group were found to be  $2459 \pm 266.1$  ng/mL,  $2609 \pm 306.4$  ng/mL and  $2633 \pm 261.6$  ng/mL. According to the analysis result, it was observed that the applications of various doses of TMZ did not make any statistically significant difference on the amount of CD63 in the EV released in both cell lines (Table 4). However, when the CD63 amounts were compared, it was found that the amount of CD63 in the EV rooted from LN229 cells was higher compared to U87-MG (Fig. 2c). Furthermore, the size of all the tested EV preparations measured by the Nanosight particle tracking system showed a peak between 100 and 300 nm (Fig. 2d). Any substantial changes in EV concentration were not observed after TMZ application in both cells (Fig. 2e). However, the concentration of EV released from the U87-MG control group was higher than the other (Fig. 2f).

Table 4  
The amount of CD63 released from U87-MG and LN229 cells as a result of TMZ administration at different concentrations

	U87-MG	LN229
C	$799.2 \pm 73.24$ ng/mL	$2459 \pm 266.1$ ng/mL
100	$729.7 \pm 81.74$ ng/mL	$2609 \pm 306.4$ ng/mL
200	$866.9 \pm 42.92$ ng/mL	$2633 \pm 261.6$ ng/mL

## **TMZ treatment leads to an increase in HSPs expression in human glioma cancer cells and EV contents.**

Western blot analyses (Fig. 3) were performed to evaluate the effect of TMZ treatment on HSF-1, Hsp90, Hsp70, Hsp60 and Hsp27 in both U87-MG and LN229 cells. In U87-MG cells, 100  $\mu$ M TMZ did not significantly alter HSF-1 and Hsp90 expression, but with 200  $\mu$ M TMZ, their levels were remarkably increased compared to untreated cells. While both 100 and 200  $\mu$ M TMZ caused a significant increase in Hsp70 expression, they did not show a statistical difference in Hsp60 expression in U87-MG cells. However, we could not obtain results for the Hsp27 protein. In LN229, the HSF-1 and Hsp70 expression levels increased in a dose-dependent manner. HSP90 expression levels also increased with 100 and 200  $\mu$ M TMZ by 60.5% and 40.7%, respectively. In addition, it was observed that Hsp60 expression did not change with 100 and 200  $\mu$ M TMZ. Also, only 200  $\mu$ M TMZ triggered a 257.8% increase in Hsp27 in LN229.

Here, we also investigated the effect of TMZ on HSPs which found in EV content (Fig. 4). In EV content originated from U87-MG, the Hsp90, Hsp70 and Hsp60 expression levels increased in a dose-dependent manner. Particularly, the change in the expression levels of the Hsp70 in the EV released from U87-MG cells was similar to the change in U87-MG cells. In the EV rooted from LN229, 100  $\mu$ M TMZ did not considerably change Hsp90 expression, but with 200  $\mu$ M TMZ, Hsp90 levels were significantly risen by 253%.

## **TMZ treatment triggers expression of RAD51 and MDM2 gene in relation to HSPs expression in cells and EV contents**

In order to demonstrate which genes have impact on HSPs response, it was determined by Real-time PCR studies to what extent the RNA levels of 86 genes were affected in both cell lines. The results of the analysis showed that while it was not determined that there was a statistically significant difference for 100  $\mu$ M TMZ application, the administration of 200  $\mu$ M TMZ produced a considerable increase in the different genes. In U87-MG cells, RAD51 gene expression levels were increased by 5.18-fold in treatment groups with 200  $\mu$ M TMZ (Fig. 5a). Fold increase of MDM2 gene expression in TMZ (200  $\mu$ M) treated LN229 cells was determined by 7.64 (Fig. 5a). Moreover, the effect of TMZ administration on the protein levels of RAD51 and MDM2 were evaluated with Western blot analysis (Fig. 5b). While any notable changes in MDM2 protein levels in LN229 cells were not observed after TMZ treatment, only 200  $\mu$ M TMZ triggered a 92.7% increase in RAD51 protein in U87-MG cells (Fig. 5c). The correlation between protein and mRNA expression levels is notoriously poor, with explanatory power hanging around 40% in several studies [16, 17]. The inconsistency is typically explained with other levels of regulation between transcript and protein product [18].

In addition, we also investigated at how TMZ affected the expression of the genes RAD51 and MDM2 in EVs. Quality and quantification of EV-RNAs isolated were evaluated with Agilent Bioanalyzer (Fig. 6). In the Real-time PCR analysis, absolute quantification analysis was performed with the standard graph obtained with the PCR products of the target gene. In the analysis results, while it was determined that the RAD51 gene was not found in EV content released from U87-MG cells, we found that the levels of MDM2

gene expressions increased in EV content released from LN229 cell line as a result of TMZ treatment (except for 100  $\mu$ M TMZ treated LN229 cells). In LN229 cells treated with 200  $\mu$ M TMZ, MDM2 gene expression level in EV content increased 9.22-fold (Fig. 5d). However, RAD51 and MDM2 proteins in EV contents originating from U87-MG and LN229 cells could not be detected with Western blot. Furthermore, similar trend was observed as regard of the change of Hsp70 expression level between EVs originated from LN229 cells and in LN229 cells. The Hsp70 expression level increased in a dose-dependent manner in EVs released from LN229 cells. What is more, the expression level of Hsp60 did not differentiate with 100 TMZ, but 200  $\mu$ M TMZ triggered a 64.3% increase in Hsp60 in EVs released from LN229 cells. All results were given in Table 5.

