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RESEARCH ARTICLE

Temozolomide Resistance is Associated with Upregulated MICB mRNA Expression and Protein Secretion in Human Glioblastoma Cells

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Abstract

BACKGROUND: Standard treatment for glioblastoma multiforme (GBM) chemotherapy with temozolomide (TMZ) is often limited by chemoresistance, which is correlated with cancer immune evasion caused by the dysregulation of natural killer group 2 member D ligands (NKG2DL). While suppression of NKG2DL facilitates cancer progression, how chemoresistance regulates NKG2DL expression remains unclear. This study was conducted to examine the association of TMZ resistance with the expression and secretion of the NKG2DL subtype, MHC class I chain-related protein B (MICB), in human GBM cells.

METHODS: An experimental *in vitro* study was conducted using TMZ-sensitive (U87MG) and intrinsically TMZ-resistant (T98G) human GBM cell lines. MICB mRNA expression was analyzed via quantitative reverse transcription polymerase chain reaction (qRT-PCR). Surface protein expression and extracellular secretion of MICB were measured using flowcytometry and enzyme-linked immunosorbent assay (ELISA), respectively.

RESULTS: Repeated TMZ exposure successfully induced resistance in U87MG-R (+TMZ) cells, yielding significantly higher cell viability ($p<0.01$) and slower proliferation rates compared to parental U87MG cells. Following 2-days TMZ treatment, both intrinsically resistant T98G and adaptively resistant U87MG-R (+TMZ) cells, demonstrated a significant increase in MICB mRNA levels ($p<0.05$ and $p<0.0001$, respectively) and extracellular secretion of soluble MICB protein ($p<0.01$ and $p<0.01$, respectively), compared to TMZ-sensitive counterparts. Conversely, MICB surface protein level after 2-days TMZ treatment was significantly reduced in both T98G ($p<0.0001$) and U87MG-R (+TMZ) ($p<0.01$) cells relative to the respective U87MG cells.

CONCLUSION: GBM cell resistance to TMZ was associated with an upregulation of MICB mRNA transcription and extracellular protein secretion, and concurrently with a suppression of MICB expression on the cell surface. These conditions may lead to a cellular adaptive mechanism to develop resistance to TMZ and may represent a strategic route to evade native host immune.

KEYWORDS: glioblastoma multiforme, temozolomide, cancer chemoresistance, MHC class I chain-related protein B (MICB), natural killer group 2D receptor (NKG2D)

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Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary malignant brain tumor (1-3), with poor prognosis despite standard treatments including surgery, radiotherapy, and Temozolomide (TMZ) chemotherapy in combination with the immunotherapy.(4,5) The common therapeutic issue in GBM patients is due to tumor recurrence and resistance to TMZ, as they can decrease the responsiveness of GBM cells to treatment and lead to therapeutic failure and eventually to patient's death.(6) TMZ resistance in GBM can be intrinsic due to factors like O6-methylguanine-DNA methyltransferase (MGMT) upregulation and enhanced base excision repair (BER), and suppression of mismatch repair (MMR) (7,8), or acquired via the induction of glioma stem cell (GSC) and increased of manganese superoxide dismutase (MnSOD) as antioxidant agent.(9,10) The former was known as a mechanism of cancer to form self-renewing cell populations, which can maintain tumor growth, generate diversity in the tumor bulk, and support cancer resistance to radiation and chemotherapy.(11)

Cancer chemoresistance within the tumor microenvironment is closely linked to immune evasion (12,13), primarily through the dysregulation of natural killer group 2 member D ligands (NKG2DL) which normally trigger natural killer (NK) and cytotoxic T cell responses.(14,15) Among these ligands, MHC class I chain-related protein B (MICB) was prioritized for this study over other NKG2DLs due to structural and clinical advantages. Unlike MICA, which features high genetic polymorphism and erratic immunological functions, MICB is a stable, clinically reliable prognostic biomarker.(16,17) Furthermore, the UL16-binding protein family (ULBP1–6) was excluded because most members lack a traditional alpha3 domain and standard transmembrane anchor, relying instead on a glycosyl phosphatidyl inositol (GPI) motif; this structural variation causes highly unpredictable shedding kinetics. In contrast, transmembrane-anchored MICB is uniformly shed by tumor cells using proteases like a disintegrin and metalloproteinase (ADAM) and matrix metalloproteinase (MMP) (18,19), or by secreting a soluble form, sMICB (20,21) that circulates to desensitize neighboring NK cells via receptor endocytosis and degradation, leading to immune escape.

