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Quercetin Inhibits the Migration and Invasion of HCCLM3 Cells by Suppressing the Expression of p-Akt1, Matrix Metalloproteinase (MMP) MMP-2, and MMP-9

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Background:

Quercetin is a natural bioactive flavonoid that is present in a wide variety of vegetables and fruits and exhibits a promising anti-metastasis property in various human cancer cells. However, the effect of quercetin on human HCCLM3 cells is unclear.

Material/Methods:

In the current study, a wound-healing assay was performed using quercetin-treated HCCLM3 cells to further explore whether quercetin affects the motility of human HCCLM3 cells. Transwell assay was used to explore the potential effect of quercetin in HCCLM3 cells on cell migration and cell invasion. Western blotting analysis was used to explore the expression of p-Akt1, MMP-2, and MMP-9 in quercetin-treated HCCLM3 cells.

Results:

The wound-healing time was delayed in quercetin-treated HCCLM3 cells, and the ability to migrate and invade was inhibited in quercetin-treated human HCCLM3 cells. Moreover, the protein levels of p-Akt1, MMP-2, and MMP-9 were down-regulated in quercetin-treated HCCLM3 cells, as detected by Western blotting.

Conclusions:


Our data show that quercetin attenuated cell migration and invasion by suppressing the protein levels of p-Akt1, MMP-2, and MMP-9 in HCCLM3 cells.

MeSH Keywords:

Carcinoma, Hepatocellular • Cell Migration Assays • Neoplasm Invasiveness • Quercetin

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Background

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer in the world and the second leading cause of cancer-related death [1]. Due to liver dysfunction, the lack of effective treatment options, and high rates of metastasis and recurrence, the survival rate of HCC patients remains low [2]. Therefore, potential molecular mechanisms involved in the progress of metastasis in HCC need to be identified.

MMP-2/MMP-9 are members of the matrix metalloproteinase (MMP) family, and both proteins are implicated in tumor migration, invasion, and metastasis in various cancers, like cervical cancer [3], prostate cancer [4], and gastric cancer [5,6]. Moreover, metastasis is inhibited when MMP-2/MMP-9 are down-regulated in the SK HEP 1 and MHcc97H HCC cell lines [7]. Conversely, induction of MMP-2/MMP-9 facilitates metastasis of HCC cells [8]. The PI3K/Akt signaling transduction pathway was reported to participate in the tumor metastasis of HCC [9–11]. In addition, the PI3K/Akt signaling transduction pathway also plays a critical role in MMP2/MMP-9-mediated tumor cell migration and invasion in various cancers [12–15].

Quercetin is a natural flavonoid from the flavonol subclass that is present in a wide variety of vegetables (e.g., onions), fruits (e.g., apples), and red wine [16]. It functions as a pleiotropic molecule with anti-proliferation, anti-oxidant, anti-inflammatory, and anti-cancer properties [17]. In addition, quercetin was recently shown to have inhibitory effects on cell migration and cell invasion in breast cancer cells [18,19], oral cancer cells [20], glioblastoma cells [21], prostate cancer cells [22], and melanoma cells [23]. Recent evidence demonstrated that quercetin can inhibit cancer cell migration and invasion by reducing the protein level of MMP-2/MMP-9 [20]. Moreover, quercetin also has been reported to regulate the ability to migrate and invade in various cancers by inhibiting Akt phosphorylation through the PI3K/Akt signaling transduction pathway [24,25].

Despite the evidence showing the anti-metastatic potential of quercetin in various tumors, the effect of quercetin on human HCCLM3 cells remains unclear. Therefore, the current study investigated the effects of quercetin on the migration and invasion of human HCCLM3 cells and explored its potential underlying mechanisms.

Material and Methods

Reagents and antibodies

Quercetin was procured from Sigma (St. Louis, MO). Akt1 antibodies (1: 1000 dilution), p-Akt1 (1: 1000 dilution), MMP-2 (1: 500 dilution), and MMP-9 (1: 1000 dilution) were all obtained

from Cell Signaling Technology, Beverly, MA. We obtained β -actin (1: 1000 dilution) from Santa Cruz (CA).