Table 5  
The effect of TMZ application on HSP proteins in both cell line and their EV content

	U87-MG		LN229		U87-MG EV		LN229 EV	
	100	200	100	200	100	200	100	200
HSF-1	-	97,6 $\uparrow$	65,7 $\uparrow$	77,9 $\uparrow$				
HSP90	-	20,4 $\uparrow$	60,5 $\uparrow$	40,7 $\uparrow$	37,3 $\uparrow$	75,2 $\uparrow$	-	253 $\uparrow$
HSP70	132,2 $\uparrow$	299,7 $\uparrow$	67,7 $\uparrow$	167,3 $\uparrow$	145,1 $\uparrow$	224,9 $\uparrow$	61,6 $\uparrow$	66,2 $\uparrow$
HSP60	-	-	-	-	51 $\uparrow$	55,7 $\uparrow$	-	64,3 $\uparrow$
HSP27			-	257,8 $\uparrow$				
RAD51	-	92,7 $\uparrow$						
MDM2			-	-				

## Discussion

This study is based on investigating the effects of TMZ on Hsp levels and several genes involved in apoptosis, DNA damage response, cell cycle regulation, stress response, tumour formation and suppression in the glioma cancer cells (U87-MG and LN229) and their EV content. Herein, our results indicated that TMZ inhibited cell growth in a dose and time-dependent manner. According to MTT assay results, U87-MG cells were more sensitive to the cytotoxic effects of the TMZ compared to LN229 cells. IC<sub>50</sub> values of TMZ in U87-MG cells were lower than IC<sub>50</sub> values of LN229 cells for 48 and 72 h treatment periods, and also IC<sub>50</sub> values of LN229 were twofold higher than the other cell line. Furthermore, the IC<sub>50</sub> value of TMZ administered for 72 hours for U87-MG cells has been found to be 397.2  $\mu$ M in one study [19]. Also, it has been reported that the IC<sub>50</sub> value of TMZ applied to LN229 cells for 72 hours is 954.2  $\mu$ M [20]. This result indicates that TMZ has a higher cytotoxic effect in the U-87 MG cell line than the LN229 cell line due to the more aggressiveness of LN229 cells than U87-MG cells [20, 21]. Hence, to evaluate the difference in cellular and EV response on TMZ treatment, we use fixed two doses (100 and 200  $\mu$ M) of

TMZ and 72 h treatment time for both the cell lines. In addition, the selected doses are among the most preferred concentrations in TMZ studies in the literature as they do not have much toxic effect [7, 20, 22].

Western blot is the most preferred method in characterisation studies of EVs. The expression of ALIX and TSG101 antibodies, which are considered to be EV markers, were demonstrated by Western blot analysis (Fig. 2a). In addition to immunological analysis, in this study, qualitative analyses of isolated EVs were performed using the PS Capture™ Exosome ELISA Kit based on the presence of CD63, which is considered a common EV marker. According to the analyses, there was no statistically significant difference in the amount of CD63 in the EVs released from the control and experimental groups of TMZ application in both cell lines (Fig. 2b). However, the results indicated a significant difference between the amounts of CD63 in the EVs released from both cells (Fig. 2c). In a study conducted by Yoshioka et al. (2013) it has been indicated that the amount of exosomal marker proteins can be different in various cell lines [23]. For this reason, different methods are preferred for EV quantification due to the variable protein contents of different EV types. In this study, we chose the NTA method capable of analysing EV parameters such as size and number. According to the results, it was not observed statistically meaningful difference in the concentration of EV released from the untreated and treated groups with TMZ application in both cell lines (Fig. 2e). Moreover, in a study conducted by Simon et al. (2018), it has been shown that an anti-cancer drug applied to U87-MG and LN18 cells cause a negligible change in the EV concentration [24]. The results of this study reveal that TMZ application affects the content of EV without changing the amount. Besides, our results indicated that there was a significant difference between the amounts of EV released from both cells (Fig. 2f). What is more, there are several studies demonstrated that the amount of EV changes depending on the cell type. Simon et al. (2018) has reported that U87-MG and LN18 cells released different concentrations of EV, Tian et al. (2014) has found that immature dendritic cells excreted a limited number of EVs, and Chen et al. (2011) has indicated that mesenchymal stem cells secreted a large number of EVs [24–26].

In order to indicate the effect of successful drug therapy on the target cell, the therapeutic index must be determined. Because HSPs have a capability to be presented as possible therapeutic targets in the treatment of GBM, we investigated the effect of TMZ on HSPs and HSF-1 (which as a transcription factor plays a substantial role in expression of heat shock protein) in cell and EV content. While HSF-1, Hsp90 and Hsp70 expression was triggered by TMZ treatment in the U87-MG cell line, Hsp60 expression did not change (Table 5). In the LN229 cell line, application of TMZ led to increased expression of HSF1, Hsp90, Hsp70 and Hsp27 (Table 5). Studies have shown that TMZ application increases Hsp70 expression in U87-MG [27], T98G and U251 cells [28]. Castro et al. (2015) have revealed that TMZ treatment increases Hsp70 expression in Gli36 cells, decreases it in DBTRG cells and does not change it in U87-MG cells [29]. The same study has determined that TMZ has increased Hsp27 expression in U87-MG, DBTRG and Gli36 glioma cell lines. The results of these studies have been shown that TMZ has different responses in different glioma cells. Furthermore, Hsp70 and Hsp27 with increased expression levels are known to be associated with poor prognosis and treatment resistance in various types of cancer (breast, cervix, hepatocellular carcinoma) [30,31]. Therefore, the results of our study provided evidence that increased HSPs levels may be associated with the TMZ resistance.

