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Demethoxycurcumin Suppresses Proliferation, Migration, and Invasion of Human Brain Glioblastoma Multiforme GBM 8401 Cells *via* PI3K/Akt Pathway

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Abstract. *Background/Aim: Demethoxycurcumin (DMC), one of the derivatives of curcumin, has been shown to induce apoptotic cell death in many human cancer cell lines. However, there is no available information on whether DMC inhibits metastatic activity in human glioblastoma cancer cells in vitro. Materials and Methods: DMC at 1.0-3.0 μM significantly decreased the proliferation of GBM 8401 cells; thus, we used 2.0 μM for further investigation regarding anti-metastatic activity in human glioblastoma GBM 8401 cells. Results: The internalized amount of DMC has reached the highest level in GBM 8401 cells after 3 h treatment. Wound healing assay was used to determine cell mobility and results indicated that DMC suppressed cell movement of GBM 8401 cells. The transwell chamber assays were used for measuring cell migration and invasion and results indicated that DMC suppressed cell migration and invasion in GBM 8401 cells. Gelatin zymography assay was used to examine gelatinolytic activity (MMP-2) in conditioned media of GBM 8401 cells treated by DMC and results demonstrated that DMC significantly reduced MMP-2 activity. Western blotting showed that DMC reduced the levels of p-EGFR$^{(Tyr1068)}$, GRB2, Sos1, p-Raf, MEK, p-ERK1/2, PI3K, p-Akt/PKBα$^{(Thr308)}$, p-PDK1, NF-κB, TIMP-1, MMP-9, MMP-2, GSK3α/β, β-catenin, N-cadherin, and vimentin, but it elevated Ras and E-cadherin at 24 h treatment. Conclusion: DMC inhibited cancer cell migration and invasion through inhibition of PI3K/Akt and NF-κB signaling pathways in GBM 8401 cells. We suggest that DMC may be used as a novel anti-metastasis agent for the treatment of human glioblastoma cancer in the future.*

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Key Words: Demethoxycurcumin (DMC), migration, invasion, NF-κB, human glioblastoma multiforme (GBM 8401) cells.

Glioblastoma multiforme (GBM) has been recognized to be the most common malignant brain tumor and the most lethal tumor in adult brain cancer patients. GBM patients have an

estimated 5-year survival rate less than 10% (1). Although the development of novel therapeutic modalities have been used, median survival patient with GBM is about 15 to 16 months (2). Currently, the major treatment interventions for GBM patients are neurosurgical resection, radiotherapy, and adjuvant chemotherapy with temozolomide (3). However, metastasis or malignant diffusion have already developed in most glioma patients at the time of diagnosis (4); thus, the possibility of remission, relapse, and metastasis are still substantial. These treatments are still unsatisfactory. Tumor cells can metastasize to vital organs through blood circulation and develop new tumors (5, 6). Tumor metastasis has been accounted for over 80% of cancer mortality (7).

The metastatic cascade exhibits a multi-step and complicated process, including tumor cell local invasion, cell entry into blood vessels, adhesion, invasion, colonization, and outgrowth of metastases in other organs. Furthermore, the metastatic process may result from the cancer cell's genetic alterations and the interaction with its tumor microenvironment (8, 9). The foremost step for tumor invasion is the basement membrane's degradation by matrix metalloproteinases (MMPs) (10, 11). Moreover, overexpression of MMPs promotes tumor cell invasion and metastasis (12). Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) both play critical roles in the metastatic cascade (12). It has been suggested that blocking MMPs is a potent strategy for inhibiting cancer cell metastasis (13, 14).

Natural products and phytochemicals have been clinically used to treat human cancers and inhibit invasion of cancer cell lines (15, 16). Phytochemicals affect cancer cell migration and invasion, which mediate multiple pathways (17). Curcumin, a natural chemical component, is the main active ingredient of turmeric, which is extracted from the Curcuma longa rhizome, and has been widely used by consumers in India and China (18, 19). Demethoxycurcumin (DMC), one of the curcumin analogs, presents similar biological properties as curcumin, exhibits anti-inflammatory, anti-proliferative, and anti-microbial effects (20-22). DMC is widely used in domestic cooking and folk medicine (23). DMC is more chemically stable than curcumin (24) and had better anti-cancer and anti-inflammatory activity than curcumin (25, 26). DMC induced apoptotic cell death *via* both mitochondria- and caspase-dependent pathways in human brain malignant glioma GBM 8401 cells *in vitro* (27) and DMC is superior to temozolomide concerning its anti-GSC (glioma stem cell) effects *in vivo* (28). Recently, DMC triggered cell apoptotic death and inhibited cell survival pathways in human glioma U87 MG cells *via* inhibiting SOD, leading to superoxide anion accumulation (29).

Although DMC has been demonstrated to induce cytotoxicity in human GBM cells, there are no reports showing anti-metastatic properties in GBM cancer cells. Therefore, in the present study, we investigated DMC's effects on cell proliferation, migration and invasion in human brain malignant glioma GBM 8401 cells for *in vitro* studies. We showed that DMC inhibited cancer cell proliferation, migration, and invasion by inhibiting PI3K/Akt pathway, NF-κB, β-catenin, MMP-2, and -9 in GBM 8401 cells *in vitro*.