Cell culture and treatment

The HCCLM3 cells (human hepatocarcinoma cell line) were received from the China Center for Type Culture Collection (CCTCC, Wuhan, China). The HCCLM3 cells were cultured in RPMI-1640 medium (Invitrogen) with 10% fetal bovine serum (FBS). Cells were incubated at 37°C in an incubator with 5% CO₂. Cells were passaged when they reached 70–80% confluence.

Wound-healing assay

HCCLM3 cells were cultured in 6-well plates at 5×10^5 , and the cells were scratched using a 200- μ L pipette tip when they grew to 90% confluence. Then, the cells were washed twice using PBS, and the RPMI-1640 medium was completely changed.

Then, we added the different concentrations of quercetin into the wounded cells and incubated them for 36 h. Photos of the wound were taken at 0 and 36 h, and 7 randomly fields were used to assess cell migration ability by using microscopy.

Transwell migration assay

The anti-migration effect of quercetin in HCCLM3 cells was detected by using Transwell assays. In the upper chamber, 4×10^4 cells were cultured in 600 μ L of RPMI-1640 medium with different concentrations of quercetin. In the lower chamber, 800 μ L of RPMI-1640 medium with 10% FBS was added. After incubation for 36 h at 37°C, the upper chamber was cleaned by using a cotton swab to clean out the cells that were not migrated. The migrated cells were routinely processed by fixing in 4% formalin for 10 min, then stained in 0.1% crystal violet for 15 min, and 5 randomly selected fields were captured using microscopy at 100 \times magnification.

Matrigel invasion assay

The upper chamber was coated with an 80- μ L mixture of Matrigel/RPMI1640 medium (1: 5), and 4×10^4 cells were cultured in 600 μ L of RPMI-1640 medium with different concentrations of quercetin. In the lower chamber, 800 μ L of RPMI-1640 medium with 10% FBS was added. After incubation for 36 h at 37°C, the upper chamber was cleaned by using a cotton swab to clean out the cells that were not migrated. The migrated cells were routinely processed by fixing in 4% formalin for 10 min, then staining in 0.1% crystal violet for 15 min, and 5 randomly selected fields were captured by using microscopy at 100 \times magnification.

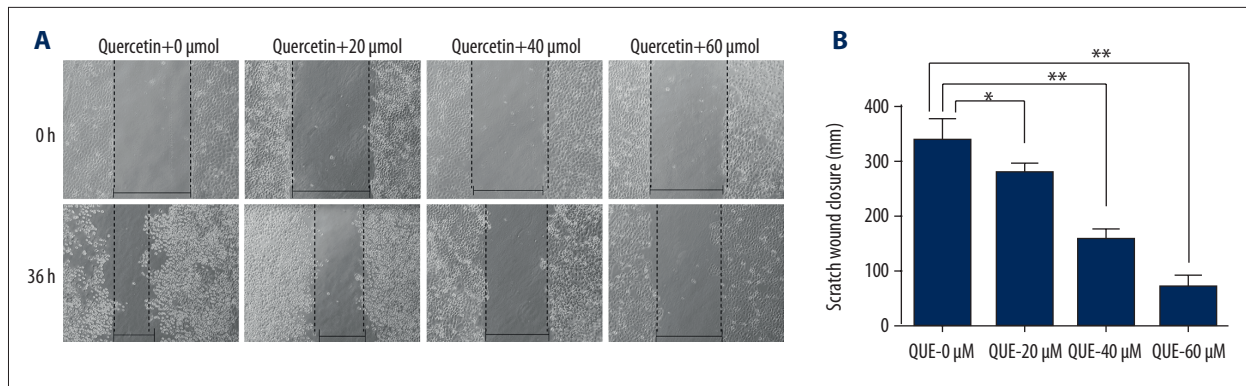


Figure 1. Quercetin delayed the wound-healing time in human HCCLM3 cells. (A) The inhibitory effects of quercetin on HCCLM3 cells were determined using a wound closure assay. The width of the scratch was measured in the control and quercetin groups, and the quantitative data are shown (B). The values are presented as means \pm S.D. All experiments were performed in triplicate (* $p < 0.05$, ** $p < 0.01$).

