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*Original Article*

# Curcumin inhibits lung cancer invasion and metastasis by attenuating GLUT1/MT1-MMP/MMP2 pathway

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**Abstract:** Glucose transporter (GLUT) 1 is found highly expressed in malignant tumors and considered a mediator inducing cancer metastasis. Curcumin is a natural product which exerts anti-invasion and metastasis effects in cancer. This study aimed at evaluating whether attenuating GLUT1 was involved in curcumin's anti-invasion and metastasis effects. In the *in vitro* part, constricted pcDNA3.1-GLUT1 vector was transfected into A549 cells. MTT assay was used to assess the curcumin's effects on proliferation in lung cancer A549 cells. Transwell assay was used to evaluate the anti-invasion effect of curcumin on A549 cells. Real-time PCR and Western-blotting were employed to examine the expression levels of GLUT1, membrane type 1-MMP (MT1-MMP) and matrix metalloproteinase (MMP) 2 in curcumin- incubated A549 cells. In the *in vivo* part, tumor weight and metastatic rate were assessed in nude mice bearing untransfected, empty vector transfected and pcDNA3.1-GLUT1 transfected A549 cells originated tumors. In this study, we found that curcumin began to show significant cytotoxicity against proliferation effect at 45 μmol/L. Curcumin inhibited invasion and expressions of GLUT1, MT1-MMP and MMP2 untransfected A549 cells in a concentration-dependent manner. pcDNA3.1-GLUT1 transfected A549 cells exhibited resistance to curcumin's anti-invasion effect by up-regulating expressions of GLUT2, MT1-MMP and MMP2. Furthermore, curcumin failed to decrease the metastatic rate in nude mice bearing pcDNA3.1-GLUT1 transfected A549 cells originated tumors. These results suggested that curcumin inhibit lung cancer invasion and metastasis by attenuating GLUT1/MT1-MMP/MMP2 pathway.

**Keywords:** Lung cancer, curcumin, glucose transporter

## Introduction

The morbidity and mortality of lung cancer is increasing rapidly worldwide which has become one of the leading causes responsible for cancer-related death [1]. It is believed that non-small cell lung cancer (NSCLC) is the most common pathological type of lung cancer, accounting for approximately 80% of lung cancer cases [2]. The prognosis of NSCLC has been proved poor which was evidenced by the 15% overall 5-year survival rate. Though there are multiple choices for NSCLC including surgery, chemotherapy, radiotherapy, immunotherapy and so on, clinical data showed that the mortality was still inevitable in most cases because of the progression and relapse due to invasion and metastasis [3]. In this regard, it is of significance to develop efficient anti-invasion reagents to decrease the vulnerability and improve the survival rate of patients with NSCLC.

In adaption to increased proliferation and invasion, compared with normal cells, malignant cells are highly energy consumptive [4]. Normal cells acquire energy via respiration chain in mitochondria, while cancer cells are more relied on aerobic glycolysis [5]. This phenomenon was known as Warburg effect which was proved associated with development and progression of cancer [6]. Increased glucose uptake was identified in various malignant cells and considered the mechanism of Warburg effect [7]. It was suggested that the glucose transporter 1

**Table 1.** Sequences of primers for PCR

| Gene name | Type | Sequences | Remark |
|---|---|---|---|
| GLUT1 | F | 5'-AGGTCTAGAACGTCCATTCTCCGTTTCAC-3' | cDNA generation |
| | R | 5'-TTATCTAAGAGAGATCCTCGGGGCTGCTG-3' | |
| GLUT1 | F | 5'-CACATGCCTTGTTCTTTGCCAAG-3' | Subclone for detection |
| | R | 5'-TCTATACACAGCAGGGCAGGA-3' | |
| MT1-MMP | F | 5'-CTTTTCCATCCCCTGACATACC-3' | |
| | R | 5'-CTGACTGAGCAACGGAGACCCT-3' | |
| MMP2 | F | 5'-AAGGATGGCAAGTACGGCTT-3' | |
| | R | 5'-CGCTGGTACAGCTCTCATACTT-3' | |
| GAPDH | F | 5'-CGGAGTCAACGGATTTGGTCGTAT-3' | |
| | R | 3'-AGCCTTCTCCATGGTGGTGAAGAC-5' | |