Materials and Methods

Chemicals, reagents, medium and antibodies. Demethoxycurcumin (DMC), was purchased from Sigma Chemical Co. (St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO). Cell culture materials including RPMI 1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were purchased from GIBCO®/Invitrogen Life Technologies (Grand Island, NY, USA). Western blotting antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA), polyvinylidene difluoride (PVDF) membrane was obtained from Millipore (Temecula, CA, USA). Transwell culture chambers (8-μm pore size) were obtained from BD Biosciences (Franklin Lakes, NJ, USA)

Cell culture. The human brain glioblastoma multiforme GBM 8401 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The GBM 8401 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotic solution in an atmosphere of 5% CO_2 and 95% air at 37˚C. The culture medium was changed every 2 days (27, 30).

Cell proliferation and cell viability. GBM 8401 cells were seeded in 12-well plates for 24 h and exposed to 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μM of DMC or were incubated with 1% DMSO (vehicle control) for 48 h. At the end of incubation, cells were measured total viability by flow cytometric assay based on cells that excluded the dye (alive cells) and stained cells (dead cells) as previously described (31) or were measured proliferation rate by MTT assay as previously described (32, 33).

DMC uptake in GBM 8401 cells. The autofluorescence of DMC can be detected by 488 nm excitation of argon laser in flow cytometry. GBM 8401 cells were plated in 12-well plates with RPMI 1640 medium overnight and incubated with 2 μM of DMC for different times (0, 3, 6, 9, 12, and 24 h). After incubation, cells were detached, collected, and measured cellular uptake by flow cytometer (Beckman Coulter, Fullerton, CA, USA) as previously described (34).

Wound healing assay. GBM 8401 cells (3×10^5 cells/well) kept at 12-well plate for grown entirely confluent. Cell monolayers were scraped from one to the other end of the individual well using a sterile 200 μl tip and washed with PBS three times. The width of the wound distance was measured and recorded as the baseline. Then cells were cultured in RPMI 1640 medium containing 0 and 2 μM of DMC for 0, 6, 12, 24 and 48 h. The healing area of cell monolayers in the plates were examined and photographed under inverted microscope as described previously (35, 36).

Cell migration and invasion assay. Cell migration and invasion assays were conducted with transwell culture chambers. In brief, the individual transwell membrane was coated with collagen in the incubator overnight for migration assay. GBM 8401 cells were suspended at a density of 4×10^5 cells/transwell in FBS-free RPMI 1640 medium. The 200 μl of low-serum medium containing

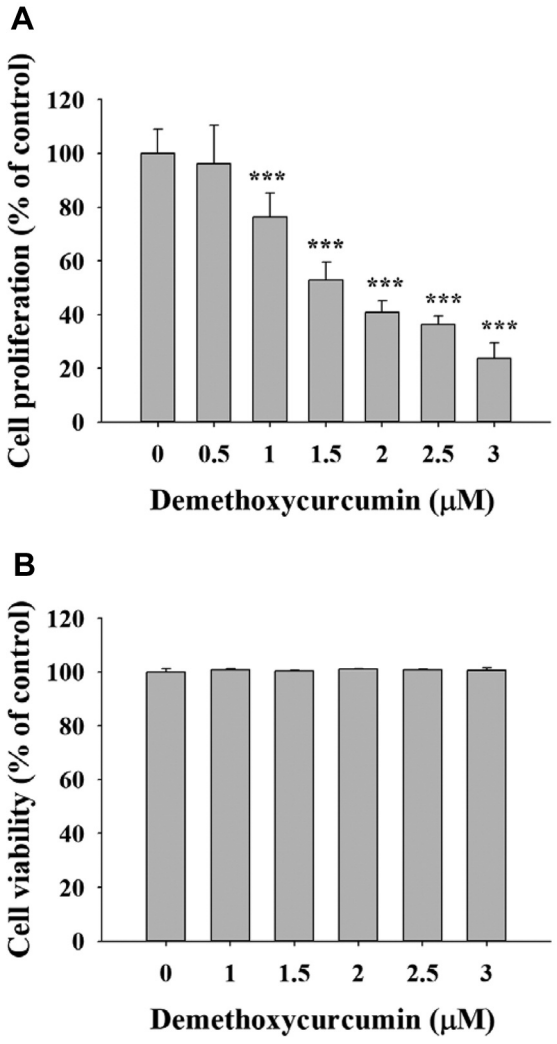

Figure 1. *Demethoxycurcumin (DMC) decreased cell viability and proliferation of GBM 8401 cells. (A). Cells were plated in 96-well plates with RPMI 1640 medium for 24 h and were incubated with DMC at final concentrations (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μM) for 48 h. After incubation, cell proliferation was measured by the MTT assay as described in Materials and Methods. (B). Cells were plated in 12-well plates with RPMI 1640 medium for 24 h and were incubated with DMC at final concentrations (0, 1.0, 1.5, 2.0, 2.5, and 3.0 μM) for 48 h. After incubation, cells were collected, counted and total cell viable cell number was measured by PI exclusion assay. ***p<0.001, significant difference between DMC-treated groups and the control as analyzed by one-way ANOVA analysis.*