Western blotting

The HCCLM3 cells were treated with different concentrations of quercetin (0 μ mol and 40 μ mol). Then, the cell lysate was collected and the protein lysates were quantified. The quantified protein lysates were electrophoresed in 10% SDS-PAGE (SDS-polyacrylamide gel) and then was transferred to the PVDF (polyvinylidene difluoride) membranes. After transferring, the PVDF membranes were incubated with the primary antibodies at 4°C overnight. Then, after washing twice with TBST buffer, the PVDF membranes were incubated with secondary antibody for 1 h. Finally, the proteins were visualization with enhanced chemiluminescence method by using the Western blot image-forming system (Tanon 5200, China). All of the experiments were repeated 3 times.

Statistical analysis

Statistical differences from the respective controls for each experimental test condition were compared by ANOVA (one-way analysis of variance) or the *t* test. The $p < 0.01$ or $p < 0.05$ represented a significant difference. Values are expressed as the mean \pm S.D.

Results

Quercetin delayed the wound-healing time in human HCCLM3 cells

A wound-healing assay was performed using quercetin-treated HCCLM3 cells to further explore whether quercetin affects the motility of human HCCLM3 cells. As shown in Figure 1, quercetin-treated HCCLM3 cells (20 μ mol, 40 μ mol, and 60 μ mol) migrated more slowly than cells in the control group (0 μ mol) at 36 h. Thus, quercetin delayed the wound-healing time in

human HCCLM3 cells and inhibited HCCLM3 cell motility in a dose-dependent manner.

Quercetin inhibited the cell migration and invasion in human HCCLM3

Furthermore, a Transwell assay was used to explore the effect of quercetin on the migration and invasion of HCCLM3 cells. As shown in Figure 2A and 2B, the number of migrated cells decreased from 219.73 ± 14.01 (0 μ mol) to 173.32 ± 14.84 (20 μ mol), 100.31 ± 25.03 (40 μ mol), and 44.33 ± 5.03 (60 μ mol) after 36 h of quercetin treatment. Thus, quercetin inhibited the migration of human HCCLM3 cells *in vitro*. Moreover, the quercetin treatment also suppressed the ability of HCCLM3 cells to invade through a Matrigel basement membrane. As shown in Figure 2C and 2D, the number of invaded cells decreased from 123.75 ± 24.11 (0 μ mol) to 85.33 ± 5.86 (20 μ mol), 45.01 ± 6.03 (40 μ mol), and 20.65 ± 8.51 (60 μ mol) after 36 h of quercetin treatment. Thus, quercetin suppressed the invasion of human HCCLM3 cells *in vitro*. Based on these results, quercetin attenuated the migration and invasion of HCCLM3 cells *in vitro*.

Quercetin down-regulated the protein levels of p-Akt1, MMP-2, and MMP-9 in human HCCLM3

According to previous reports, the PI3K/Akt1 signaling pathway is positively correlated with tumor metastasis [26,27], and quercetin was also reported to inhibit p-Akt1 expression in various cancers [12,28]. Accordingly, Western blotting was used to explore whether the PI3K/Akt1 signaling transduction pathway was necessary for the anti-migration and anti-invasion effects of quercetin on HCCLM3 cells. As shown in Figure 3A and 3B, the administration of 40 μ mol quercetin significantly reduced p-Akt1 levels compared with the 0 μ mol group ($p < 0.05$).