Though it is reported that suppression of NKG2DL facilitates cancer progression, but how chemoresistance regulates NKG2DL expression remains unclear. Therefore, this study was conducted to analyze the association of TMZ resistance with MICB expression and secretion in human GBM cell lines, T98G and U87MG. Mapping the precise MICB expression and secretion profiles across both intrinsically and adaptively resistant GBM cells is therefore a vital prerequisite for designing targeted chemo-immunotherapy combinations capable of blocking sMICB-mediated NK cell desensitization and restoring host anti-tumor immunity.

Methods

GBM Cell Culture

The cell lines used for this study were the GBM cells with intrinsic TMZ resistance (T98G/CRL-1690) and the cells with TMZ sensitivity (U87MG/HTB-14). To authenticate the characteristic and the TMZ sensitivity of the cells, analysis for any morphological and proliferation changes were performed. All GBM cell lines were grown in Dulbecco's modified eagle medium (DMEM; Cat. No. 12100046; Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA), supplemented with 3.7 g/L sodium bicarbonate ($NaHCO_3$; Cat. No. S6014; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 10% fetal bovine serum (FBS; Cat. No. A5670701; Gibco; Thermo Fisher Scientific Inc.), 1% penicillin-streptomycin (P/S; Cat. No. 15140122; Gibco; Thermo Fisher Scientific Inc.), and 1% amphotericin B (Cat. No. 15290026; Gibco; Thermo Fisher Scientific Inc.) under standard cell culture condition (37°C, 5% CO_2, and 20% $O_{2)}$. Those culture conditions were used both for routine maintenance and for treatment. The passage number of cells used was within range from P7-P10 for U87MG, P1-P3 for U87MG-R (+TMZ), and P37-P40 for T98G. To minimize the risk of mycoplasma contamination, all reagents used for cell culture were certified free from mycoplasma by the manufacturer, cell culture medium were filtered with 0.22 µm syringe filter (Cat. No. 99722; TPP Techno Plastic Products AG, Trasadingen, Switzerland) prior to use, and the aseptic methods were practiced during culture.

TMZ Treatment

For TMZ treatment, about $1x10^5$ cells were treated with cytotoxic-50 (CC_{50}) dose of TMZ (26 µM) in DMEM medium without FBS for two days. Afterwards, cells were harvested and prepared for analysis of MICB expression and secretion using quantitative reverse transcription polymerase chain reaction (qRT-PCR), flow cytometry, and enzyme-linked immunosorbent assay (ELISA).

Cell Viability Assay

Following cell harvesting, the number of viable cells was determined using trypan blue solution (Cat. No. 15250061; Gibco; Thermo Fisher Scientific Inc.) and an automated cell counter (LUNA-II™; Logos Biosystems; Aligned Genetics Inc., Anyang, South Korea). Cell viability was calculated by comparing the number of live cells in the treated samples to the number of live cells in control.

Establishment of TMZ-acquired Resistance of U87MG GBM Cells

To induce TMZ acquired resistance, about 1x10^5 U87MG cells/well in a 12-well plate were grown in 1 mL DMEM medium with complete supplementation (10% FBS, 1% P/S, and 1% amphotericin B) under standard cell culture condition and repeatedly treated with the CC50 dose of temozolomide (TMZ; Product Reg. No. DKI1918602901B1; TEMOTERO; PT Amarox Pharma Global, Bekasi, Indonesia) every two days for 62 days. To assess changes in cell sensitivity to TMZ, percentage of cell viability was calculated by comparing the lived cell number between TMZ-treated and untreated (control). For further use, treated cells with reduced sensitivity to TMZ, labelled as U87MG-R (+TMZ), were cultured in DMEM medium with complete supplementation containing CC_{50} dose of TMZ.

qRT-PCR

Total RNA was extracted from approximately 1x10^5 to 3x10^5 cells using Tripure Isolation Reagent (TRIpure reagent; Cat. No. ATB2700; PT Indogen Intertama, Jakarta, Indonesia). The isolated RNA was then used for RT-qPCR analysis with SensiFAST SYBR No-ROX One-Step qPCR KIT (Cat. No. BIO-72005; Meridian Bioscience Inc., Newtown, OH, USA) and the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.). The primer sequences used for this experiment were provided on the Table 1. The thermocycling conditions were as follows: 45°C for 10 minutes and 95°C for 2 minutes in the holding stage, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds in the cycling stage, and then 95°C for 15 seconds, 60°C for 1 minute, 95˚C for 15 seconds, and 60°C for 15 seconds in the melt curve stage. The relative mRNA expression was analyzed using Livak's formula ($2^{-\Delta\Delta Cq}$), and the Cq values of each sample were normalized to the Cq value of U87MG cells without TMZ, labeled as U87MG (-TMZ).