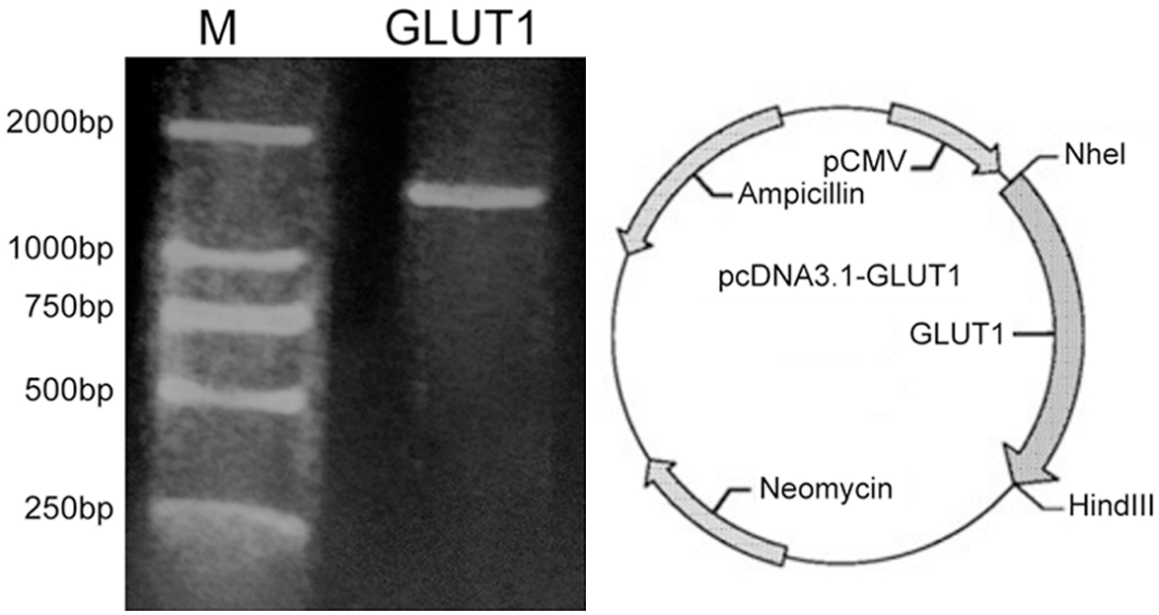


**Figure 1.** Construction and identification of pcDNA3.1-GLUT1. The left part of this figure demonstrates the PCR analysis of GLUT1 full length cDNA fragment. Lane M indicates DNA marker. The right part of this figure shows the pcDNA3.1-GLUT1 vector construction diagram.

(GLUT1) is the rate-limiting transporter for cell glucose uptake and correlated with anaerobic glycolysis in cancer cells [8]. Furthermore, GLUT1 was found over-expressed and treated as a prognosis indicator in many cancers including colon cancer, breast cancer, esophageal cancer, lung cancer and so on [9, 10]. Several recent studies found the GLUT1 overexpression was also associated with lymph node/blood vessel metastasis and invasion depth in malignant tumors [11, 12].

Because of their activity in degradation of basement membrane and extracellular matrix (ECM), the matrix metalloproteinases (MMPs) which belong to zinc-dependent proteinases family were generally accepted as the contributors in metastasis and invasion abilities of cancer [13]. By secreting MMPs, malignant cells would facilitate themselves migrating into adjoining tissue, blood vessels and lymph vessels, resulting in local infiltration, distant organ metastasis and lymph node metastasis [14]. Among the identified MMPs, MMP2 (gelatinase B) was selected as the indicator of tumor cell malignancy because it's unique enzymatic activity to degrade type-IV collagen which is the main composition of basement membrane [15]. In a previous study, the co-expression of GLUT1 and MMP2 was identified along with the