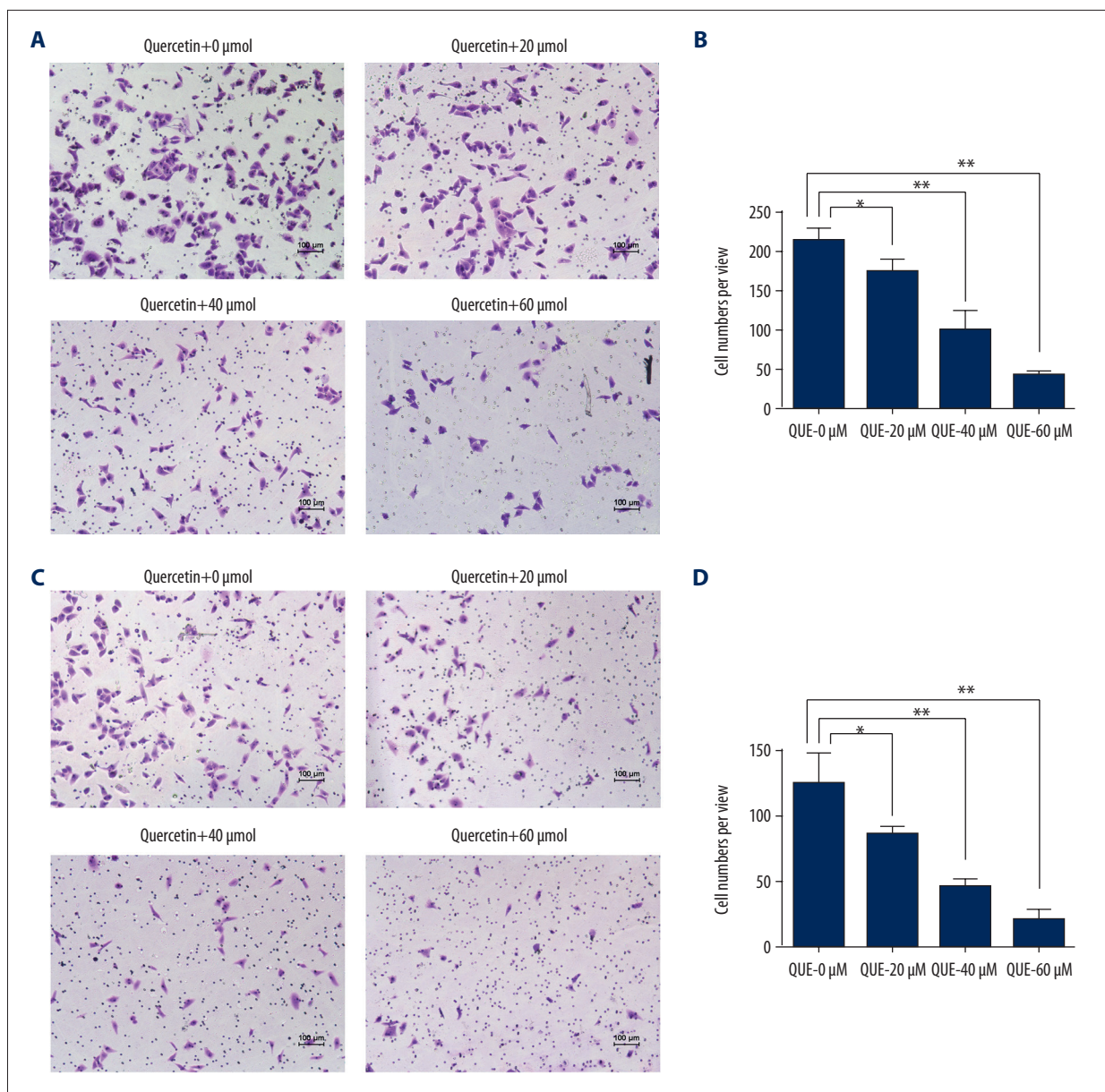


Figure 2. Quercetin inhibited the migration and invasion of human HCCLM3 cells. **(A)** The inhibitory effects of quercetin on the migration of HCCLM3 cells were determined using a Transwell assay, and the quantitative data are shown **(B)**. **(C)** The inhibitory effects of quercetin on the invasion of HCCLM3 cells were determined using a Transwell assay, and the quantitative data are shown **(D)**. The values are presented as means \pm S.D. All experiments were performed in triplicate (* $p < 0.05$, ** $p < 0.01$).