Flowcytometry

After harvesting, approximately 1x10^6 cells were resuspended in a staining buffer containing FACSFlow Sheath Fluid (Cat. No. 342003; BD Biosciences, San Jose, CA, USA) and 2% fetal bovine serum (FBS; Cat. No. A5670701; Gibco; Thermo Fisher Scientific Inc.). The cells were then treated with the MICA/MICB antibody, anti-human, PE (Cat. No. 130-100-889; Clone 6D4; Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany). The antibody incubation was carried out in the dark at 4°C for about 15 min. Following incubation, the cells were washed with staining buffer to remove any excess antibodies and were resuspended in phosphate-buffered saline (PBS; Cat. No. 003002; Invitrogen; Thermo Fisher Scientific Inc.) before being analyzed using the BD FACSCanto™ II Clinical Flow Cytometry System (BD Biosciences). As much as 1x104 cells (events) per sample were acquired for the data analysis using FlowJo. The median fluorescence intensity (MFI) of the antibody-stained cells was then compared to the MFI of the unstained cells to assess differences in MICB surface protein expression between samples.

MICB ELISA

The collected secretome or conditioned medium (CM) from the GBM cell line culture was collected using centrifugation in 1000 x g for 20 min, followed by centrifugation using an Amicon® Ultra-4 Centrifugal Filter Unit with a 10 kDa MWCO column (Cat. No. UFC8010; Millipore; Merck KGaA, Germany) and Microsep™ Centrifugal Filters with a 10 kDa MWCO (Cat. No. 17104751; PALL; Pall Corporation, USA) in 5000 rpm for 15 min to concentrate the protein concentration of the samples as well as to reduce protein interference from FBS through separation of smaller FBS molecules from the samples. The MICB concentration in the CM concentrate was then measured using a MICB ELISA based on the sandwich principle, with the Human MICB ELISA Kit (Cat. No. E-EL-H2458; Elabscience; Elabscience Bionovation Inc., Wuhan, China). The intensity of the resulting color was measured by absorbance at a

Table 1. Primer sequences of MICB as the target gene and of 18S RNA as the reference gene.

Target Gene	Forward Primer	Reverse Primer
MICB	5’-CTG AGA AGC TGG CGA CGT A-3’	5’-CGA AGA CTG TGG GGC TCA-3’
18S RNA	5'- AAA CGG CTA CCA CAT CCA AG -3’	5'- CCT CCA ATG GAT CCT CGT TA -3’

wavelength of 450 nm (A450) using the Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific Inc.). The MICB concentration in each sample was determined by plotting the A450 value on a standard curve of known concentrations.

Statistical Analyses

Data were shown as the mean±standard deviation (SD) of the mean. Differences between treatment and control groups were assessed using a one-way ANOVA test followed by Tukey's post hoc test. Statistical analysis was carried out using GraphPad Prism 8.3.0 software (Dotmatics, Boston, MA, USA).

Results

U87MG Cells Acquired Resistance After Repeated TMZ Exposure

Administering the CC_{50} dose of TMZ (26 μM) to U87MG cells every two days resulted in the reduction of cell sensitivity to TMZ, as shown by changes in cell viability over 60 days following the initial treatment (Figure 1A). Percentage of cell viability was 77.841±5.596% two days after TMZ treatment, reducing to 35.252±5.048% by day 6. However, by day 13, percentage of cell viability increased to 77.470±12.720%, then eventually reaching nearly 100% afterwards (103.387±15.241% on day 32; 102.121±10.997% on day 42; 105.446±4.637% on day 62).

Furthermore, U87MG cells that had developed resistance to TMZ (U87MG-R (+TMZ)) were continuously cultured and treated repeatedly with TMZ every two days to maintain their resistance. Further analysis of parental U87MG cells showed a significant reduction (p=0.0043) in cell viability post 48 h-TMZ treatment compared to U87MG cells without treatment (control) (Figure 1B). Additionally, Figure 1B also demonstrated that the TMZ-resistant GBM cells, showed a significant increase in cell viability of both the intrinsic (T98G) and acquired (U87MG-R) cells compared to their sensitive counterparts, with p=0.0054 and p=0.0063, respectively, after TMZ treatment for 2 days.

