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Exploring nano curcumin as a potential therapeutic alternative for glioblastoma multiforme via downregulation of growth factors and induction of apoptosis

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ABSTRACT: Glioma is a type of brain tumor that start from neuroglial stem cells. Despite advancements in surgery and additional adjuvant therapy such as Temozolomide (TMZ), treating this tumor continues to present a challenge with notable side effects, such as toxicity and resistance to treatment. Therefore, the investigation of natural remedies becomes intriguing. This study examines the potential of nano-particle-formulated curcumin, a compound derived from *Curcuma longa* L as a promising antitumor agent. The research was carried out in vitro by treating Glioblastoma multiforme (GBM) cell with various concentrations (25, 50, and 100 µg/mL) of nano-particle of curcumin (NC) and TMZ 300 µM. qRT-PCR was employed to assess the relative expression of mRNA Caspase 3 (Casp-3), Insulin-like Growth Factor Binding Protein 2 (IGFBP-2), Epidermal Growth Factor Receptor (EGFR), and Extracellular Signal-Regulated Kinases (ERK). While the proportion of live, necrotic and apoptotic cells was employed utilizing flow cytometry. GBM shows a high expression of growth factors and a low expression of apoptotic gene. The treatment using NC reduced the expression of IGFBP-2, EGFR, and ERK genes, while increasing Casp-3. GBM cells shows higher apoptotic activities and lower necrotic activities after the addition of NC. In comparison to TMZ, NC demonstrates a promise as an anti-tumor agent, particularly for brain tumors, with the optimal dosage identified to be 25 µg/mL.

KEYWORDS: Apoptosis; glioblastoma multiforme; growth factor; nano-curcumin; temozolomide.

1. INTRODUCTION

Glioblastoma is a malignant brain tumor with 41% survival a year [1]. Gliomas, which originate from the glial cells in the central nervous system, are the most prevalent type of primary intracranial tumors, constitute a significant majority, approximately 70-80% of all brain tumors [2]. According to the World Health Organization (WHO), gliomas are categorized into four grades (I-IV), with Glioblastoma multiforme (GBM) being categorized as grade IV glioma [3] which known for its extreme aggressiveness and high invasiveness, rapidly spreading to other part of the brain.

The rapid spread of GBM is driven by the abundant presence of growth factors within the tumor. Insulin Like Growth Factor Binding Protein-2 (IGFBP-2) was reported to be highly expressed in GBM and

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promotes cell migration and invasion [4]. A high level of Epidermal Growth Factor Receptor (EGFR) in GBM, which activated by Epidermal Growth Factor (EGF) ligand, triggering various signalling pathway such as Phosphoinositide 3-Kinases (PI3K), Extracellular Regulated Kinase/Mitogen Activated Protein Kinase Kinase (ERK/MAPK), and Signal Transducer and Activator of Transcription-3 (STAT-3), playing a crucial role in cell communication and function [5]. The ERK/MAPK pathway, which responds external signals, governs crucial cellular processes like proliferation and division. Along with PI3K/AKT/mTOR, it fosters GBM cell growth by stimulating protein synthesis and inhibiting cell death/apoptotic pathways [6]. One of the genes involved in activating proteolytic function for initiating apoptosis in cells is Caspase-3 (Casp-3) [7]. This gene is also found at low levels in cancer cells, allowing them to proliferate further [8]. Targeting these genes could offer a solution for more effective GBM therapy.

The diverse nature of gliomas and their invasive spread pose significant challenges for developing effective treatment strategies [9]. The typical treatment for GBM involves surgically removing the tumor followed by a combination of chemotherapy and radiotherapy, utilizing temozolomide (TMZ) [10]. Since its clearance for clinical application in 2005 as the exclusive chemotherapeutic remedy for GBM, TMZ has only resulted in an average survival period of 15 months for patients [11]. Many patients also experience tumor recurrence and develop resistance to TMZ, offering limited effectiveness as an alternative treatment option [12]. This presents a challenge for treatment plans to achieve optimal drug levels with minimal toxicity and fewer side effects.