MMP-2/MMP-9 are regulated by the PI3K/Akt1 signaling transduction pathway [29,30], and both MMPs have been implicated in the invasion of hepatocellular carcinoma [31,32]. Therefore, the protein level of MMP-2/MMP-9 was examined in control cells and quercetin-treated cells. After 36-h quercetin treatment, the quercetin-treated cells (40 μ mol) expressed a much lower level of the MMP-2/MMP-9 proteins than did control cells (0 μ mol). Based on these results, quercetin inhibited the protein levels of p-Akt1, MMP-2, and MMP-9 in the HCCLM3 cell line.

Discussion

Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related death. Although the therapeutic approaches have improved, the 5-year survival rate of HCC patients after surgical resection remains low due to the high rates of metastasis and recurrence [2]. Quercetin is a natural bioactive flavonoid, widely present in vegetables and fruits [16], and has exhibited promising anti-metastasis properties in various

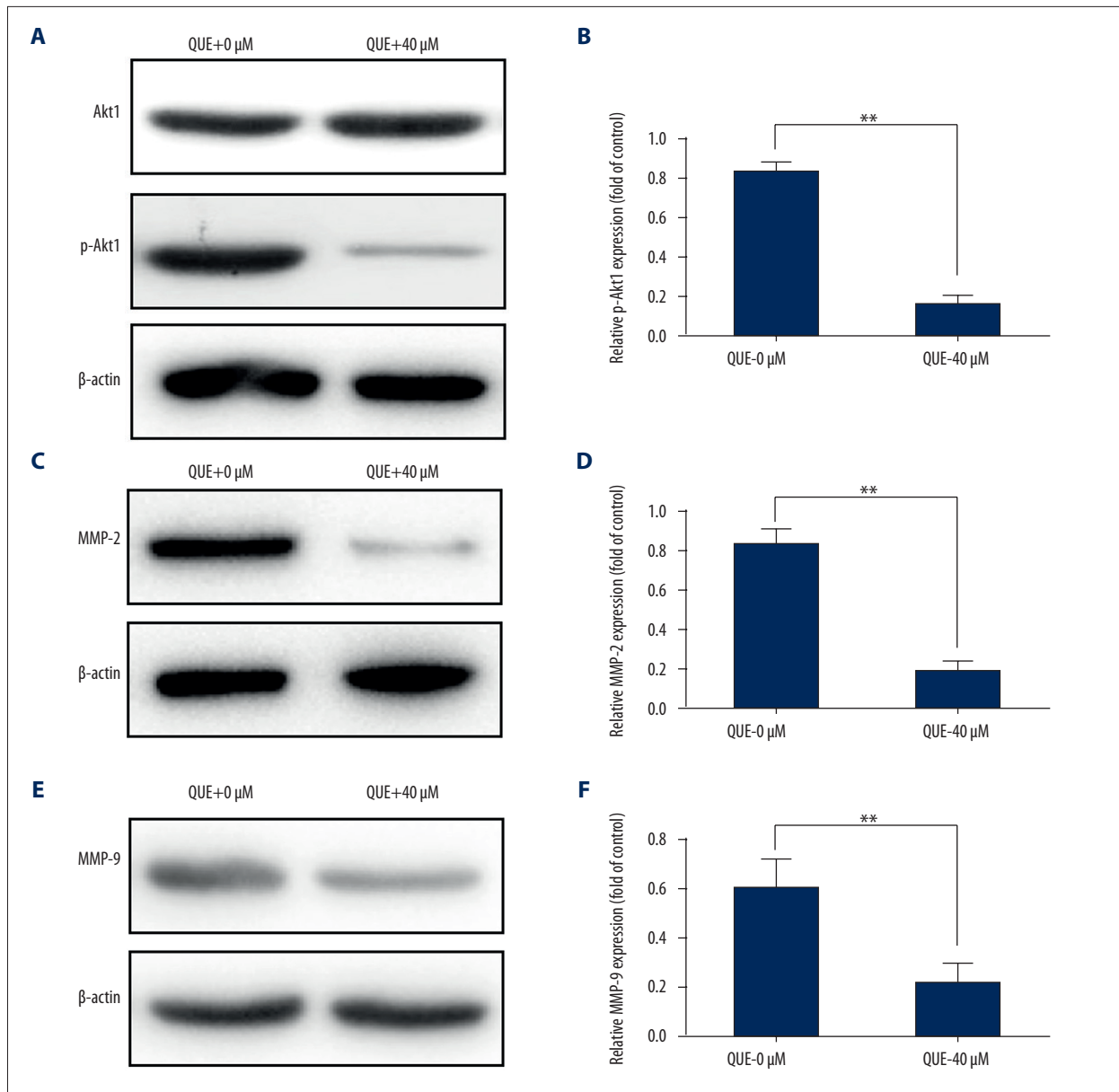


Figure 3. Quercetin markedly decreased p-Akt1, MMP-2, and MMP-9 expression in human HCCLM3 cells. (A) Akt1 and p-Akt1 levels were detected by Western blotting, and the quantitative data are shown (B). (C) MMP-2 expression was detected by Western blotting, and the quantitative data are shown (D). (E) MMP-9 expression was detected by Western blotting, and the quantitative data are shown (F). The values are presented as means \pm S.D. All experiments were performed in triplicate (* $p < 0.05$, ** $p < 0.01$).

human cancer cells, both *in vitro* or/and *in vivo*, including breast cancer [18,19], oral cancer cells [20], glioblastoma [21], prostate cancer [22] and melanoma [23]. Evidences from human head and neck squamous cell carcinoma shows that quercetin dose-dependently delayed wound-healing time in both HSC-3 and FaDu cells [33]. However, the effect of quercetin on cell migration and invasion has been less clearly defined. In the present study, quercetin delayed the wound-healing time in HCCLM3 cells (Figure 1, $p < 0.05$), indicating the inhibitory effects