To compare the growth rates of U87MG, U87MG-R (+TMZ), and T98G cells, the proliferation curves and doubling times of each GBM cell line were analyzed after 3-, 5-, 7-, and 9-days culture (Figure 1C and 1D). The proliferation data indicated that the growth rates of the three GBM cell lines were similar until day 5 (Figure 1C). Afterwards, the TMZ-sensitive U87MG cells began to proliferate faster than the TMZ-resistant U87MG-R (+TMZ) and T98G cells. Doubling time analysis (Figure

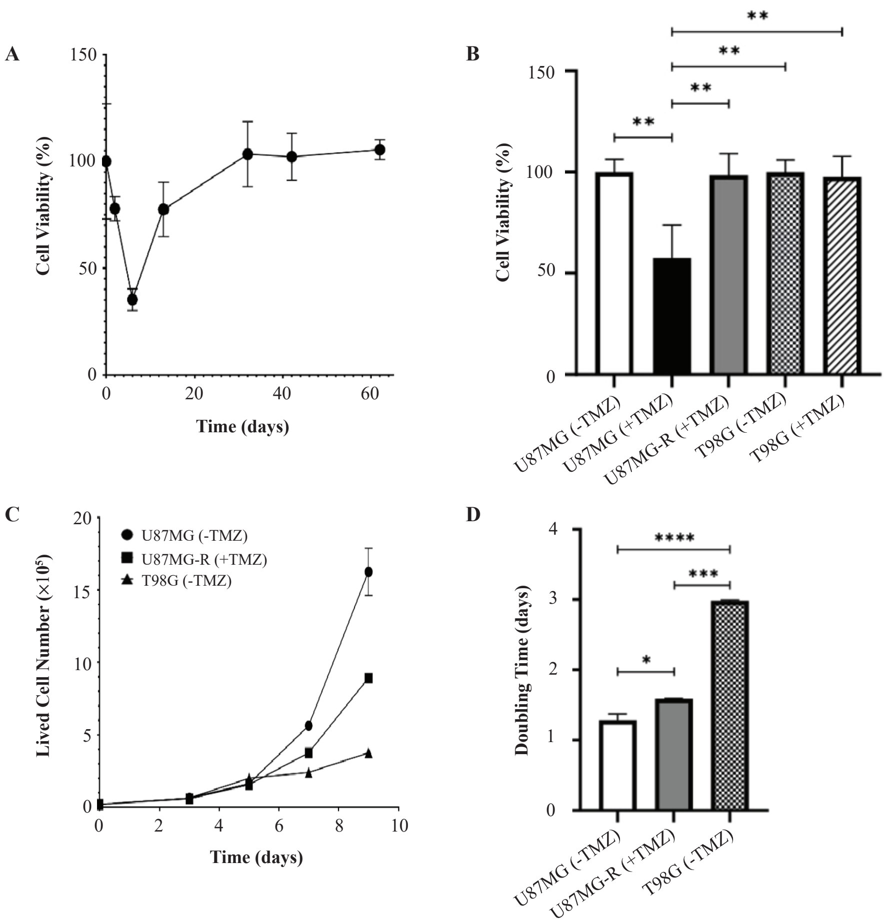

Figure 1. Establishment of TMZ-resistant cells. A: Percentage of U87MG cell viability in day 2, 6, 13, 32, 42, and 62 after repeated TMZ treatment every two days. B: Percentage of U87MG, U87MG-R (+TMZ), and T98G cell viability post 2-days treatment with 26 μM TMZ; **p<0.01. C: Proliferation of GBM cell lines were assessed by counting the number of viable cells over a 9-days culture period. D: Doubling time of GBM cell lines were assessed by counting the number of viable cells over a 9-days culture period; *p<0.05, ***p<0.001, ****p<0.0001. Data were shown as mean±SD. Statistical significance was evaluated using one-way ANOVA and Tukey's post hoc test. (-TMZ) indicates untreated samples, while (+TMZ) denotes samples treated with TMZ.

1D) also revealed that U87MG cells grew significantly faster than U87MG-R (+TMZ) (*p*=0.0193) and T98G cells (*p*≤0.0001). Interestingly, a significant difference in growth rate was observed between the intrinsically resistant T98G cells and the adaptively resistant U87MG-R (+TMZ) cells (*p*=0.0002).