Curcumin, the main compound of the rhizomes of turmeric (*Curcuma longa* L.) have received immense attention due to their bio-functional properties such as anti-tumor, antioxidant, and anti-inflammatory [13]. Extensive studies can be found assessing curcumin as an anticancer agent. It has been shown to prevent carcinogenesis by suppressing cancer cell metastasis and induces cancer cell apoptosis [14]. A study by Astinfeshan et al. [15] revealed that curcumin can inhibit angiogenesis through VEFGR and PI3K/Akt signalling pathway. Other studies have explained that curcumin was able to cross the Blood-Brain Barrier (BBB), inhibits STAT-3 and Nuclear Factor-kappaB (NF-kB) pathway, and also activating Casp-3 in malignant glioblastoma cells (U-251) [16].

Despite its potential to influence immune cells and impede cancer cell survival, natural curcumin's effectiveness as a cancer treatment is hampered by its limited intestinal absorption and its bioavailability [17]. To overcome this problem, a new form of curcumin with sufficient absorption should be developed. Formulating curcumin in its nano-particle form present a promising solution. Nanoparticles are technically defined as particles with one dimension less than 100 nm [18]. Nanoparticle based drug delivery system have reflected benefits in cancer treatment by demonstrating good pharmacokinetics, precise targeting, reduced side effects, and drug resistance [19]. Therefore, this study aims to assess the efficacy of nano-curcumin (NC) as a therapeutic alternative for GBM, by evaluating the expression of growth factor genes such as IGFBP-2, EGFR, and ERK, as well as the apoptosis gene, Casp-3, using qualitative RT-PCR instrumentation. The quantification of live, necrotic, and apoptotic cells was also investigated using Flow cytometry to see the impact of NC against GBM cells.

2. RESULTS

2.1. Casp-3, IGFBP-2, EGFR, and ERK relative expression on GBM cells treated with NC

The mRNA relative expression of GBM cells treated with NC was conducted to several target genes that play an important role in cancer cell growth and cell death. The results of this test are presented in Figure 1. GBM is shown to have an abundance of growth factor genes, such as IGFBP2 (1b), EGFR (1c), and ERK (1d). After the treatment with NC, the expression of these growth factors is significantly downregulated ($p < 0.05$). Meanwhile, in Figure 1a, GBM cells show low expression of apoptotic gene (Casp-3), and the addition of NC to GBM cells significantly upregulate this gene ($p < 0.05$). Compared to TMZ, NC demonstrates promising potential as an alternative therapy for glioma, with the optimum concentration of NC being 25 µg/mL.

2.2. The quantification of apoptotic, live, and necrotic cells

The efficacy of NC as an anti-cancer agent was evaluated against GBM cells via flow cytometry instrument. Figure 2 and 3 shows the apoptotic, live, and necrotic of GBM cells after the treatment with TMZ and NC for 24 hours, which led to a decrease of live cells and an increase of early apoptotic cells. It was found that treatment with NC 25 µg/mL shows a satisfactory outcome, closely resembling the positive control in GBM cells treated with TMZ at 300 µM.