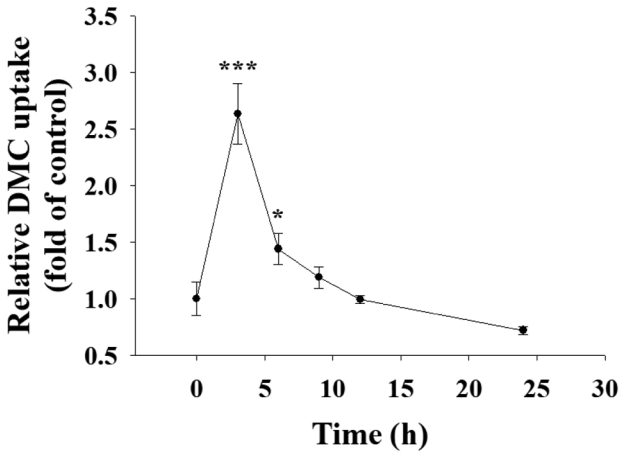

Figure 2. *Internalization of Demethoxycurcumin (DMC) by GBM 8401 cells. Cells were grown in 12-well plate and incubated with 2.0 μM of DMC for various time periods and harvested for measuring DMC uptake as described in Materials and Methods. *p<0.05, ***p<0.001 significant difference between DMC-treated groups and the control as analyzed by one-way ANOVA analysis.*

suspended cells (4×10^4 cells) and DMC solution (0 and 2 μM) were pipetted into the transwell upper chamber, while 800 μl of RPMI 1640 medium with 10% FBS were placed on the lower chamber. After 24 and 48 h incubation, cells on the upper chamber's side (non-migrated cells), were scrubbed with cotton swabs. Subsequently, the lower surface with migrated cells was fixed with 4% cold formaldehyde and stained with 0.1% crystal violet. Cells from each treatment were photographed and counted under microscopy. Cell invasion assay were conducted as migration assay, however, the transwell culture membrane was coated with Matrigel as described previously (31, 37).

Gelatin zymography assay. GBM 8401 cells (4×10^5 cells/well) were maintained in 12-well plates to reach around 100% confluency and washed with serum-free RPMI 1640 medium, cells were incubated with 2 μM of DMC for 24 and 48 h. The individual conditioned medium from each treatment was collected and 12 μl of conditioned medium was loaded into 10% SDS-PAGE containing 0.2% gelatin as described previously (37). After washed, the gel was immersed in substrate buffer (50 mM Tris HCl, 5 mM $CaCl_2$, 0.02% NaN_3, and 1% triton X-100; pH 7.4) while shaking for 18 h at 37˚C. Coomassie blue (0.2% in 10% acetic acid/50% methanol; Bio-Rad, Hercules, CA, USA) was used to stain the gel. Finally, the gel was destained with methanol/acetic acid destaining buffer for the bands on the gel appeared clear. In a dark blue field, a clear zone (MMP-2 gelatinolytic activities) were examined and photographed and the clear bands were further analyzed using ImageJ software (NIH, USA) as described previously (38).

Western blot assay. GBM 8401 cells (2×10^6 cells) in 10-cm dishes were incubated with DMC (0 and 2 μM) for 24 h. Cells were lysed in buffer containing 40 mM Tris-HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonide P-40 with protease inhibitors for 10 min at 4˚C and then centrifuged to extract cellular proteins and measured the total protein. Equal amount of protein (30 μg) was loaded into 10% SDS-PAGE gels, followed by electroblotting onto polyvinylidene difluoride (PVDF) membranes. The blot was blocked and probed with different primary antibodies at 4˚C overnight, washed, and probed with HRP-conjugated secondary antibody at room temperature for 1 h. And bands signals were enhanced chemiluminescence (GE Healthcare) and were detected by using ECL reagent.