Expression of MICB mRNA and Protein on The Surface of TMZ-resistant Cells

Analysis of MICB mRNA and surface protein expression was performed in TMZ-resistant GBM cells compared to the sensitive cells, either without or with the CC_{50} dose of TMZ treatment for 2 days. The results of the mRNA relative expression analysis (Figure 2A) revealed a significant decrease in the MICB expression post TMZ-treatment for 2 days, both in the U87MG cells and T98G cells, with *p*=0.0487 and *p*=0.0027, respectively. In addition, Figure 2A also revealed that MICB expression was significantly higher in adaptively TMZ-resistant U87MG-R (+TMZ) cells, as well as in intrinsically TMZ-resistant T98G cells, compared to their sensitive counterparts after TMZ-treatment for 2 days (U87MG-R (+TMZ) *vs.* U87MG (+TMZ), *p*<0.0001); T98G (+TMZ) *vs.* U87MG (+TMZ), *p*=0.0461).

The analysis of MICB surface protein expression (Figure 2B) showed a significant reduction of MICB surface protein levels in TMZ-resistant GBM cells compared to their sensitive counterparts (U87MG-R (+TMZ) *vs.* U87MG (+TMZ), *p*=0.0023; T98G (+TMZ) *vs.* U87MG (+TMZ), *p*<0.0001). The effect of TMZ treatment over 2 days showed differing effects on U87MG and T98G cells. In U87MG cells, there was a tendency for MICB surface protein expression to increase following TMZ treatment. Meanwhile, in T98G cells, MICB surface protein expression tended to decrease, although, these changes were not statistically significant.

TMZ-resistant Cells Tended to Elevate MICB Secretion

To evaluate MICB protein secretion in GBM cells, MICB concentrations were measured using ELISA in the conditioned medium (CM) with and without 2-days TMZ exposure. The results (Figure 2C) showed significant increase of MICB secretion both in intrinsically resistant T98G cells and in adaptively resistant U87MG-R (+TMZ) cells compared to TMZ-sensitive U87MG cells, respectively with *p*=0.0021 and *p*=0.0004. Additionally, TMZ treatment for 2 days did not have a significant impact on MICB secretion levels in either U87MG or T98G cells.