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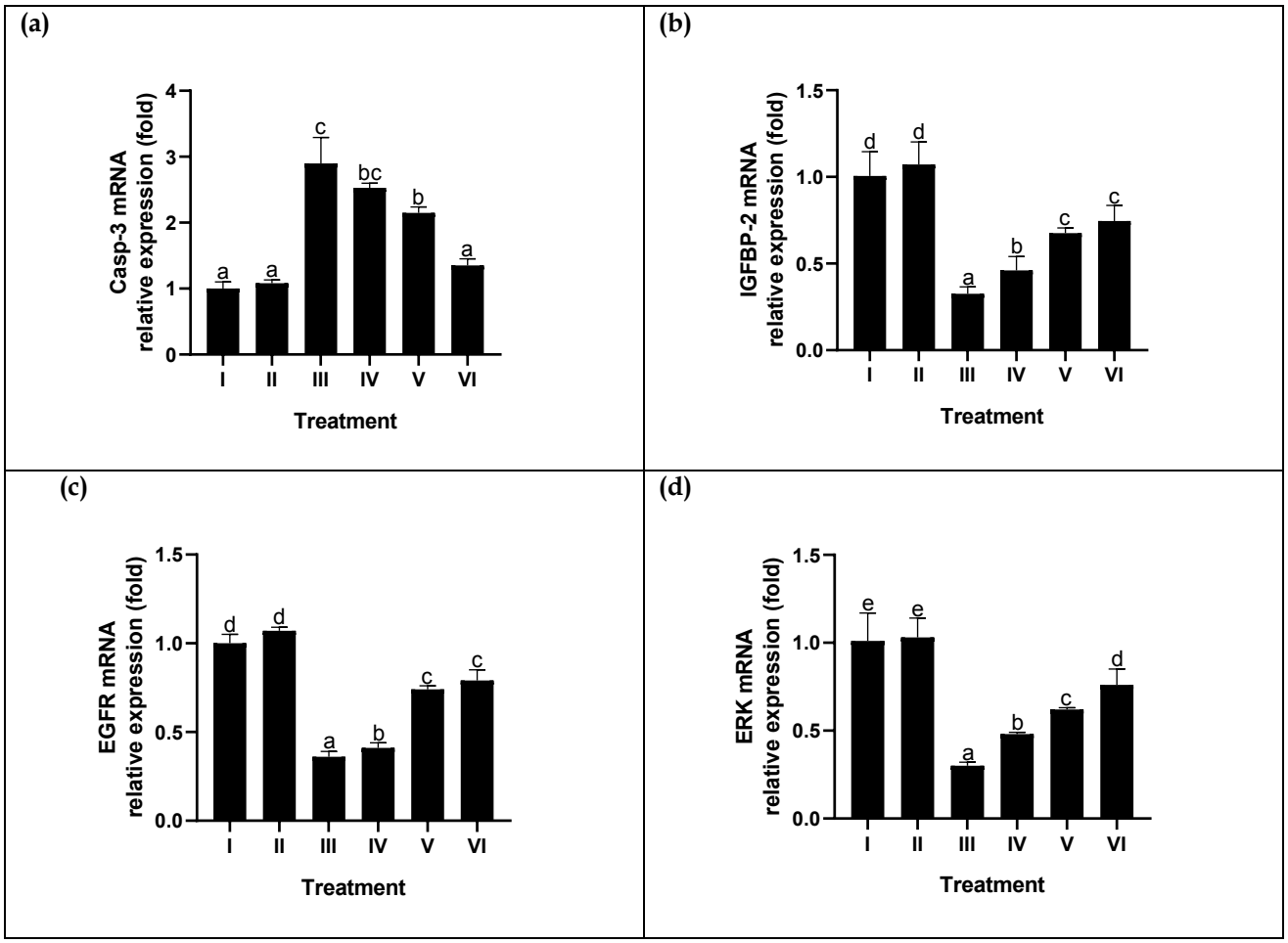

Figure 1. The impact of various NC concentrations towards mRNA relative expression of Casp-3, EGFR, IGFBP-2, ERK on GBM cells

(a) Casp-3, (b) IGFBP-2, (c) EGFR, and (d) ERK. Various treatment (I, II, III, IV, V, and VI) indicates: I): Untreated cells, II): Cells + DMSO 1%, III): Cells +TMZ 300 µM, IV): Cells + NC 25 µg/mL, V): Cells + NC 50 µg/mL, and VI): Cells + NC 100 µg/mL. Data are presented as mean ± standard deviation. Different letters (a, b, bc, c) in Figure 1a indicate statistically significant differences ($p < 0.05$) based on Tukey's post hoc test. Different letters (a, b, c, d) in Figure 1b and 1c indicate statistically significant differences ($p < 0.05$) based on Mann-Whitney test. While different letters (a, b, c, d, e) in Figure 1d indicate statistically significant differences ($p < 0.05$) based on Mann-Whitney test.