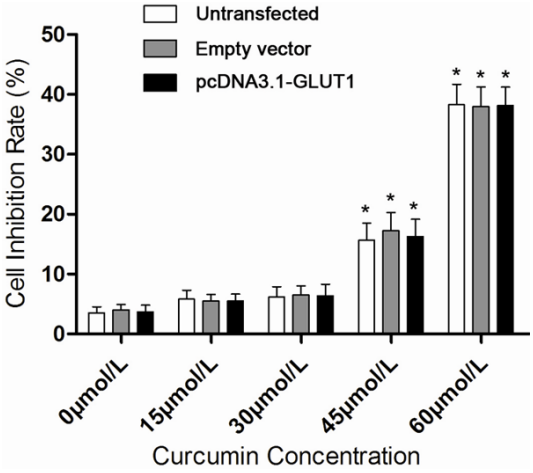


**Figure 2.** Curcumin's proliferation inhibition effect on A549 cells. Columns in this figure indicated the cell inhibition rate of untransfected, empty vector transfected and pcDNA3.1-GLUT1 transfected A549 cells when incubated with curcumin at serial concentrations (0, 15, 30, 45 and 60 μmol/L). Values are presented as (mean ± SD). *differences are significant from lower concentrations.

overexpression of membrane type 1-MMP (MT1-MMP) which is accepted as the extensive activator of MMP2.

Since the effects regular therapies including surgery, chemotherapy and radiotherapy are limited, anti-cancer activities of natural products such as curcumin, matrine and ginsenoside, etc. have become novel therapeutic alternatives in cancer treatment [16-18]. Curcumin, also with the name of 1,7- bis (4-hydroxy-3methoxyphenol)- 1,6-heptadiene-3,5-dione, is a natural compound extracted from roots of *Curcuma logna*. Previously, curcumin was known for its multiple biological and pharmacological activities against proliferation, invasiveness and metastasis in various human cancers [19-21]. Several signaling pathways were proved involved in but the underlying mechanisms for anti-cancer effects are still not fully investigated.

In this context, we suggest a possible mechanism of curcumin's anti-invasiveness in this study. By investigating curcumin's effect on GLUT1 over-expressed lung cancer cells, curcumin was supposed to exert anti-invasion effect by modulating GLUT1/MT1-MMP/MMP2 pathway. We believe the results in this study would be helpful in understanding the mechanism of curcumin's anti-cancer effects, providing theoretical basis for the potential clinical application of curcumin-related drugs in the future.

## Materials and methods

### *Cell culture and curcumin treatment*

Human lung cancer cell line A549 used in this study was purchased from Cell Resource Center of Chinese Academy of Sciences. Cells were grown in culturing medium containing RPMI1640 (Hyclone) supplemented by 10% fetal bovine serum (FBS, Gibco), 100 μg/mL streptomycin (Sigma), 100 U/ml penicillin (Sigma) and 2 mmol/L glutamine (Sigma) in culturing dishes. Cells were maintained in an incubator with humidified environment containing 95% fresh air and 5% $CO_2$. Cells were incubated with curcumin (Sigma) at serial concentrations at 0, 15, 30 45 and 60 μmmol/L for 24 hours.

### *GLUT1 expression vector construction*

The process of construction of GLUT1 expression vector was in accordance with the protocol described previously [22]. Total RNA was extracted from A549 cells by using RNeasy Mini Kit (Invitrogen) according to the manufacturer's instructions. The GLUT1 first-strand complementary DNA (cDNA) was generated by using Maxima™ First Strand cDNA Synthesis Kit (Fermentas) and amplified with the primers designed by TaKaRa (**Table 1**). PCR products and pcDNA3.1 (+) vector (TaKaRa) were digested by HindIII (TaKaRa) and XbaI (TaKaRa). Specific primers (**Table 1**) were used to subclone the full length coding sequence of GLUT1 into pcDNA3.1 (+) vector to generate pcDNA3.1-GLUT1 (**Figure 1**). The plasmid was digested by NheI (TaKaRa) and HindIII to confirm the correct construction of pcDNA3.1-GLUT1 (**Figure 1**).

### *pcDNA3.1-GLUT1 transfection*

Cultured A549 cells were planted into 6-well plate ($1\times10^6$/well) 24 hours before transfection. After the original medium was replaced by serum-free medium, 4 μg empty pcDNA3.1 vectors or 4 μg pcDNA3.1-GLUT1 were transfected into A549 cells with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The medium was replaced by medium containing RPMI1640 supplemented by 10% FBS, 100 μg/mL streptomycin, 100 U/