In our study, we also showed that TMZ affected HSPs, which are found in EV content originated both cell lines. HSP expression exhibited a similar pattern in EV and cell contents as well as TMZ application led to increased expression of Hsp90, Hsp70, and Hsp60. Moreover, we found that Hsp70, especially, was correlated in EV and parental cells in both cell lines as a result of TMZ treatment (Table 5). However, EV-HSF1 and EV-Hsp27 expression in both cell lines could not be determined. In a study, it has been shown that TMZ affects the content of EV proteins, and secreted EVs after TMZ application play a role in intercellular communication [32]. It has been indicated in different studies that HSP, whose expression levels are increased, especially in EVs released from cancer cells, can be used as biomarkers in cancer diagnosis [33–35]. Moreover, these HSPs may be associated with drug resistance, poor prognosis, response to treatment, migration, and invasion [36]. In a study conducted by Lv et al. (2012), it has been determined that anti-cancer drugs increase the release of Hsp-containing EVs from hepatocellular carcinoma cells. These EVs play a role in cytotoxic responses [37].

It is widely known that different genes may contribute to increases in expression levels of HSPs. In this context, to figure out which genes are responsible for the rises, we investigated the effects of TMZ applied to U87-MG and LN229 cells on a variety of genes such as cell cycle regulation, DNA damage response, apoptosis, tumour formation and suppression with Real-time PCR analyses. Herein, although there was a change in expression level of several analysed genes at different doses, expression levels in genes without primer dimer formation which may be caused by the use of SYBR-Green Supermix were examined. In the result of the analyses, we found that the expression of the RAD51 gene, which plays a role in the DNA repair mechanism, increased approximately 5 times and the protein level of RAD51 enhanced by 92.7% in U87-MG cells treated with 200  $\mu$ M TMZ. These results suggest that this increase may be associated with resistance. It has been reported in many studies that the RAD51 expression level was high in numerous tumour types such as prostate cancer and ovarian cancer [38, 39]. The high level of RAD51 also has explained the resistance against DNA damaging reagents such as chemotherapy, as it causes an increase in homologous recombination. In addition, the expression level of the RAD51 gene has increased in various GBM cells treated with TMZ, and this increase was associated with resistance [40, 41]. Therefore, different treatment approaches targeting the RAD51 gene have been tested in order to increase the sensitivity of TMZ in GMB cells [42]. Nevertheless, why and how RAD51 overexpression occurs in GBM cells remains unclear. Moreover, there are studies revealing the relationship between both RAD51 and HSPs. After the creation of single or double-strand breaks, DNA Damage Response Pathway (DDR) occurs, and DDR proteins, such as RAD51, are kept active by Hsp70 and Hsp90 [43, 44]. However, the role of Hsp27 in the DNA repair mechanism is still under investigation [45]. All these results have suggested that the increase in RAD51 gene expression may associated with the rise of expression level in HSPs and this may lead to TMZ resistance.

Besides these consequences, we found that 200  $\mu$ M TMZ administration led to increased expression of the MDM2 oncogene, but it was not caused any changes in the MDM2 protein level in LN229. MDM2, mediating the degradation of the p53 protein under normal conditions, acts as a negative regulator to suppress the activity of the p53 protein. In the studies, overexpression of the MDM2 gene with the p53 mutation has been observed [46,47]. It has been reported that tumours with both the p53 mutation and

high MDM2 expression level are associated with a worse prognosis [48]. Moreover, MDM2 whose expression level is increased has also been demonstrated to provide resistance to anticancer drugs such as cisplatin and doxorubicin in breast cancer [49, 50]. In another study conducted by Sato et al. (2011) have shown that MDM2 inhibition increased p53 expression and stem cell-like GBM cells became more sensitive to TMZ [51]. In this study, it was shown for the first time that TMZ application to LN229 cells with mutant p53 activity increased MDM2 expression. Furthermore, there is a study that show a link between MDM2 and HSPs. HSPs also prevent the degradation of mutant p53s by MDM2. Molecular chaperones assist in folding mutant p53 intermediates and stabilise their interaction with p73. When the MDM2 oncogene is over-expressed, HSPs are displaced, and a stable multi-protein complex comprising of mutated p53-TAp73 $\alpha$ -MDM2 is formed, additionally amplifying cancer cells chemoresistance [52]. Taking into account all of the data, it's possible that this increase in MDM2 contributes to HSPs expression in LN229 cells and is linked to TMZ resistance.