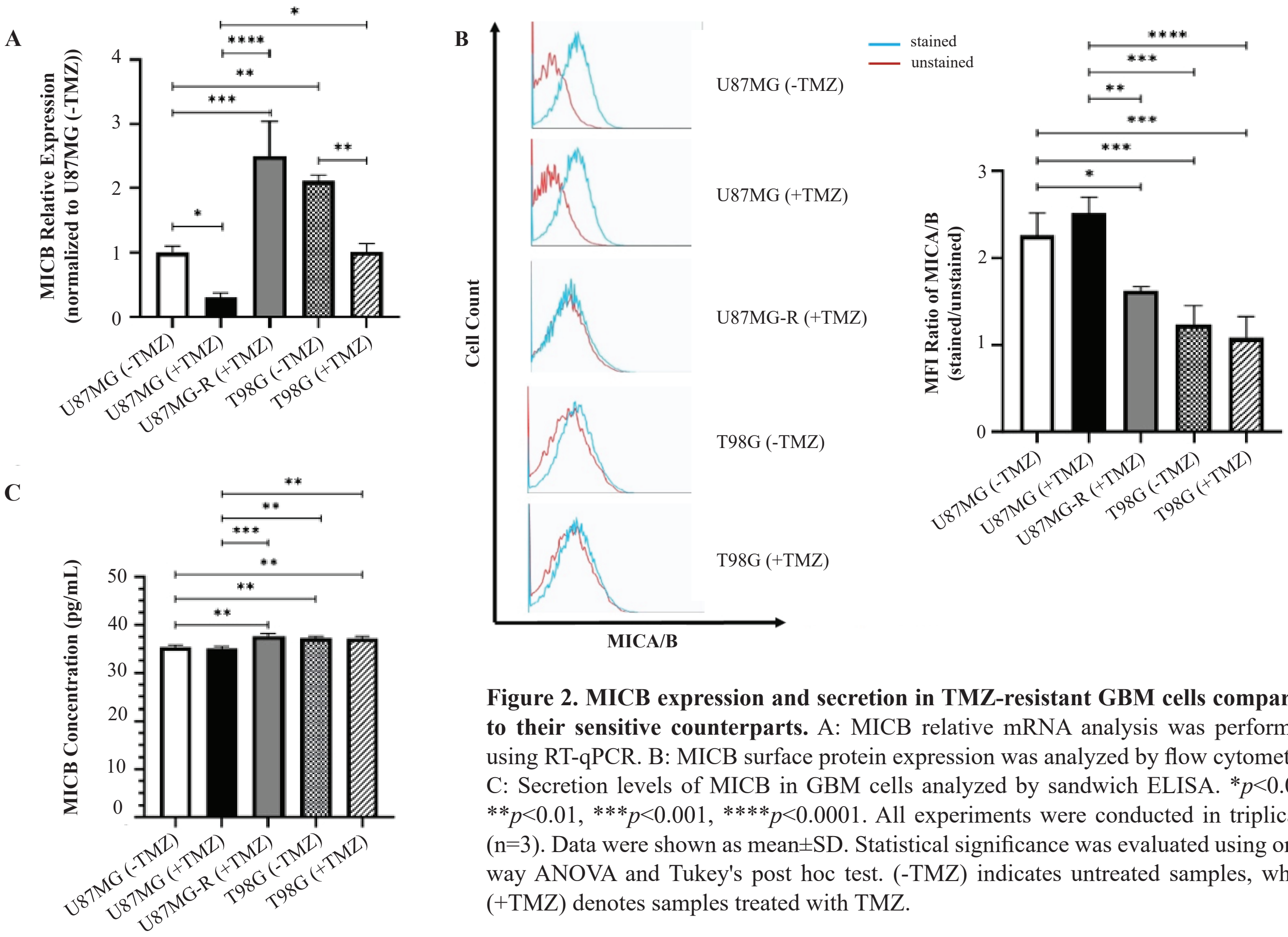

Figure 2. MICB expression and secretion in TMZ-resistant GBM cells compared to their sensitive counterparts. A: MICB relative mRNA analysis was performed using RT-qPCR. B: MICB surface protein expression was analyzed by flow cytometry. C: Secretion levels of MICB in GBM cells analyzed by sandwich ELISA. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. All experiments were conducted in triplicate (n=3). Data were shown as mean±SD. Statistical significance was evaluated using one-way ANOVA and Tukey's post hoc test. (-TMZ) indicates untreated samples, while (+TMZ) denotes samples treated with TMZ.

Discussion

To date, chemoresistance remains the primary obstacle to effective cancer therapy, manifesting either intrinsically or adaptively. Previous studies have suggested that the drug response of cancer cells is closely related with the cancer immune evasion due to the factors of tumor microenvironment (TME).(22,23) Immunosuppresive properties of TME can affect immune surveillance of cancer and facilitate drug resistance. This study proposes that TMZ resistance in GBM cells correlates with the immune surveillance mechanisms of NK cells by regulating the expression and secretion of MICB, a known NKG2D ligand. MICB, likewise MICA, is a protein expressed on the cell surface that has a molecular structure similar to that of other MHC class I molecules. The molecular structure of MICA/MICB consists of a cytoplasmic domain, a transmembrane domain, and α1-3 domains located on the extracellular side of the cell.(14) MICB is usually absent or present in low levels on normal cells, but its expression increases when cells are stressed, damaged, or transformed.(15) MICB acts as ligand for cells that express the NKG2D receptor and signals NK cells and T cells to "kill me". NKG2D is a homodimer receptor that consists of two type II transmembrane glycoproteins with a C-type lectin-like structure on the extracellular side of the cell membrane.(24)

In this study, two GBM cell lines with contrasting TMZ sensitivities were utilized: U87MG cells (sensitive to TMZ) and T98G cells (intrinsically resistant to TMZ). U87MG is a cell line with epithelial morphology and derived from human grade IV glioma.(25) On the other hand, T98G display a fibroblast-like cell morphology and was isolated from the brain of a glioblastoma multiforme, 61-year-old, Caucasian male.(26) U87MG cells are among the most sensitive GBM cells to TMZ treatment, with a decrease in cell viability of about 75%, while T98G cells are inherently resistant to TMZ.(27) We repeatedly treated U87MG cells with TMZ at the CC_{50} concentration until their sensitivity to TMZ declined to approximately 95-100% viability. This condition was maintained for more than two months under continuous TMZ treatment, indicating that these cells have begun to acquire TMZ resistance and are subsequently referred to as U87MG-R (+TMZ) cells. This study highlights the effects of acquired TMZ resistance in U87MG-R (+TMZ) cells on the regulation of MICB expression and secretion, in comparison to those observed in T98G cells.