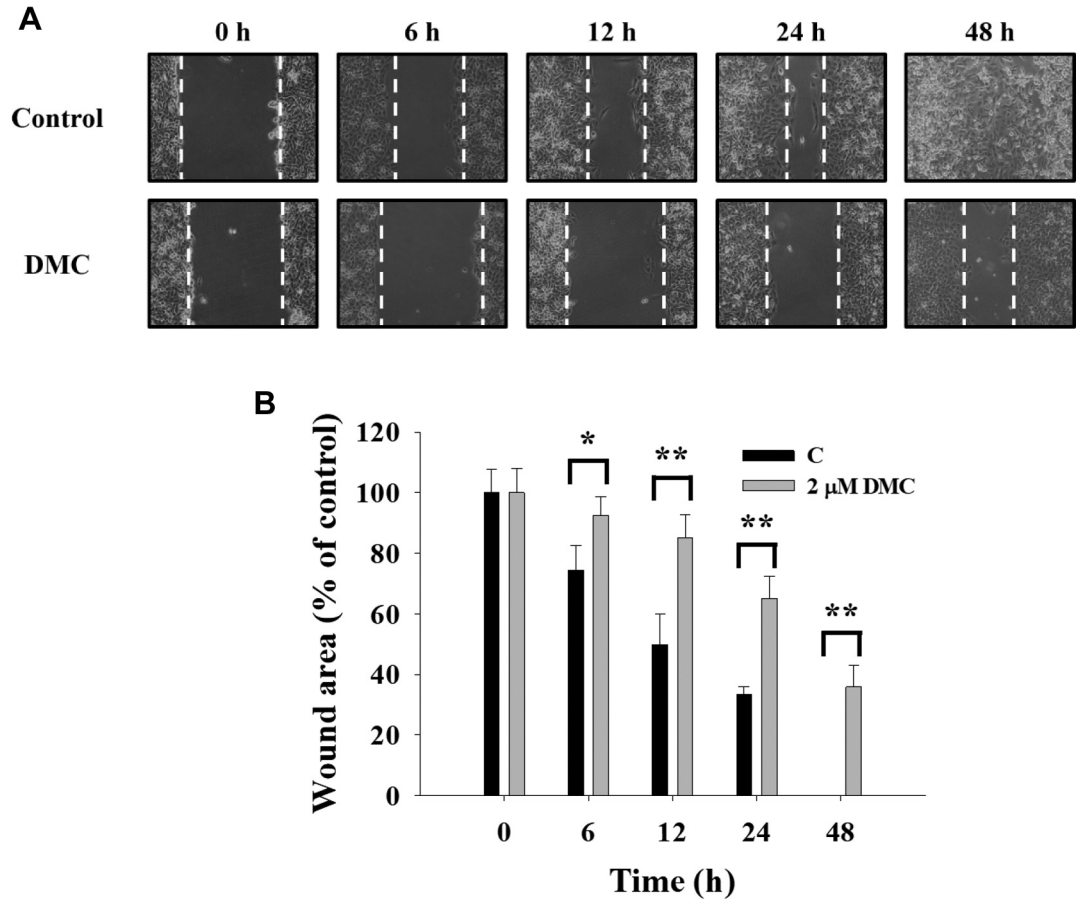

Figure 3. *Demethoxycurcumin (DMC) decreased cell mobility of GBM 8401 cells. Cells were growth in 12-well plates until cell monolayer formation, were scraped, incubated in medium containing 0 and 2 μM of DMC for 0, 6, 12, 24 and 48 h. After incubation, cells were scraped and RPMI 1640 medium containing DMC was added to cells as described in Materials and Methods. (A) The representative figures for closures of scrape area; (B) The percentage of inhibition was calculated. *p<0.05, **p<0.01, significant difference between DMC-treated groups and the control as analyzed by one-way ANOVA analysis.*

Statistical analysis. Data are expressed as the median (interquartile range) (means±SD.). the student's *t*-test was used when comparing two groups. A value of $p<0.05$ was considered statistically significant.

Results

DMC decreased cell proliferation and cell viability of GBM 8401 cells. For cell proliferation assays, cells were treated with DMC (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μM) for 48 h, and incubated with the MTT reagent. We measured cell proliferation by a spectrophotometer, and results are shown in Figure 1A. Results indicated a significant dose-dependent reduction of cell number with DMC at 1.0-3.0 μM concentrations for 48-h treatment. Cells were treated with different DMC doses (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μM) for 48 h, we then harvested, and cell viability was measured by PI exclusion assay. As shown in Figure 1B, DMC did not reduce cell number with DMC at 0.5-3.0 μM concentration for 48-h treatment. Thus, we selected 0 and 2.0 μM of DMC for cell migration and invasion experiments.

DMC uptake of GBM 8401 cells. Cells were treated with 2.0 μM of DMC for 3, 6, 9, 12, and 24 h for determining cellular uptake of DMC by flow cytometric assay. Results (Figure 2) showed that after 3 h treatment, DMC was significantly internalized into GBM 8401 cells, however, after 4-24 h treatment, its levels were decreased.

DMC decreased cell mobility. A wound-healing assay was performed to evaluate cell mobility. GBM 8401 cells were grown in 12-well plates until cell monolayer formation. Cells were wounded by scraping and incubated with the RPMI 1640 medium containing 0 and 2.0 μM of DMC for 6, 12,