Herein, we also examined the expression level of RAD51 and MDM2 genes as a result of TMZ application in EVs originating from both glioma cells. Although the RAD51 gene expression level was altered by TMZ treatment in U87-MG cells, when EV-RAD51 was examined, primer dimer formation was determined depending on the SYBR green. Therefore, TaqMan probe was preferred to prevent primary dimer formation caused by SYBR-green to precisely detect expression levels of EV-RAD51. As a result, RAD51 was found to not carry in EV content in this study first time. Additionally, it was found for the first time that TMZ administration increases the MDM2 gene in EV content and MDM2 mRNA levels have an excellent correlation in EV and parental cells (Fig. 5c). TMZ therapy has been known to cause changes in the expression levels of numerous genes in GBM cells; however, little information has been found that it may also affect EV content. In a study, the mRNA expression level of many genes thought to be responsible for TMZ resistance such as GSTp1, MGMT, APNG, ERCC1, ERCC2, MVP, ABCC3, CASP8, and IGFBP2 have correlated in EV and parental cells, and it has suggested that these genes can be potential biomarkers for TMZ resistance [53]. In this scope, according to the results of our study, MDM2 mRNA is thought to be a potential EV-mRNA marker related to TMZ resistance.

In conclusion, TMZ application causes resistance in U87-MG and LN229 cells, and despite the fact that TMZ is utilised as a chemotherapeutic in the treatment of GBM with no alternatives, it may not be the only good anticancer agent in the therapy of GBM. For this purpose, using TMZ in combination with different drugs can be an excellent strategy to increase the effectiveness of treatment. In addition, the fact that EVs correlate with expression levels in the cell at both the mRNA level and the protein level suggests that EVs in the treatment of GBM may have potential biomarkers that can be used to investigate the treatment response. However, different studies are required to determine how some mRNA and Hsp, whose expression levels are increased with TMZ treatment, may affect the recipient cells.

## **Declarations**

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### **Competing Interest**

The authors declare no competing interest

### **Availability of data and materials**

The related detailed data from this study is available from the corresponding author.

### **Code availability**

Not applicable

### **Author's contributions**

EK designed the study. EK, ZA and HT performed the study and EK analysed the results. EK wrote the manuscript. ZA and EOU drafted the manuscript. All authors read and approved the final manuscript.