Firstly, the present study verified the differences in the response to TMZ treatment between TMZ sensitive and resistant GBM cells. The results of this study demonstrate differences in the response to TMZ treatment between TMZ sensitive and resistant GBM cells. After 2 days of TMZ treatment at the concentration of 26 μM, there was no significant difference in viability between U87MG-R (+TMZ) and T98G cells, both of which maintained approximately 100% viability; in contrast, U87MG cells exhibited a significant decline in viability, dropping to approximately 50%. This finding indicates that the U87MG-R (+TMZ) cells are insensitive to TMZ treatment, similar to T98G cells. Furthermore, U87MG cells significantly exhibited the fastest growth rate and thus displayed the lowest doubling time among the three cells. Metabolic differences between U87MG and T98G cells that may influence their proliferation rates was also has been reported.(28) In U87MG cells, an increase in enzymes associated with glycolysis, such as triosephosphate isomerase (TPIS) and lactate dehydrogenase B (LDHB), was observed, whereas T98G cells showed increased expression of Glucose-6-Phosphate 1-Dehydrogenase, an enzyme that plays a role in redirecting metabolism toward the Pentose Phosphate Pathway. These metabolic differences may be a key factor determining the proliferation rates of GBM cells.

In addition to assessing cell viability, this study also observed differences between TMZ sensitive and resistant cells in MICB expression, specifically in both surface and soluble proteins. In cells undergoing stress, such as malignant transformation or DNA damage caused by chemotherapy, the immune surveillance system mediated by NK cells can be activated to recognize NKG2DL such as MICB on tumor cell membranes.(29) This recognition leads to the activation of various intracellular signaling pathways, including phosphatidylinositol 3-kinase, growth factor receptor-bound protein 2, and c-Jun N-terminal kinase, which may induce NK cell cytotoxicity.(30) Additionally, the interaction between MICB and NKG2D activates Janus kinase 2/signal transducer and activator of transcription 4 (JAK2/STAT5) signaling, which triggers NK cells to release cytokines, thereby enhancing the elimination of tumor cells.(15) Interestingly, constitutive activation of STAT signaling, can deliver the opposite outcome in malignancies, as it was reported that STAT3 promotes proliferation, survival, and angiogenesis of cancer cells.(31)

The expression of NKG2DL can be inhibited by factors such as proteolytic enzymes and exosomal secretion. Cancer cells can shed NKG2DL into sNKG2DL, which may induce endocytosis and lysosomal degradation of NKG2D receptor; thereby disrupting NKG2D immune surveillance function. Additionally, proteolysis of NKG2DL can interfere with recognition, migration, cytotoxicity capabilities, and homeostasis of NK cells, allowing cancer cells to escape detection by NK cells and T cells.(15,32) Higher levels of sMICB are often found in serum of patients with cancer.(33) The expression of sNKG2DL is correlated with therapy outcome of metastatic melanoma patients.(34)

Our results reveal that, prior to TMZ treatment, the TMZ-resistant T98G cells expressed significantly higher level of MICB mRNA, lower level of MICB surface protein, and higher level of MICB soluble protein compared to the TMZ-sensitive U87MG cells, suggesting a dysregulation of MICB protein expression and secretion. The discrepancy between MICB mRNA and surface protein levels may stem from post-translational regulatory mechanisms, such as proteolysis, ubiquitination and sumoylation, which contribute to intracellular degradation of MICB protein and decrease its expression on the cell membrane.(35,36) Other mechanisms contributed to the dysregulation of NKG2DL expression in cancer immune escape have been previously reviewed at the transcriptional, translational, and post-translational levels through transcription factors such as p53, miRNAs such as miR-20a, miR-93, miR-106, and miR-10b, and proteolytic enzymes or exosomes, respectively.(14)

Following TMZ treatment, the mRNA, the surface protein, and also the soluble protein level of MICB were also determined (Figure 3). We found that the TMZ-sensitive U87MG cells maintained both surface and soluble MICB protein levels similar to the untreated cells. However, these cells exhibited a capacity to decrease the MICB mRNA synthesis as a compensatory response to TMZ treatment, indicating a negative feedback mechanism for cell survival. Subsequently, repeated TMZ treatment induced the development of acquired resistance in U87MG-R (+TMZ) cells, as evidenced by lower surface protein levels and higher soluble protein levels of MICB compared to the untreated counterparts. These protein expression profiles were similar to those observed in T98G cells, suggesting an association with TMZ resistance properties that may facilitate immune evasion by TMZ-resistant GBM cells. Moreover, TMZ-resistant GBM cells significantly showed higher MICB secretion than the TMZ-sensitive cells, potentially affecting the immune system's ability to recognize GBM cells. Several studies have demonstrated that cancer progression often results in increased NKG2DL secretion which associated with decreased survival rates in patients.(37,38) Interestingly, we observed opposing MICB mRNA expression levels between U87MG-R (+TMZ) and T98G cells following TMZ treatment. In U87MG-R (+TMZ) cells, increased MICB mRNA accompanied with decreased level of MICB surface protein, reveals that after prolonged TMZ treatment, the cells can adapt and activate the regulatory mechanisms to reduce their recognition by immune cells through increased MICB secretion while lowering MICB expression on cell surface. Conversely,