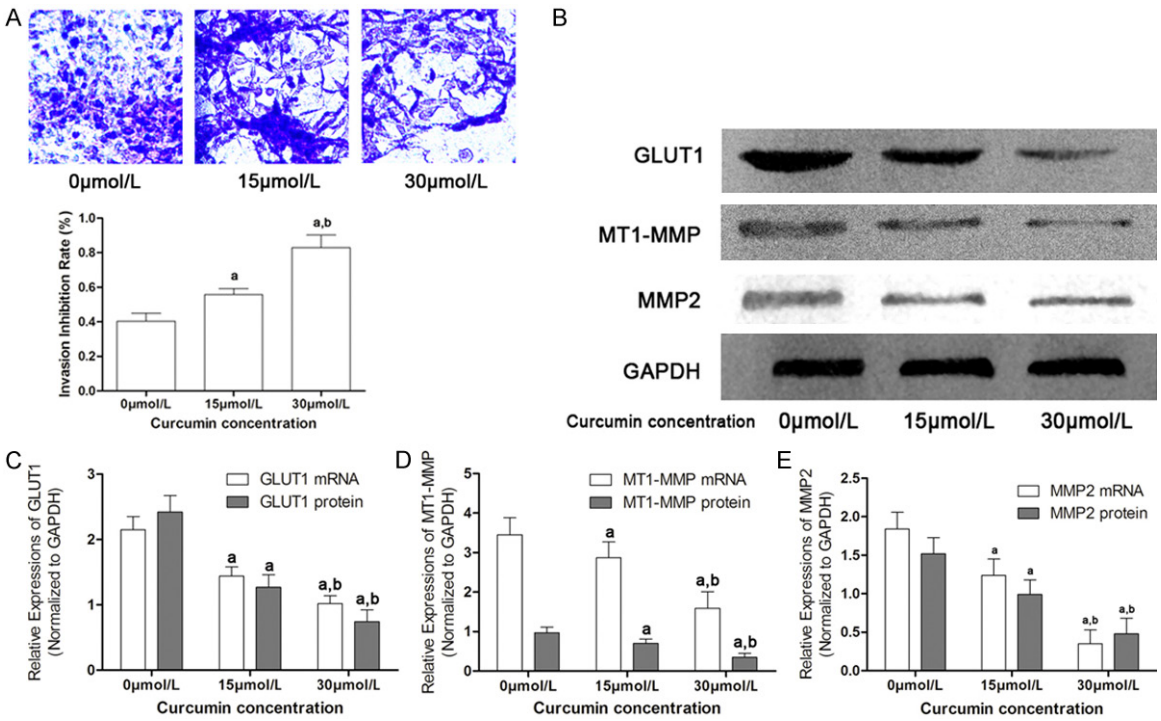


**Figure 3.** Effects of curcumin incubation on expressions of GLUT1, MT1-MMP and MMP2 and cell invasion in A549 cells. (A) The upper part demonstrates the results acquired from transwell assay when A549 cells were incubated with curcucmin of concentrations at 0, 15 and 30 μmol/L. Columns at lower part demonstrates the invasion inhibition rate when A549 cells were incubated with curcucmin of concentrations at 0, 15 and 30 μmol/L. (B) Immunoblots of GLUT1, MT1-MMP, MMP2 and GAPDH from A549 cells incubated with curcucmin of concentrations at 0, 15 and 30 μmol/L are showed. Columns on (C-E) indicate the relative mRNA and protein expression levels of GLUT1, MT1-MMP and MMP2 in A549 cells incubated with curcucmin of concentrations at 0, 15 and 30 μmol/L. Values are presented as (mean ± SD). A differences are significantly from 0 μmol/L; B differences are significantly from 15 μmol/L.

mL penicillin and 2 mmol/L glutamine and the cells were amplified in cell culture dishes to reach confluence over 80%.

*Cell viability assessment*

Cell viability was assessed by colorimetric 3-(4,5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) assay. After A549 cells were planted on a 96-well plate, MTT (Sigma) at concentration of 5 mg/ml was used to incubate the cells for 4 hours at 37°C. Then 150 μL dimethylsulfoxide (DMSO, Sigma) was added to the wells after cells were washed by PBS. A plate reader (Bio-Rad) was used to detect the absorbance at 540 nm (A540). Cell viability was expressed as inhibition rate which was calculated as [1-A540 (experimental well)/$A_{540}$ (control well)] ×100%.