3. DISCUSSION

This study further explains the potential of NC as a new neo-adjuvant alternative for GBM through its influence on several gene targets in GBM cells. As depicted in Figure 1a, the expression level of Casp-3 was significantly low in untreated GBM cell, consistent with previous findings indicating similarly low Casp-3 expression in colon cancer [8]. Curcumin at 25 µM was also found to significantly ($p < 0.01$) inhibit cell viability and induce apoptosis in lung cancer via the activation of Casp-3 and Casp-9 [20]. The level of Casp-3 in this study was also significantly upregulated ($p < 0.05$) after the addition of NC at 25 µg/mL when compared to untreated GBM cells. Upregulating these gene has emerged as a critical focus in anticancer due to its ability to degrade proteins and trigger programmed cell death [21].

In concurrence with the low expression of apoptosis genes, an abundance of growth factor genes is observed in GBM cells (Figure 1b-d), elucidating the rapid proliferation and dissemination of GBM cells. The treatment utilizing NC significantly downregulates these genes ($p < 0.05$). Similar findings revealed that the curcuminoid compound successfully downregulating the expression of pro-metastatic genes like IGFBP-2 [22] and EGFR [23] in cancer. The phosphorylation of ERK1/2 expression was also found to be inhibited in glioma cells after treatment with curcumin [24]. The proposed mechanism of NC towards GBM cells is depicted in Figure 4.

In GBM therapy, the utmost priority lies in halting the growth and dissemination of these cells. Signalling pathway serve as a crucial route for transmitting information in signal transduction, gene