of quercetin on HCCLM3 cell motility. Moreover, the results from the Transwell assay also suggest that quercetin inhibits cell migration and invasion in HCCLM3 cells (Figure 2, $p < 0.05$).

A previous study indicated that quercetin had remarkable effects in suppressing cell migration in renal cell carcinoma cells by altering AKT-mTOR-ERK1/2 signaling pathways [28]. However, whether the expression of p-Akt1 was altering by treating with quercetin in HCCLM3 cell has been unclear. Accordingly, we assessed the

expression of p-Akt1 by Western blotting in HCCLM3 cell after treating with quercetin for 36 h. Similar to the effect of quercetin in renal cell carcinoma cells, the expression of p-Akt1 was significantly decreased in HCCLM3 cell (Figure 3A, $p < 0.05$), suggesting that quercetin inhibits p-Akt1 expression in HCCLM3 cells.

Moreover, MMP-2 and MMP-9 have been found to be involved in tumor migration, invasion, and metastasis in various cancers, and a previous study indicated that these inhibitory effects of quercetin on cell migration are due to suppressing the expression of MMP2 or/and MMP9 through altering the PI3K/Akt signaling pathway. Previous studies indicated that quercetin significantly inhibited cell migration and invasion in breast cancer cells [19] and glioma cells [24] by down-regulating the expression of p-Akt and MMP9. In the present study, as shown in Figure 3B and 3C, the expression of MMP2 and MMP9 was significantly decreased in quercetin-treated HCCLM3 cells ($p < 0.05$). These results suggest that these inhibitory effects of quercetin on cell migration and invasion in HCCLM3 cell are due to its inhibitory effects on MMP2 and MMP9 through altering the PI3K/Akt signaling pathway.

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Conclusions

Our study provides evidence that quercetin inhibits cell migration and invasion in HCCLM3 cells *in vitro* by down-regulating the expression of MMP-2 and MMP-9 through the PI3K/Akt signaling pathway. Therefore, quercetin may be a novel agent for treatment of hepatocellular carcinoma.

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