### **Ethics approval and consent to participate**

Not applicable

### **Patient consent for publication**

Not applicable

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## Figures

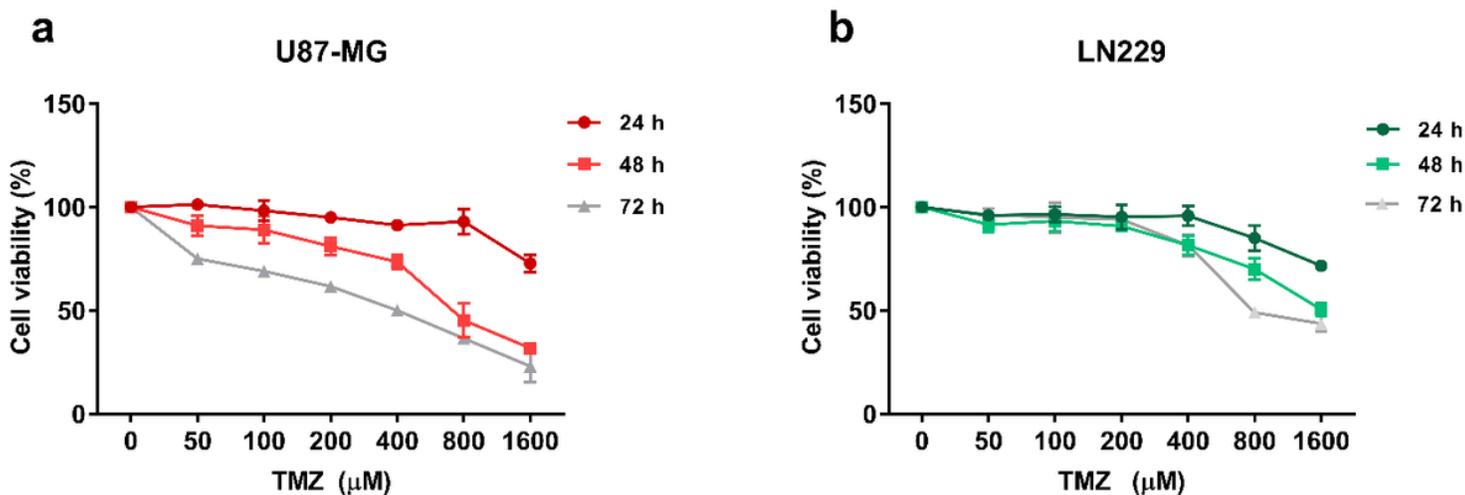
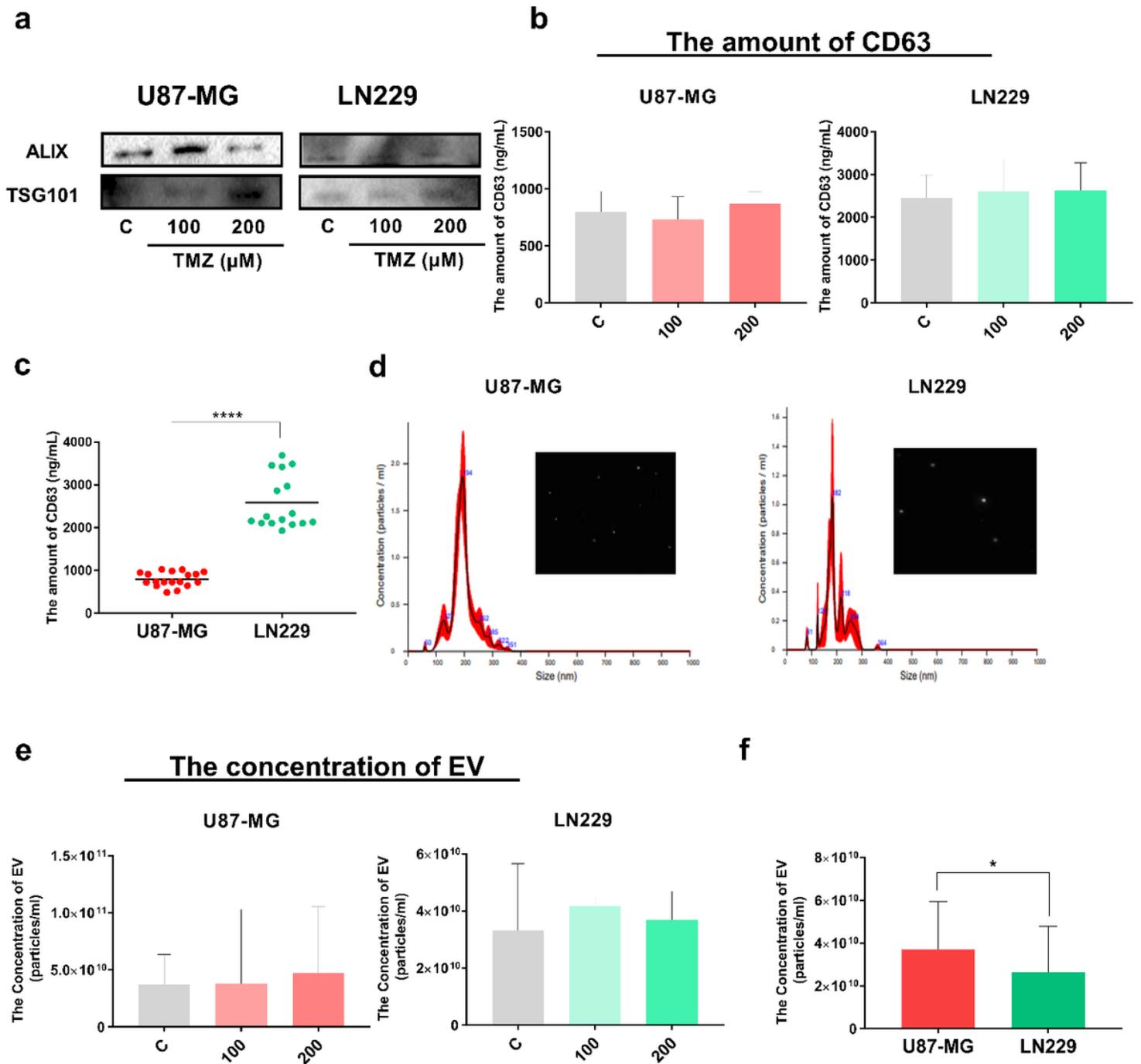