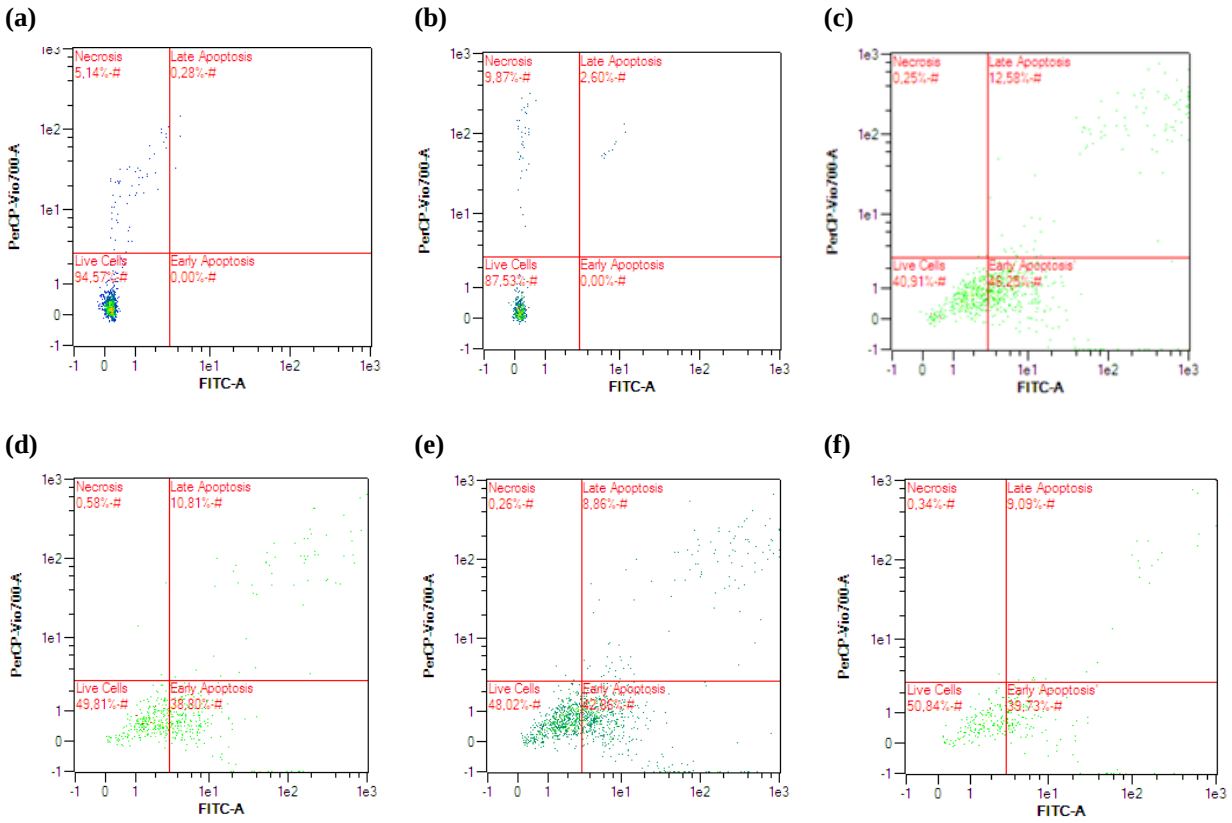

Figure 2. Dot blot representation of the apoptotic level of GBM after treated with different concentrations of NC.
(a) Negative control, (b) DMSO, (c) TMZ, (d) NC 25 µg/mL, (e) NC 50 µg/mL, and (f) NC 100 µg/mL.

expression, and cell metabolism and activity. Their abnormal activation can result in dysregulated expression of downstream target genes, contributing significantly to the initiation and progression of tumors [25]. One of the main signalling pathways that is commonly dysregulated in GBM are the activation of P13K pathway [26]. EGFR and other growth factor proteins, as a part of p85 subunits, works as an active initiators of PI3K/AKT pathway, which stimulate cell cycle progression, promoting cell growth, inhibits apoptosis [27] and inhibiting p53, a tumor suppressor gene [25]. Curcumin is also proven to reduce the malignant potential of cancer via PI3K/AKT pathway. This observation reinforces the potential of curcumin as a viable therapeutic alternative for GBM. Analysis of relative expression target in Figure 1 reveals that treatment of GBM cells with NC at a 25 µg/mL concentration elicits effects closely resembling those of TMZ treatment, which was a comparison control.

To enhance cancer treatment efficacy, an understanding of diverse cell death mechanism is imperative. Consequently, live, apoptotic, and necrotic cells were measured using flow cytometry. Notably, NC at 25 µg/mL concentration exhibited a substantial reduction in live brain tumor cells, as depicted in Figure 3. Apoptosis is a natural process in the body where are programmed to self-destruct if they are damaged, infected, or no longer needed. It acts as a protective mechanism against the development of cancer. However, in cancer, cells can evade or resist to apoptosis [28]. As shown in Figure 3c and 3d, GBM cells exhibit low level of both early and late apoptotic activity. Following the addition of NC, apoptotic levels in GBM cells increased, yielding effects nearly comparable to those observed with TMZ treatment. Conversely, necrosis was found to be elevated in GBM cells (6.67%). Unlike apoptosis, necrosis is a type of cell death that occurs uncontrollably, as a response to external factor like injury, hypoxia, or inflammation. When a cell undergoes necrosis, it typically swells and ruptures, spilling its content into the surrounding area and trigger inflammation and tissue damage [29]. One key player in this process is a protein called nuclear factor-κB (NF-κB), which is involved in regulating inflammation via activation of TNF-α and TNFR [30]. Therefore, the necrotic activity in GBM cells needs to be reduced. NC has been shown to effectively decrease necrotic activity in GBM, at 100 µg/mL concentration, NC is able to reduce the necrotic activity to 0.21% as illustrated in Figure 3b, even better than treatment using TMZ (0.28%). This further demonstrates that NC can be used as an adjuvant therapy for brain tumors. Nevertheless, further investigation regarding

the potential of NC is warranted. Despite numerous studies on the anticancer effect of NC, clinical trials remain infrequent.