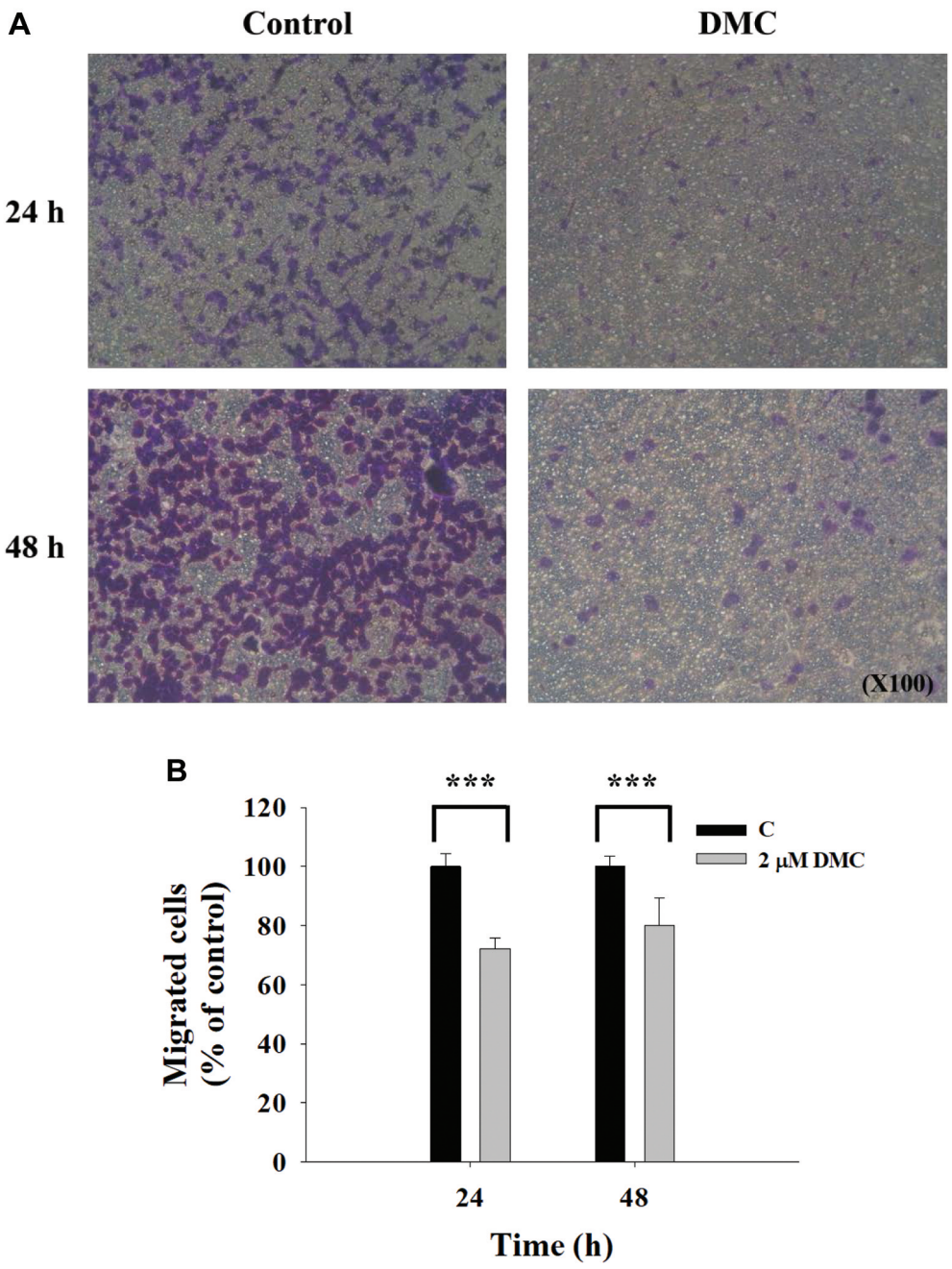

Figure 4. *Demethoxycurcumin (DMC) inhibited the cell migration of GBM 8401 cells. Cells were treated with DMC and grown in transwell plates coated with collagen for 24 and 48 h for invasion assays. (A) Representative images of migrated cells in transwell. (B) The number of migrated cells. ***p<0.001, significant difference between DMC-treated groups and the control as analyzed by one-way ANOVA analysis.*

24, and 48 h. As shown in Figure 3, the healing of the scraped area in the DMC treatment was lower than that of control and it showed a time-dependent manner. DMC significantly reduced cell mobility (Figure 3A) and the reduced rate was up to 45.8% at DMC in 48 h when compared to control (100%) (Figure 3B).

DMC suppressed cell migration and invasion. For measuring cell migration and invasion, the transwell cell migration and invasion assays were conducted in GBM 8401 cells *in vitro* after exposure to DMC. As shown in Figures 4 and 5, DMC suppressed cell migration with 76% and 91% at 24 and 48 h treatment, respectively (Figure 4A and B) ($p<0.05$). DMC inhibited cell