*Cell invasion assay*

The invasive ability of A549 cells was evaluated in a membrane transwell system in accordance with previous studies [23]. Transwell membrane chamber (Corning) coated by Matrigel (BD) was used in this study. A549 cells ($2\times10^4$/well) were seeded to the upper wells of transwell chambers. Same medium with 10% FBS was contained in the lower wells of the chamber. After incubated humidified environment with 5% $CO_2$ atmosphere at 37°C for 48 hours, the cells passed through Matrigel were fixed by methanol. Then the crystal violet staining applied to indicate the invasion under a light microscope (Motic).

*Quantitative real-time PCR*

A549 cells were collected by centrifugation and total RNA was extracted by using RNeasy Mini Kit (Invitrogen) according to the manufacturer's instructions. SuperScript III Reverse Transcriptase (Invitrogen) was used to perform the reverse transcription and synthesize cDNA. All-in-one™ qPCR kit (GeneCopoeia) was used to perform quantitative real-time PCR according to protocols provided by the manufacturer. The

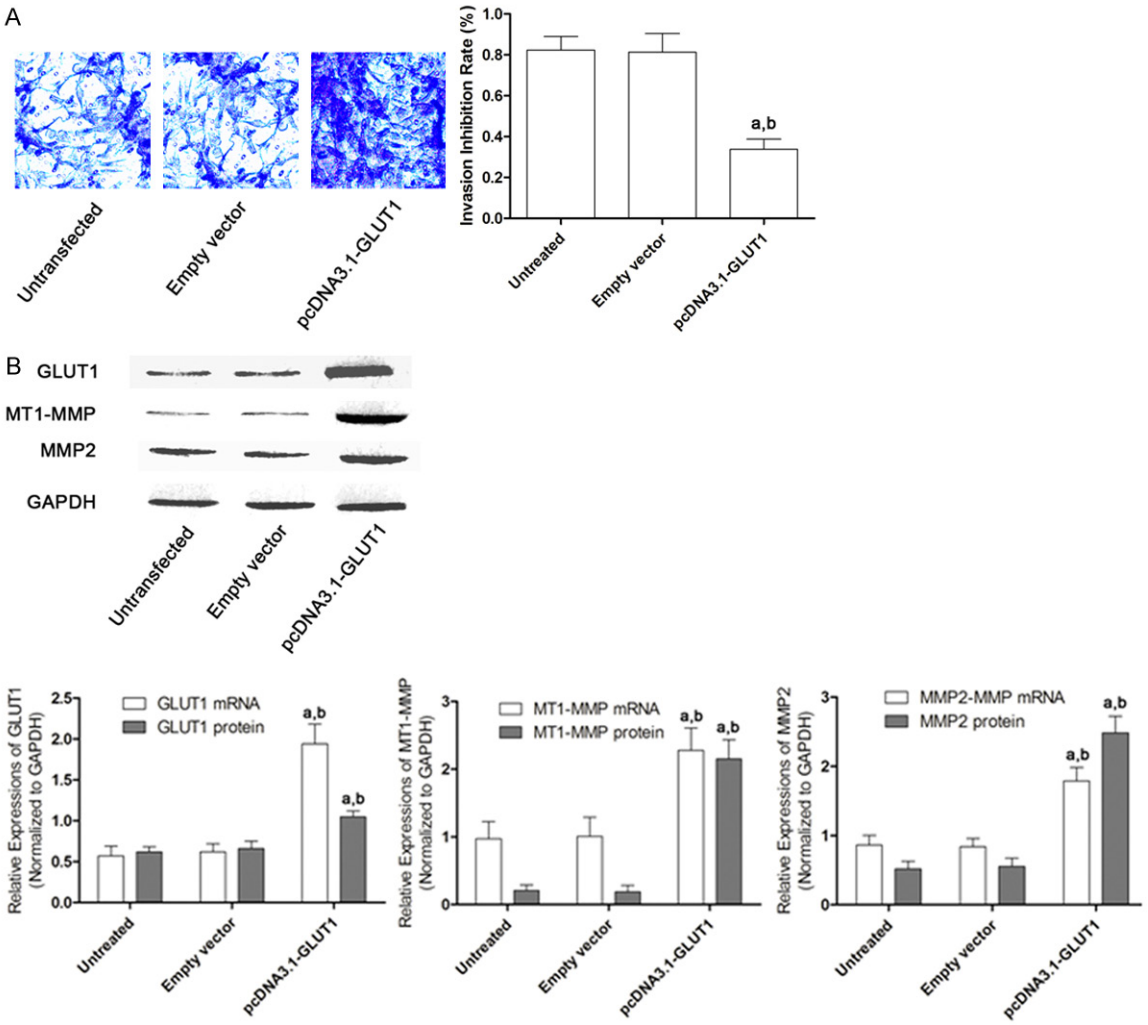


**Figure 4.** Effects of pcDNA3.1-GLUT1 transfection on expressions of GLUT1, MT1-MMP and MMP2 and cell invasion in A549 cells. A. The left part showed the captured image of transwell assay of untrasfected, empty vector transfected and pcDNA3.1-GLUT1 transfected A549 cells incubated with curcumin at 30 μmol/L. Columns at right part indicated the invasion inhibition rate of untrasfected, empty vector transfected and pcDNA3.1-GLUT1 transfected A549 cells incubated with curcumin at 30 μmol/L. B. The left part showed the immunoblots of GLUT1, MT1-MMP, MMP-2 and GAPDH in untrasfected, empty vector transfected and pcDNA3.1-GLUT1 transfected A549 cells incubated with curcumin at 30 μmol/L. Columns on the right part indicate the relative mRNA and protein expression levels of GLUT1, MT1-MMP and MMP2 in untrasfected, empty vector transfected and pcDNA3.1-GLUT1 transfected A549 cells incubated with curcumin at 30 μmol/L. Values are presented as (mean ± SD). A differences are significantly from untransfected; B differences are significantly from empty vector.

primers for GLUT1, MT1-MMP, MMP2 and GAPDH were shown in **Table 1**. GAPDH was introduced as the internal reference.

*Western blotting*