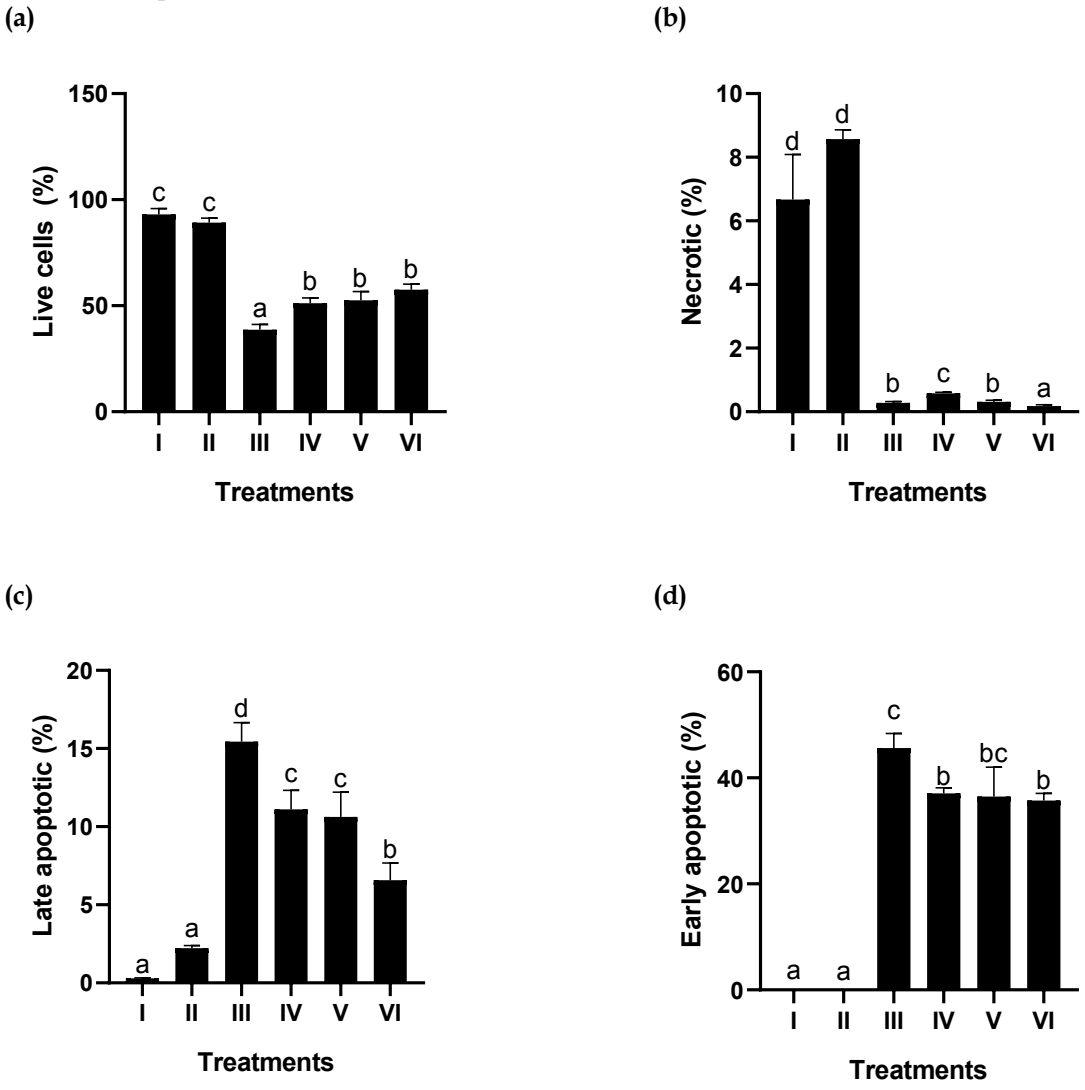

Figure 3. The effect of various concentrations of NC toward live cell, necrotic, and apoptotic levels.
(a) Live cells level, (b) Necrotic level, (c) Late apoptotic level, (d) Early apoptotic level. Various treatment (I, II, III, IV, V, and VI) indicates: I): Untreated cells, II): Cells + DMSO1%, III): Cells +TMZ 300 µM, IV): Cells + NC 25 µg/mL, V): Cells + NC 50 µg/mL, and VI): Cells + NC 100µg/mL. Data are presented as mean ± standard deviation. Different letters (a, b, c) in Figure 3a indicate statistically significant differences ($p < 0.05$) based on Tukey's post hoc test. Different letters (a, b, c, d) in Figure 3b and 3c indicate statistically significant differences ($p < 0.05$) based on Tukey's post hoc test and Mann-Whitney test respectfully. While different letters (a, b, bc, c, d) in Figure 3d indicate statistically significant differences ($p < 0.05$) based on Mann-Whitney Test.