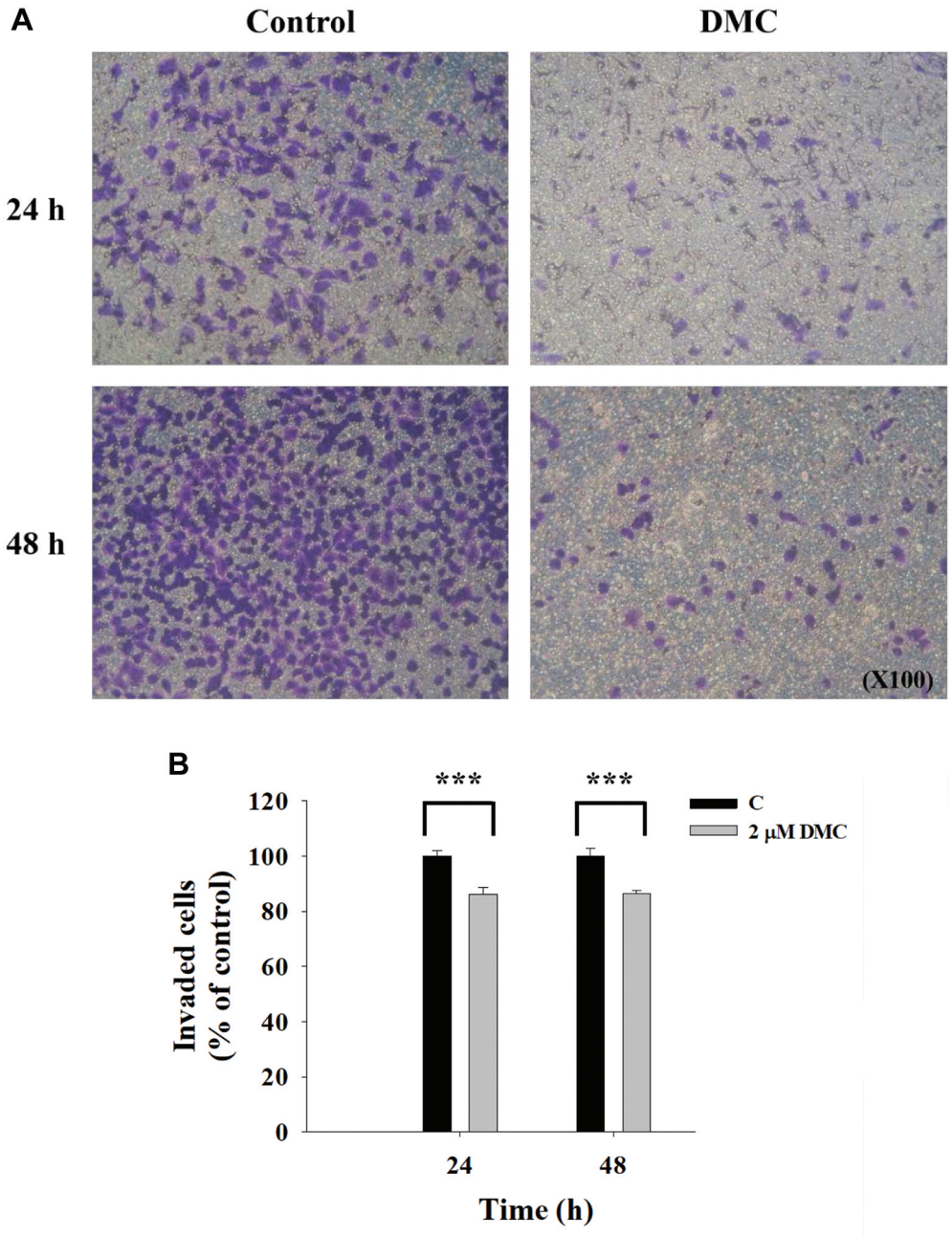

Figure 5. *Demethoxycurcumin (DMC) inhibited the cell invasion of GBM 8401 cells. Cells were treated with DMC and grown in transwell plates coated with Matrigel for 24 and 48 h for invasion assay. (A) Representative images of invaded cells in transwell. (B) The number of invaded cells. ***p<0.001, significant difference between DMC-treated groups and the control as analyzed by one-way ANOVA analysis.*

invasion by 79% and 87% at 24 h and 48 h treatment, respectively after comparison to control cells (Figure 5A and B) (p<0.001). Based on these results, DMC significantly inhibited cell migration and invasion of GBM 8401 cells.

DMC inhibited the activity of MMP-2 in GBM 8401 cells. Gelatin zymography was used for measuring the gelatinolytic activity of MMP-2 from the conditioned media, after GBM 8401 cells were treated with DMC. Cells were treated with DMC (0 and 2 μM) for 24 and 48 h, and the gelatinolytic activity of MMP-2 was assayed. Results indicated that DMC significantly inhibited the gelatinolytic activity of MMP-2 at 24 and 48 h treatment, compared to the control (0 h) (Figure 6).

DMC altered cell migration and invasion-associated proteins in GBM 8401 cells. To further investigate whether the expression of migration and invasion-associated proteins were influenced in GBM 8401 cells after exposure to DMC. The metastasis- and EMT-associated proteins in GBM 8401 cells were analyzed by western blotting and the results are shown in Figure 7. DMC reduced the levels of p-EGFR$^{(Tyr1068)}$, GRB2, Sos1 (Figure 7A), p-Raf, MEK, p-ERK1/2 (Figure 7B), PI3K, p-Akt/PKBα$^{(Thr308)}$, p-PDK1, NF-κB, TIMP-1, MMP-9, MMP-2 (Figure 7C), GSK3α/β, β-catenin, N-cadherin, and Vimentin (Figure 7D) at 24 h treatment but it elevated Ras (Figure 7B) and E-cadherin (Figure 7D) at 24 h treatment. Based on these observations, DMC inhibited cancer cell proliferation, migration and invasion through PI3K/Akt pathway, β-catenin, NF-κB, MMP-2, and -9.

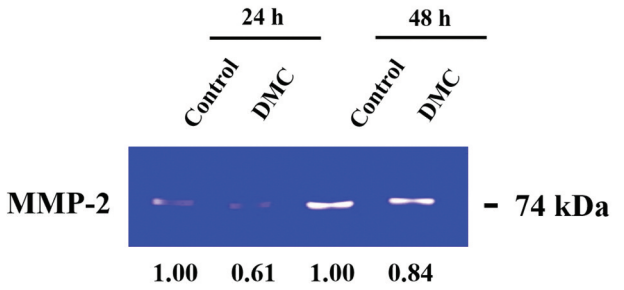

Figure 6. *Demethoxycurcumin (DMC) inhibited the activity of MMP-2 in GBM 8401 cells. Gelatin zymography was performed to detect the MMP-2 gelatinolytic activity in conditioned media of GBM 8401 cells treated with DMC. GBM 8401 cells were incubated with DMC (2.0 μM) for 24 and 48 h, and the gelatinolytic activity of MMP-2 was measured as described in Materials and Methods.*

Discussion

Genomic rearrangements and various mutations in GBM are associated with radio- and chemoresistance (39). Currently, clinical treatment for GBM patients is still unsatisfactory. Thus, one of the potential strategies for anti-cancer effects is to target cancer cell metastasis. Although DMC has been shown to induce apoptotic cell death and induce cell-cycle arrest in many human cancer cell lines; however, no reports have shown that DMC inhibited human GBM cell migration and invasion *in vitro*. In the present study, we investigated DMC's effects on GBM 8401 cell migration and invasion *in vitro*. Numerous studies with epidemiological evidence have demonstrated that diets enriched with naturally occurring substances have been recognized to significantly reduce the risk for many human cancers (40-42).

In this study, the first experiment was to evaluate the effects of DMC (0-3 μM) on cell proliferation and cell viability of GBM 8401 cells. Results indicated that DMC at 1-3 μM significantly inhibited cell proliferation (Figure 1A), but did not reduced total viable cells (Figure 1B). Thus, the 2 μM of DMC was used for further experiments. Besides, we investigated the cellular uptake of DMC and results showed that DMC was highly internalized into cells after 3-h treatment (Figure 2). Thus, we used scrape wound healing assay to examine cell's mobility after exposure to DMC at 2 μM for different periods in GBM 8401 cells. Results indicated that DMC significantly inhibited cell mobility (Figure 3). Scrape wound healing assay has been known for examining chemicals that inhibited cancer cell mobility *in vitro* (43, 44).

Furthermore, Transwell-assay was used to investigate migration and invasion of GBM 8401 cells *in vitro*. Thus, cells treated with and without DMC were layered in the transwell, which filters coated with collagen or Matrigel for examining cell migration and invasion, respectively, and results indicated that DMC repressed cell migration (Figure 4) and invasion (Figure 5). Transwell-assay is one of the major protocols for examining cell migration and invasion (45, 46).