A549 cells were lysed by RIPA lysis buffer (Beyotime) on ice and total protein was extracted by protein extraction kit (Beyotime) according to the manufacturer's instructions. A BCA kit (Thermo) was used to detect the concentration of extracted protein. 50 μg proteins were electrophoresed vertically through sodium dodecylsulfate- polyacrylamide gels and the separated proteins were transferred electronically to poly vinylidene difluoride (PVDF) membranes. Then the non-specific interactions were blocked by 5% defatted milk- TBST solution incubation at 37°C for 1 hour. After washing, specific antibodies against GLUT1 (Abcam), MT1-MMP (Daiichi Fine Chemical), MMP2 (Abcam) were applied to incubate the membranes at 4°C for 12 hours to detect corresponding proteins. After washing, membranes

**Table 2.** Tumor weight and metastasis in in vivo animal experiments

| Tumor origin | Tumor weight (g) | Metastasis | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Metastatic organs | | | | | No. of animals | |
| | | Lung | Kidney | Brain | Gum | Liver | Total | Metastatic rate % |
| Untreated A549 cells | 6.35 ± 1.20 | 8 | 6 | 5 | 10 | 6 | 20 | 50.00 |
| A549 cells transfected with empty vector | 6.77 ± 1.57 | 7 | 9 | 4 | 9 | 5 | 17 | 52.94 |
| A549 cells transfected with pcDNA3.1-GLUT1 | 6.58 ± 1.39 | 16 | 11 | 10 | 9 | 11 | 19 | 84.21[a,b] |

[a]Differences are significant when compared with "Untreated A549 cells"; [b]differences are significant when compared with "A549 cells trasfected with empty vector".

were then incubated with horseradish peroxidase- conjugated second antibodies (Santa Cruz) at 37°C for 1 hour. Immunoblots were then detected by ECL reagents (Invitrogen). Software ImageJ2x (NIH) was used to analyze relative protein expressions while GAPDH was introduced as the internal reference.

*In vivo animal study*

5-6 weeks old balb/c nude mice (Slac Laboratory Animal) were maintained under sterile conditions. Cell suspension of $2\times10^6$ untreated, empty pcDNA3.1 or pcDNA3.1-GLUT1 A549 cells were injected into left dorsa region of mice subcutaneously. The length and width of tumor were measured by calipers. Volume of tumor was calculated by formula: length×width$^2$/2. 2 weeks after injection, when the volume of tumor reached approximate 60 mm$^3$, mice were treated by curcumin (200 μg/Kg bodyweight) by inraperitoneal injection once per day for continuous 4 weeks. Mice were then sacrificed and organs including lung, kidney, brain and liver. The organ samples were fixed in formalin, serially sectioned and stained by hematoxylin and eosin fro microscopic observations. Organ sections were counted positive when metastasis was observed.