4. CONCLUSION

The efficacy of nanocurcumin treatment for glioma therapy has been utilized, modulating several target genes in GBM. Nanocurcumin at an optimal concentration of 25 µg/mL, led to the reduction in relative expression of IGFBP-2, EGFR, and ERK, while upregulating Casp-3. Additionally, nanocurcumin decreased the proportion of live cells and necrotic cells in GBM, while significantly increasing both late apoptotic and early apoptotic cell population. These result underscore the therapeutic potential of nanocurcumin as an adjuvant therapy alternative for brain cancer.

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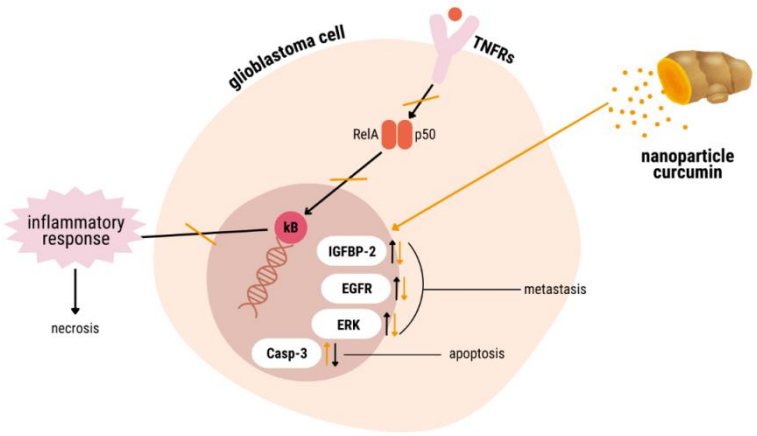

Figure 4. Proposed mechanism of NC as an anti-tumor in GBM
Treatments of NC in GBM cells are able to inhibit the proliferation and metastasis gene (EGFBP-2, EGFR, ERK) while activating the apoptosis gene (Casp-3). Uncontrollable necrosis usually happened in GBM via the activation of TNFRs, and NC are proven to inhibit the process.

5. MATERIALS AND METHODS

5.1. Preparation of nano curcumin

Curcumin was acquired from Plamed Green Science Limited, China, based on Good Manufacturing Practices (GMP). The formulation of NC was conducted following the method by Rahmat et al. [31], utilizing chitosan compound and constant stirring at 300 rpm. After that, NC was initially diluted in 100% dimethyl sulfoxide (DMSO), and then further diluted resulting in a final concentration containing 1% DMSO.

5.2. GBM cells cultivation

GBM cell line (ATCC HTB-14TM) was obtained from Aretha Medika Utama, BBRC, Bandung, Indonesia. Cells were removed from liquid nitrogen tanks (-196°C) and thawed at 37°C for 2 min. The cells were grown in Roswell Park Memorial Institute (RPMI 1640) (Biowest, L0500) culture medium consist of 10% (v/v) Fetal Bovine Serum (Biowest S1810-500), 1% amphotericin B (Biowest, L0009-050), 1% Antibiotic-antimycotic (Biowest, L0010-100), 1% MEM vitamins 100x (Biowest, X0556-100), and 0.1% gentamicin (Gibco, 12750060). The incubation took place in a humidified atmosphere with 5% CO_2 at 37°C, with a regular medium replacement every 2 days. The confluent cells were collected for subsequent analysis by using Trypsin 0.25% EDTA (Biowest, L0931-500) [32,33]. The cells were then plated onto a 6-well-plate for treatment, which was divided into 6 groups, namely: I) Untreated cells; II) Cells + DMSO 1%, III) Cells + TMZ 300 µM, IV) Cells + NC 25 µg/mL, V) Cells + NC 50 µg/mL, and VI) Cells + NC 100µg/mL for relative expression and apoptotic cells measurement. This concentration was chosen based on the cytotoxicity test results of NC on GBM conducted in previous research.