We also used gelatin zymography assay for examining the MMP-2 activity in GBM 8401 cells after exposure to DMC and results showed that DMC suppressed the MMP-2 activity at 24 and 48 h treatment. Recently, MMPs could be drug targets for anti-cancer metastasis (47). However, MMP-2 and MMP-9 have been recognized to be the most vital enzymes for the degradation of the extracellular matrix and basement membrane of cells (48, 49).

To further investigate whether or not DMC suppresses cell migration and invasion of GBM 8401 cells *in vitro*, we examined the levels of proteins associated with cell migration and invasion. Results from western blotting indicated that DMC decreased p-EGFR$^{(Tyr1068)}$, GRB2, Sos1 (Figure 7A), p-Raf, MEK, p-ERK1/2 (Figure 7B), PI3K, p-Akt/PKBα$^{(Thr308)}$, p-PDK1, NF-κB, TIMP-1, MMP-9, MMP-2 (Figure 7C), GSK3α/β, β-catenin, N-cadherin, and Vimentin (Figure 7D) but increased E-cadherin at 24 h treatment of GBM 8401 cells. GAB2 (GRB2-associated binding protein 2) has been shown to play an essential role in the proliferation and migration of various cancers (50). Sevenless homolog-1 (Sos1) is also involved in cell migration and invasion (51). The inactivation of PI3K/Akt signaling pathways could suppress cancer cell migration and invasion (52, 53). Furthermore, PI3K/Akt signaling pathway involved cell migration and invasion in glioblastoma (54) and medulloblastoma (55) cells, consistent with our findings. NF-κB, a transcription factor, affects cell survival, proliferation, and metastasis (56) and its overexpression promotes cancer cell migration (57). Therefore, down-regulation of NF-κB could lead to hindering cancer cell migration and invasion (58).

Based on these observations, DMC diminished the level of NF-κB in GBM 8401 cells; thus, we suggest that DMC

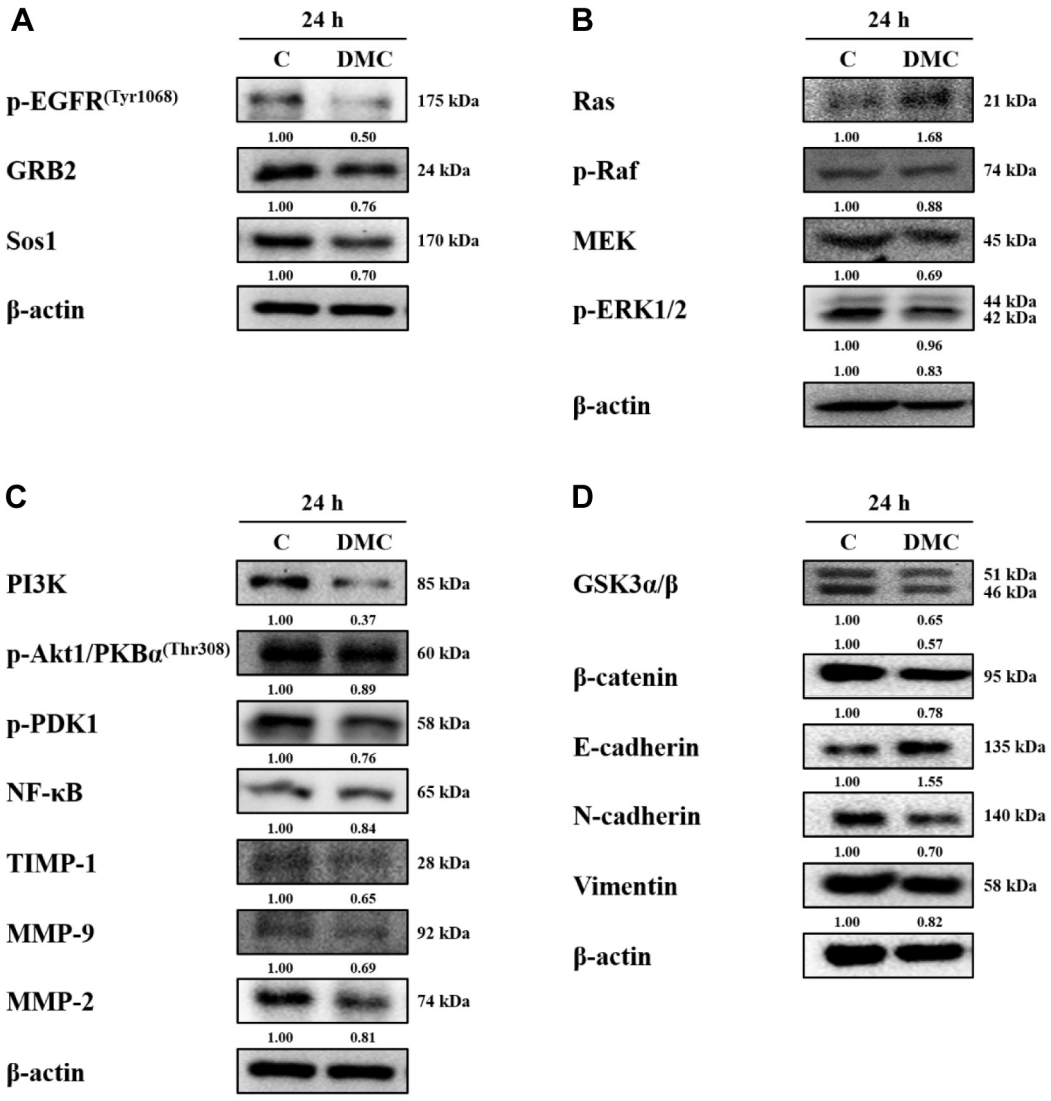

Figure 7. *Demethoxycurcumin (DMC) altered the levels of proteins associated with migration and invasion of GBM 8401 cells. Cells were treated with 2 μM of DMC for 24 h and then total proteins were quantitated and metastasis associated proteins were examined by western blotting as described in Materials and Methods. (A) p-EGFR(Tyr1068), GRB2, and Sos1; (B) Ras, p-Raf, MEK, and p-ERK1/2; (C) PI3K, pAkt/PKBα(Thr308), p-PDK1, NF-κB, TIMP-1, MMP-9, and MMP-2; (D) GSK3α/β, β-catenin, E-cadherin, N-cadherin, and Vimentin. β-actin was used as an internal control.*