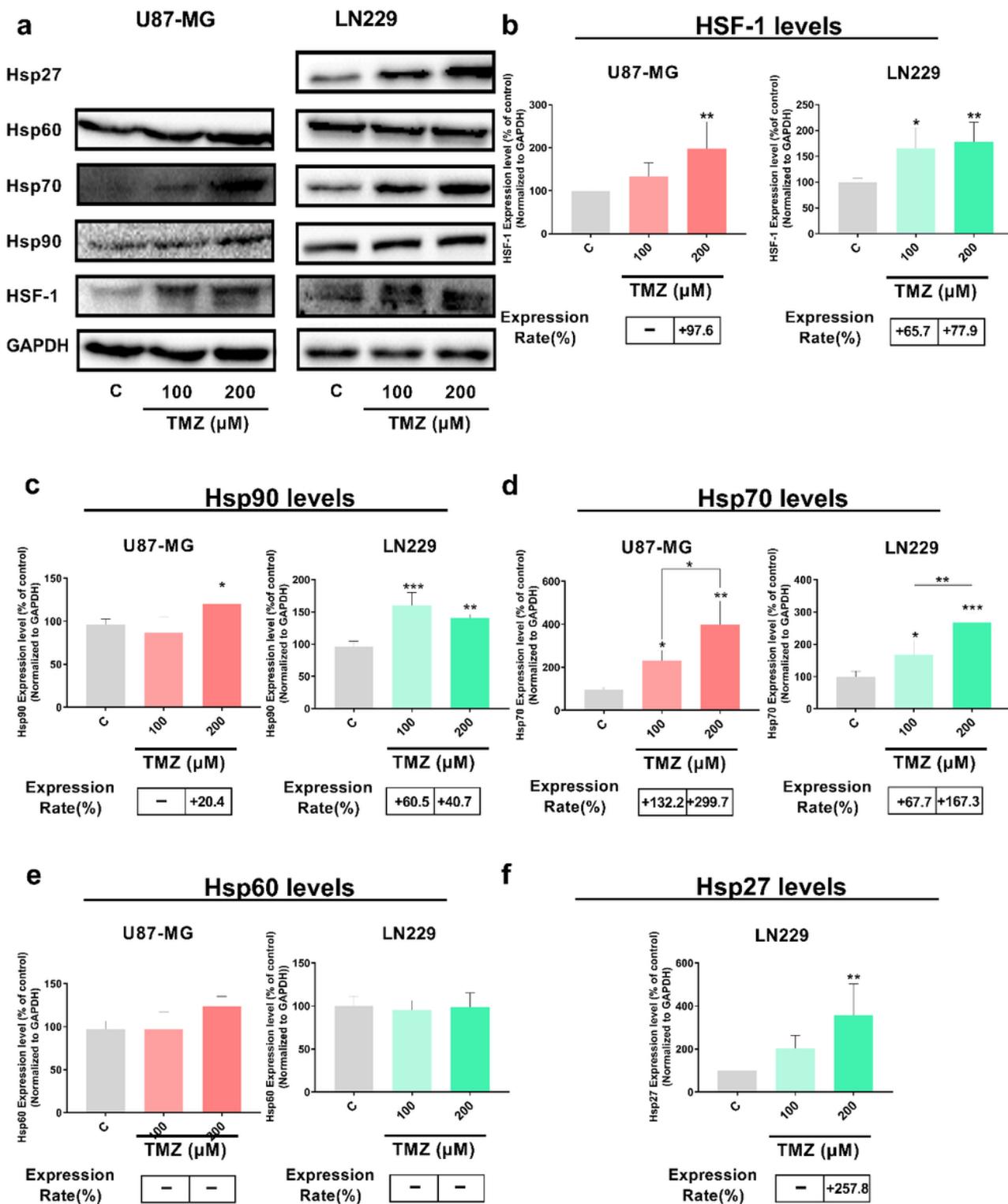
Figure 1

(a) Effects of TMZ on the viability of human glioma cancer cells U87-MG and LN229. (b) TMZ treatments were associated with an increase in cell death in a dose- and time-dependent manner.