5.3. mRNA relative expression measurement

The gene expression of Casp-3, ERK, EGFR, and IGFBP was quantified with RT-PCR. The total RNA of GBM cells was extracted using TRIzol reagent (Zymo research) then processed into cDNA with a synthesis kit from Meridian Bioscience (BIO-65054). The RNA and cDNA purity was assessed using a microplate reader, and the corresponding data are presented in Tables 1. cDNA that had been synthesized was amplified using PCR for 40 cycles (ESCO SCR-2A1). The PCR mixture contained Nuclease Free Water (NFW), SensiFAST SYBR NO-ROX (Meridian Bioscience BIO-98005), Primer forward, Primer reverse, and cDNA template. The antisense fragments design of IGFBP2, EGFR, ERK, and Casp-3 were obtained from NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov/nucleotide) (Table 2). The initial step of amplification was denaturation at 95°C for 5 min, followed by 40 thermal cycles of 94°C for 50 s, 40 cycles of 58-60°C for 50 s, and 72°C for 50 s, with a final extension at 72°C for 5 min. Finally, RT-PCR results were evaluated through detection techniques such as qPCR (quantitative PCR) using the kit from Clontech Biosciences, Advantage™ RT-for-PCR [34–36].

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Table 1. RNA and cDNA concentration and purity

Sample	RNA		cDNA	
	Concentration (ng/µL)	Purity (λ260/λ280)	Concentration (ng/µL)	Purity (λ260/λ280)
I	30.16	1.95	649.12	2.04
II	27.52	1.93	578.24	2.00
III	18.32	1.67	564.32	2.04
IV	30.24	1.82	662.24	2.02
V	31.52	1.93	757.52	2.08
VI	26.72	1.90	911.20	2.03

The various samples (I, II, III, IV, V, VI) indicates I): Untreated cells, II): Cells + DMSO 1%, III): Cells +TMZ 300 µM, IV): Cells + NC 25 µg/mL, V): Cells + NC 50µg/mL, and VI): Cells + NC 100µg/mL

5.4. The quantification of apoptotic, live, and necrotic cells

GBM cells were harvested and centrifuged for 5 minutes at 1600 rpm. The resulting sediment was washed with Annexin Binding Buffer 1x (Miltenyi Biotec, 130-0920820) and underwent another centrifugation. Repeat the washing process and stained the cells with Annexin V-FITC (BioLegend, Part79998) and Propidium Iodide (PI; BioLegend, Part79997). The stained cells were placed in a dark room with temperature of 4°C. To evaluate the quantification of apoptotic, live, and necrotic cells, MACSquant Analyzer 10 (Miltenyi Biotec) was utilized [37,38].

Table 2. Primer sequence design of target gene in GBM cells

Gen	Primer Sequence (5' - 3')	Product length (bp)	Annealing (°C)	Cycle	Reference
Human GADPH	F: GCCAAAAGGGTCATCATCTC R: TGAGTCCTTCCACGATACCA	178	58	40	NM_001357943.2
Human Casp-3	F: AGAACTGGACTGTGGCATTGAG R: GCTTGTCGGCATACTGTTTCAG	191	58	40	NM_001354783.2
Human ERK	F: TATTCGAGCACCAACCATCG R: TGCTGAGGTGTTGTGTCTTC	101	59	40	NM_002745.5
Human EGFR	F:C CTTACTTTCCTTCGACCTCTG R: GTCAGTTCCTGGAAGACCTTAC	173	60	40	NM_005228.5
Human IGFBP-2	F: TGTTTGGGTCTAGCTTGGTC R: TTCAGTCGGCTCATACCAAC	133	60	40	NM_001313992.2

Data were obtained from The NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov/nucleotide)

5.5. Statistical analysis

The sample was conducted in three replications and statistically tested using Statistical Package for the Social Sciences (SPSS) application (version 20.0). Tukey's post hoc and T-Test were performed on parametric data, while Kruskal Wallis followed by Mann-Whitney were performed on non-parametric data with a significance level of $p < 0.05$ [39].

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