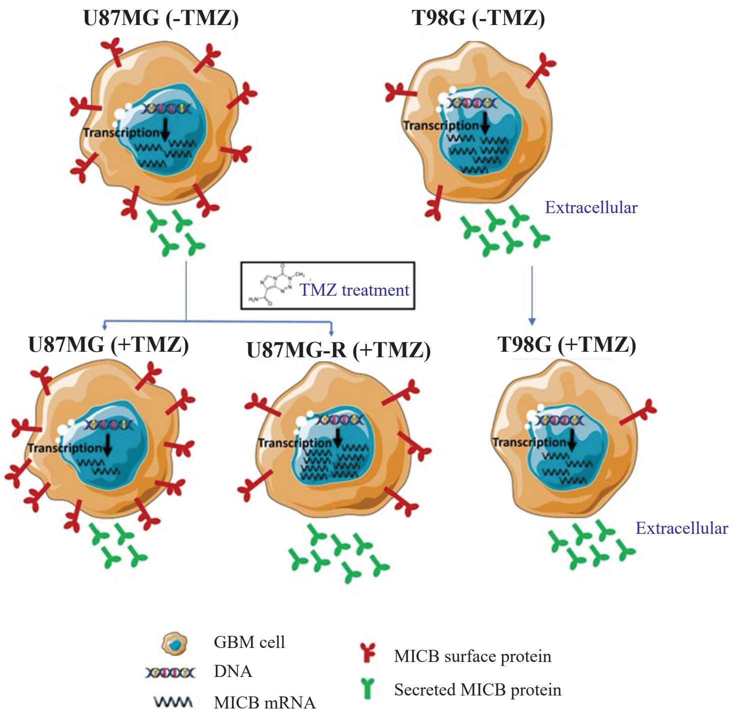

Figure 3. Regulation of MICB mRNA and protein in GBM cells pre- and post-TMZ-treatment. TMZ treatment to U87MG cells did not lead to significant changes of surface and soluble MICB protein levels in comparison to the untreated cells. However, these cells tend to decrease the MICB mRNA expression as a survival mechanism under TMZ treatment. Unlike U87MG cells, adaptively TMZ-resistant U87MG-R (+TMZ) cells showed elevated MICB mRNA expression alongside reduced MICB surface protein levels compared to the untreated counterparts, following TMZ-treatment for 2 days. On the other hand, intrinsically TMZ-resistant T98G cells exhibited a concurrent decrease in both MICB mRNA and protein expression due to intrinsic factors supporting their resistance to TMZ. Furthermore, TMZ treatment led to higher MICB protein secretion in TMZ-resistant GBM cells (U87MG-R (+TMZ) and T98G) compared to TMZ-sensitive GBM cells (U87MG).

T98G cells downregulated MICB mRNA synthesis after TMZ treatment to permanently maintain low surface protein level according to their nature as intrinsic TMZ-resistant cells. This property is influenced by intrinsic factors, either genetic or epigenetic factors (39), as well as signaling pathways associated with intrinsic TMZ resistance. Though this study added knowledge on how this secretory mechanism served as a crucial immune-evasion strategy, but further studies are required to explore and to deepen understanding of the mechanisms underlying GBM chemoresistance in order to enhance patient immune system and develop more effective GBM treatment management.

Conclusion

The resistance of GBM cells to TMZ is associated with a marked increase of the NKG2D MICB mRNA expression and protein secretion. Conversely, TMZ-resistant GBM cells exhibited a reduction in MICB ligand expression on the cell surface, suggesting an increase in extracellular shedding. This enhanced secretory mechanism may serve as a crucial immune-evasion strategy, allowing resistant cells to hinder host innate immune surveillance.

Acknowledgments

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Authors Contribution

SIW and FCI contributed to the concept and design of this study, as well as data validation and analysis. SIW was responsible for managing the funding and critical revision of the manuscript. KJK performed the experiments and wrote the manuscript. Along with KJK, RA performed the establishment of TMZ-acquired resistance of U87MG cells. All authors read and approved the final manuscript.

Ethical Statement

This study was ethically approved by the Research Ethical Committee Faculty of Medicine, Universitas Indonesia/ Dr. Cipto Mangunkusumo National General Hospital (No.: KET-1864/UN2.F1/ETIK/PPM.00.02/2023).

Conflict of Interest

The authors declare that they have no competing interests.

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