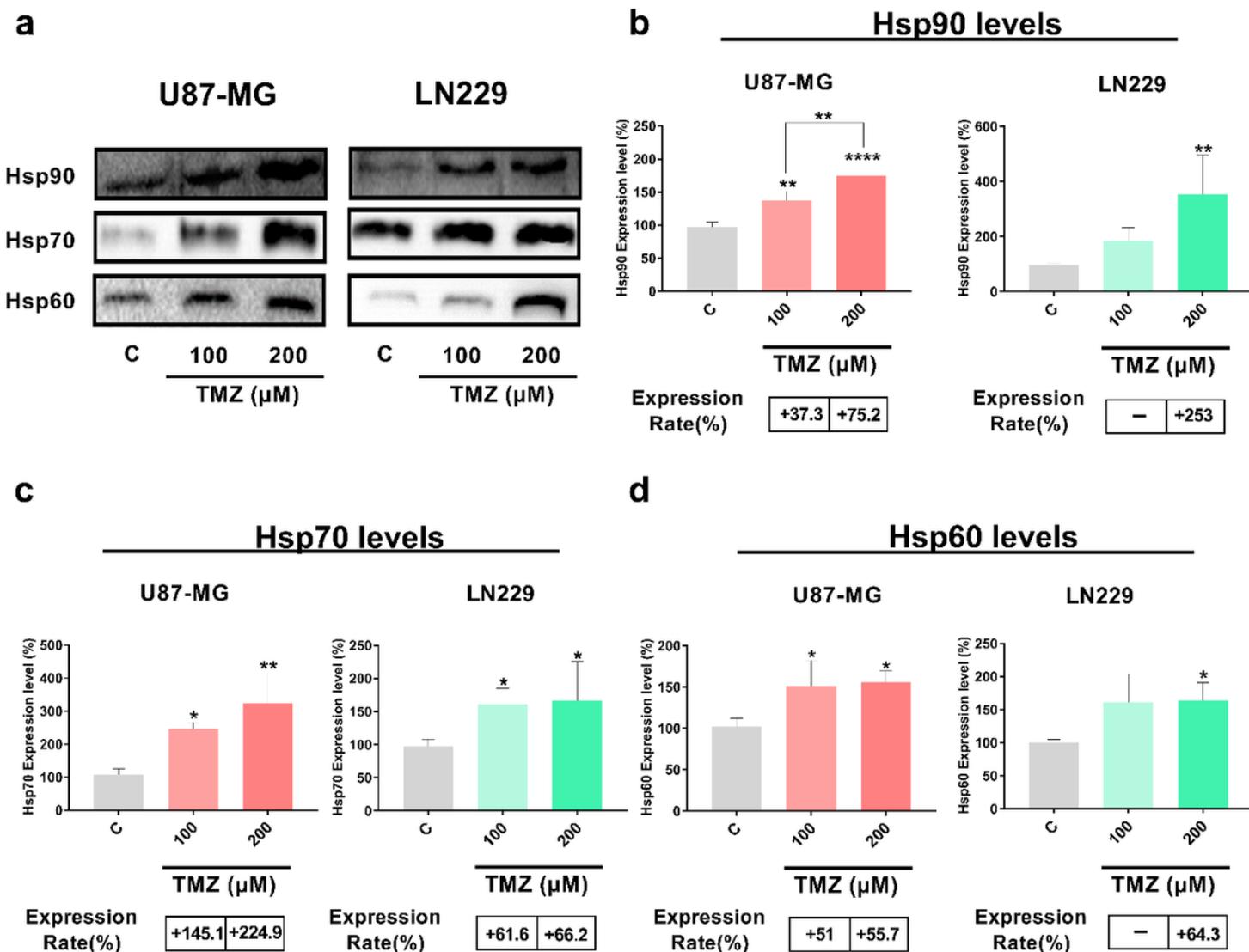
**Figure 2**

**(a)** Representative western blots showing ALIX and TSG101 level in isolated EV from U87-MG and LN229 human glioma cell lines. **(b)** The amount CD63 in the EVs secreted by the both control and TMZ administration group of U87-MG and LN229 cell lines. **(c)** Quantitative difference between CD63 in the EVs originating from U87-MG and LN229 cells ( $****P < 0.0001$ ) **(d)** Analysis of the size distribution in the EVs derived from U87-MG cell line by the NanoSight particle tracking system. **(e)** The concentration of EV from both cell lines. **(f)** The difference of concentration of EV released both cells ( $*P < 0.5$ ).



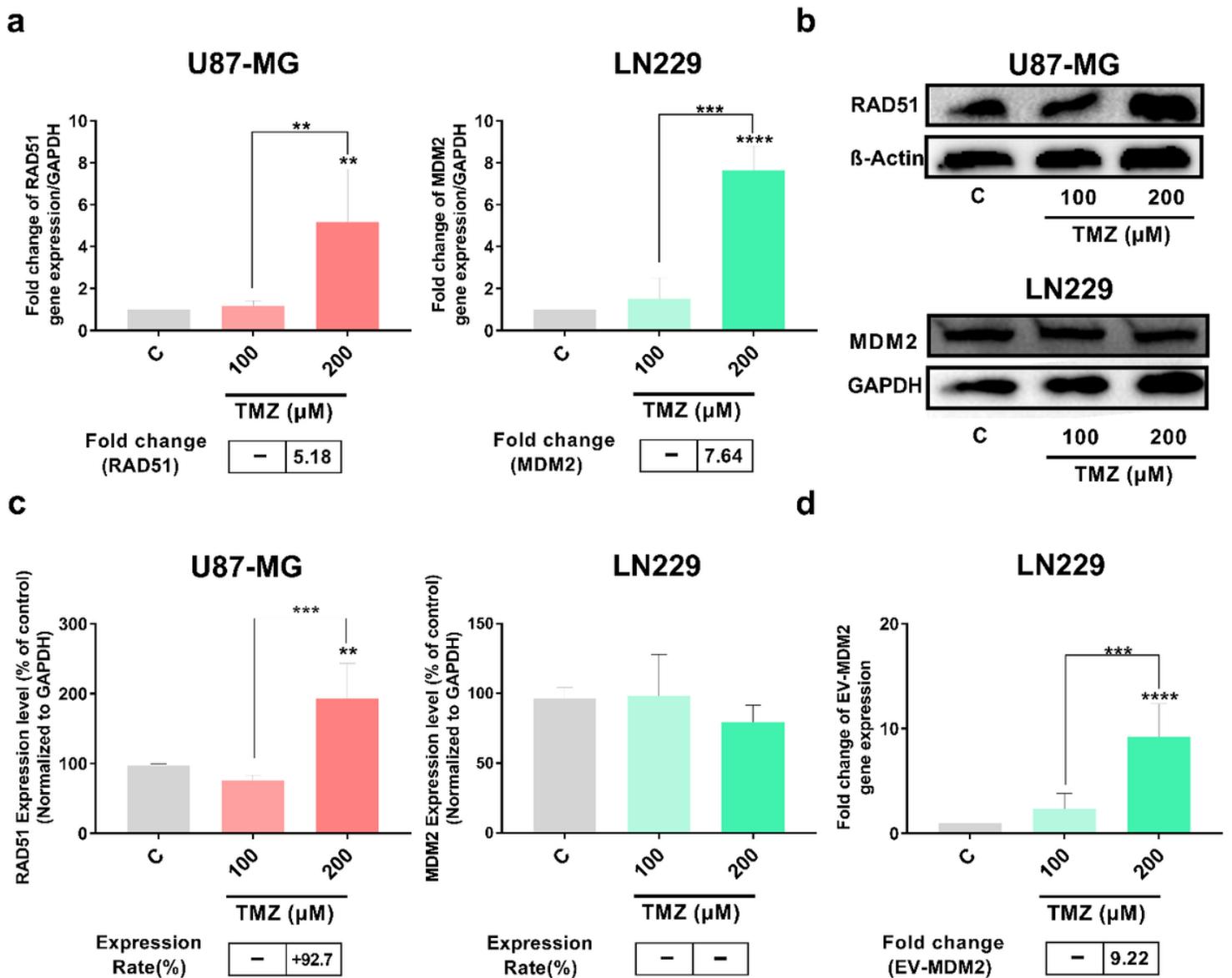
**Figure 3**

Expression of Hsps in response to TMZ treatment in human glioma cancer cells. U87-MG and LN229 cells were treated with 100 and 200 μM of TMZ for 72 h. Western blot analysis (**a**) showed that TMZ caused an increment in the expression of (**b**) HSF-1, (**c**) Hsp90, (**d**) Hsp70, (**e**) Hsp60 and (**f**) Hsp27. GAPDH was used as a loading control (\* $P < 0.5$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ ).