*Statistics*

The statistic analysis carried out in this study was performed by software SPSS (ver.16.0). Values in this study was presented as a (mean ± SD) manner. The significance of differences were analyzed by mixed model ANOVA or Student's t tests followed by Bonferroni post hoc tests. P <0.05 was considered to be significant.

## Results

*Transfection identification*

Real-time PCR and Western blotting assays were used to detect the transcriptional and translational products of GLUT1 in A549 cells to judge whether the vectors were transfected into A549 cells. As shown in **Figure 2**, compared with untransfected A549 cells and A459 cells transfected with empty pcDNA3.1 vector, both of GLUT1 mRNA and protein expression levels were dramatically elevated. This result indicated that the pcDNA3.1-GLUT1 was successfully transfected into A549 cells and increased GLUT1 gene expression.

*Curcumin inhibited proliferation of untransfected, empty pcDNA3.1 vector transfected and pcDNA3.1-GLUT1 transfected A549 cells*

As demonstrated in **Figure 2**, effect of serially diluted curcumin (concentrations at 0, 15, 30, 45 and 60 μmmol/L) incubation on proliferation of A549 cells was evaluated by MTT assay. Curcumin began to show significant cytotoxicity to inhibit proliferation of untransfected, empty pcDNA3.1 vector transfected and pcDNA3.1-GLUT1 transfected A549 cells at concentration of 45 μmmol/L. Thus, in order to investigate the anti-invasive effect of curcumin, concentration below 45 μmmol/L were appropriate the subsequent experiments concerning curcumin's inhibitory against invasion of A549 cells.

*Curcumin administration decreased invasion and expression of GLUT1, MT1-MMP and MMP2 in A549 cells in a concentration- dependent manner*

The transwell assay was used to determine the invasive ability of A549 cells as demonstrated

in **Figure 3**. The invasion rate was decreased by curcumin incubation in a concentration-dependent manner. Curcumin of concentrations at 0, 15 and 30 μmmol/L were selected to incubate A549 cells. Expression levels of GLUT1, MT1-MMP and MMP2 at both transcriptional and translational levels were reduced by curcumin incubation in a concentration-dependent manner (**Figure 3**).

*GLUT1 overexpression impaired curcumin's anti-invasive effect and up-regulated expression levels of MT1-MMP and MMP2 in curcumin- treated A549 cells*

**Figure 4** demonstrated that curcumin's anti-invasive effect was affected by GLUT1 overexpression in A549 cells by pcDNA3.1-GLUT1 transfection. Untransfected, empty pcDNA3.1 vector transfected and pcDNA3.1-GLUT1 transfected A549 cells were incubated with curcumin at concentration of 30 μmmol/L. The invasive rate of A549 cells was significantly higher in pcDNA3.1-GLUT transfected A549 cells compared with both untransfected and empty pcDNA3.1 transfected cells. As shown in **Figure 4**, compared with both untransfected and empty pcDNA3.1 transfected cells, correspondingly, expressions of MT1-MMP and MMP2 were significantly lower in pcDNA3.1-GLUT1 transfected A549 cells.

*Effects of curcumin on metastasis of untransfected, empty pcDNA3.1 vector transfected and pcDNA3.1-GLUT1 transfected A549 cells original primary tumor in vivo*

As demonstrated in **Table 2**, after 4-week curcumin intraperitoneal administration, there were no significant differences of tumor weight among untransfected, empty pcDNA3.1 vector transfected and pcDNA3.1-GLUT1 transfected A549 cell originated tumor. However, also demonstrated in **Table 2**, the metastatic rate of pcDNA3.1-GLUT1 transfected A549 originated tumor was dramatically lower than untransfected and empty pcDNA3.1 vector transfected A459 originated tumors *in vivo*.