inhibition of GBM 8401 cell migration and invasion may also occur through the NF-κB pathway. The other reason is that it was reported that suppressing NF-κB pathway will lead to inhibition of the invasion of glioblastoma cells (59). Moreover, the essential role of NF-κB in cell migration has been known *via* activating snail and repressing E-cadherin (60, 61). It is well documented that the EMT process is involved in the decrease of E-cadherin (cell adhesion molecule) and increase of vimentin and N-cadherin, that are mesenchymal markers (62, 63). After tumour cell membrane receptors binding with growth factors and extracellular matrix molecules, cascades of intracellular signals are generated to promote down-regulation of E-cadherin (64). Many studies have demonstrated that both MMP-2 and MMP-9 are associated with cancer invasion and metastasis (65, 66) and both expressions were confirmed in this study. Overall, DMC suppressed the EMT activation through down-regulation of N-cadherin, vimentin, MMP-2, and MMP-9 and up-regulation of the E-cadherin, leading to decreasing cell proliferation, migration, and invasion (67).

In conclusion, DMC inhibited PI3K/Akt pathway, β-catenin, and NF-κB leading to suppression of GBM 8401 cell

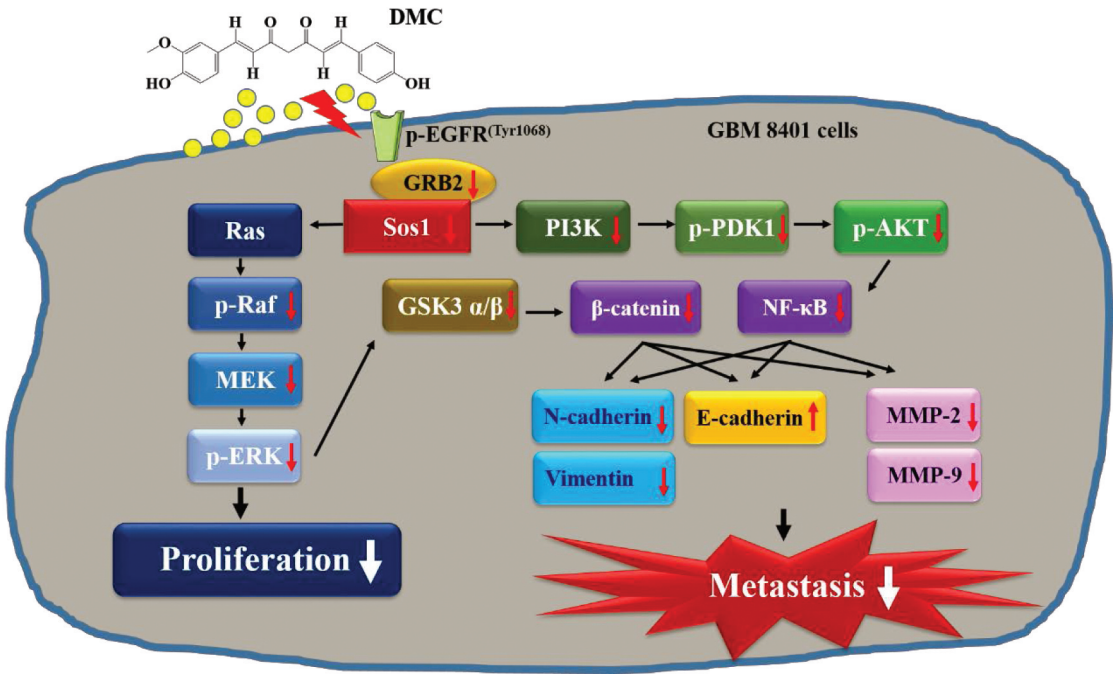

Figure 8. *The possible signaling pathways for demethoxycurcumin (DMC) inhibiting cell migration and invasion in human glioblastoma multiforme GBM 8401 cells.*

mobility, migration, and invasion *in vitro*. DMC suppressed metastasis-associated protein expression (MMP-2, MMP-9, N-cadherin, and Vimentin) in GBM 8401 cells. Therefore, the possible pathways involved in DMC activities are summarized in Figure 8. DMC may be a potential candidate compound against human GBM cancer metastasis in future.

Conflicts of Interest

The Authors have no conflicts of interest to disclose.

Authors' Contributions

R.Y. Su, S.C. Hsueh, M.Y. Yeh and Y.P. Huang conceived and designed the experiments; R.Y. Su, C.Y. Chen and M.J. Hsu performed the experiments; H.F. Lu, S.F. Peng, P.Y. Chen, J.C. Lien, Y.L. Chen and F.S. Chueh analyzed the data; J.G. Chung contributed reagents/materials/analysis tools; R.Y. Su, S.C. Hsueh, M.Y. Yeh and Y.P. Huang wrote the paper.

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