**Figure 4**

Expression of Hsps in response to TMZ treatment in EV content released from human glioma cancer cells. U87-MG and LN229 cells were treated with 100 and 200 μM of TMZ for 72 h. Western blot analysis (a) showed that TMZ caused an increment in the expression of (b) Hsp90, (c) Hsp70, (d) Hsp60 (\* $P < 0.5$ , \*\* $P < 0.01$  and \*\*\*\* $P < 0.0001$ ).



**Figure 5**

Effects of TMZ treatment on different genes in the cells and EV content. **(a)** Effects of TMZ administration on RAD51 (U87) and MDM2 (LN229) genes expression in the cells. (\*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$ ). **(b)** Expression of RAD51 and MDM2 proteins in response to TMZ treatment. **(c)** Effects of TMZ administration on RAD51 (U87) and MDM2 (LN229) protein expression in the cells. (\*\* $P < 0.01$  and \*\*\* $P < 0.001$ ). **(d)** Effects of TMZ administration to LN229 cells on MDM2 gene expression level in EV content (\* $P < 0.5$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ ).

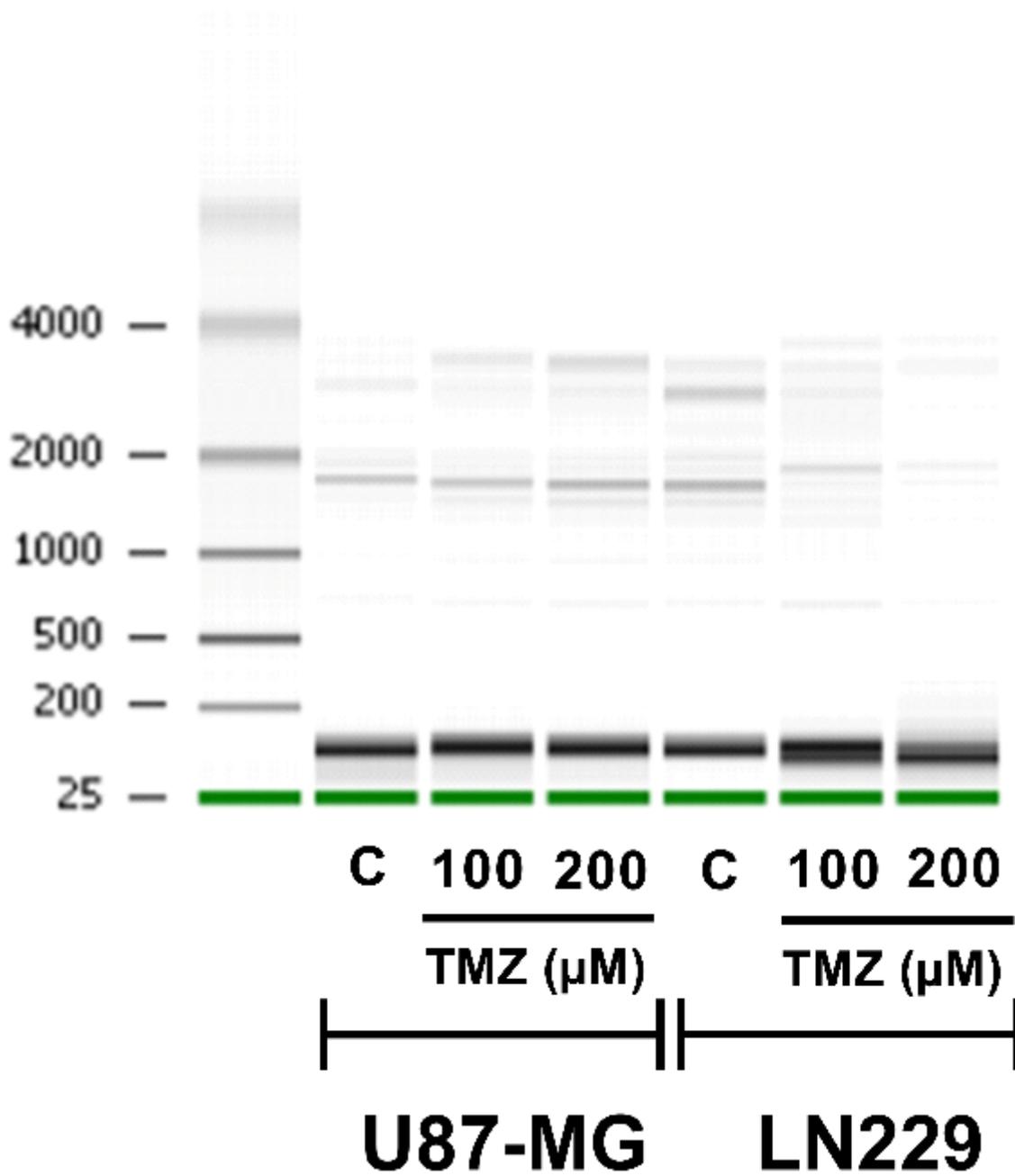


Figure 6

Quality of EV-RNAs isolated were evaluated with Agilent Bioanalyzer