## Discussion

In accordance with previous studies, results in this present study reconfirmed the inhibitory effects on proliferation and invasion of lung cancer A549 cells. Furthermore, we investigated the role of GLUT1/MT1-MMP/MMP2 in curcumin's anti-invasion effect in lung cancer. After the GLUT1 DNA was cloned from cDNA extracted from A549 cells, it was inserted into pcDNA3.1 vector to generate pcDNA3.1-GLUT1. The GLUT1 expression was up-regulated in pcDNA3.1-GLUT1 transfected A549 cells. Notably, curcumin's anti-invasion effect was impaired in GLUT1 over-expressed A549 cells, in which MT1-MMP and MMP2 expressions were also up-regulated simultaneously. In order to further support our speculation, the *in vivo* animal experiment was implemented. Untreated, empty pcDNA3.1 vector transfected and pc-DNA3.1-GLUT1 vector transfected A549 cells were inoculated before nude mice were administrated with curcumin. Results turned out that metastasis ability of pc-DNA3.1-GLUT1 vector transfected A549 originated tumor was less inhibited by curcumin. To the best of our knowledge, it is the first study suggesting curcumin could inhibit invasion of lung cancer by suppressing GLUT1/MT1-MMP/MMP2 signaling.

Because of the uncontrolled cell cycle, the excessive proliferation could be found in tumor cells, which is a high-energy demanding biological behavior. The inadequate supply of oxygen and glucose from blood would induce ischemia and hypoxia in tumor, resulting in elevated expression of several specific genes including GLUT1 [24]. The up-regulated GLUT1 would facilitate transportation of glucose into cancer cells to increase glucose utilization by glycolysis to provide energy, an effect called Warburg effect [25]. GLUT1 has been shown to be highly correlated with tumor malignancy [26]. These studies also suggest GLUT1 expression was a negative biomarker of the prognosis for lymph node and distal organ metastasis in several human cancers [27].

It was believed that GLUT1 played a role in promoting invasion and metastasis by affecting the expression of MT1-MMP/MMP2 [22]. It is now generally accepted that MMP2 is a biomarker for tumor invasion and metastasis because of its enzymatic activity in degrading type IV collagen which is the main component of base membrane [28]. Malignant cells would easily invade into lymph and blood vessels through damaged base membrane. As the upstream molecule of MMP2, MT1-MMP is considered as the inducer of MMP-2 by activating pro-MMP2 [29]. A recent study suggested that

GLUT1 was up-regulated in Hep-2 cells, further up-regulating the expression of MT1-MMP and MMP2 which are the hallmarks of cancer invasion and metastasis [22]. In the *in vitro* experiment, GLUT1 was over-expressed by transfecting pcDNA3.1-GLUT1 vector into A549 cells. We found that the expressions of MT1-MMP and MMP2 were elevated in GLUT1 over-expressed cells. As a result, the invasion ability of A549 cells was also enhanced, indicating that GLUT1/MT1-MMP/MMP2 plays an important role in invasive and metastatic potential of lung cancer cells.

From ancient times, turmeric (*C. longa*) has a long history being used as coloring agent, food spice and traditional medicine in Eastern and Southeastern Asia. Modern studies considered the natural polyphenol, curcumin, was one of the main effective components extracted from turmeric. It is believed that curcumin has various activities such as anti-inflammatory, anti-fibrosis, anti-oxidant and anti-cancer properties [30-32]. Previous studies suggested several mechanisms involved in curcumin's anti-cancer effects, and recently curcumin's inhibitory effects on invasiveness and metastasis was suggested in several human cancers including prostate cancer, breast cancer, colorectal cancer and lung cancer [33-36], however the exact mechanism are still unclear. In the *in vitro* study, we found that curcumin's effective concentration of proliferation inhibition were similar in pcDNA3.1-GLUT1 transfected or untransfected A549 cells. In the *in vivo* study, after curcumin administration, differences of reduction of graft tumor weight and volume between GLUT1 over-expressed or un-over-expressed tumor were found not significant, suggesting GULT1 was not associated with curcumin's anti-proliferation effect. However, GLUT1 over-expressed A549 cells exhibited more resistance to curcumin's inhibitory effects against invasion *in vitro* and metastasis *in vivo*.

Based on the above result, we conclude that curcumin inhibits invasion and metastasis of lung cancer by modulating GLUT1/MT1-MMP/MMP2 pathway. However, as a preliminary study, there are still limitations in this study. We will further investigate this mechanism in more lung cancer cell lines. Moreover, the regulatory effect of curcumin on GLUT1 should be investigated. As expression of GLUT1 was depressed by curcumin, study on the association between curcumin, GLUT1 and tumor hypoxia, ischemia and energy metabolism would be of potential value.

## Disclosure of conflict of interest

None.

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