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Quercetin suppresses the migration of hepatocellular carcinoma cells stimulated by hepatocyte growth factor or transforming growth factor-α: Attenuation of AKT signaling pathway

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ABSTRACT

Flavonol, which is found abundantly in plants such as fruits and vegetables, belongs to the family of flavonoid, natural polyphenols. Quercetin, one of the flavonol, reportedly has anti-cancer effects and prevents the proliferation of various cancer cells, including hepatocellular carcinoma (HCC). However, the effects of quercetin on HCC cells migration have not yet been clarified. We have previously shown that the migration of human HCC-derived HuH7 cells induced by hepatocyte growth factor (HGF) or transforming growth factor-α (TGF-α) is mediated through p38 MAPK and AKT. In this study, we investigated whether quercetin affects the HGF- or TGF-α-induced migration of HuH7 cells. Quercetin significantly suppressed both HGF- and TGF-α-induced migration of HuH7 cells in a dose-dependent manner. In addition, myricetin, another flavonol, also showed significant inhibition of the cell migration. Each HGF- and TGF-α-induced autophosphorylation of receptors were not affected by quercetin or myricetin. Quercetin did not suppress HGF- or TGF-α-induced p38 MAPK phosphorylation. On the contrary, quercetin and myricetin inhibited the growth factors-induced phosphorylation of AKT. Our results strongly suggest that quercetin suppresses the growth factor-induced migration of HCC cells by inhibiting the signaling pathway of AKT but not p38 MAPK.

1. Introduction

Flavonoids, natural polyphenolic substances, are widely distributed in dietary plants, such as onions, apples, berries, apricots, and broccoli [1–5]. Flavonoids chemically consist of two benzene rings linked by a heterocyclic oxygen ring, and more than 4000 types of flavonoids are found in nature [2,4,6]. Flavonoids are classified into various families according to the degree of oxidation of the oxygen heterocycle, such as flavones, flavonols, flavanones, flavanonols, isoflavones, and flavanols [2,4,6]. Among flavonoids, flavonols exhibit multiple biological functions, including anti-inflammatory, anti-microbial, anti-thrombotic, and antioxidant effects [3,4,7,8]. The epidemiological studies revealed that high intake of flavonols correlates with the decreasing of the death rate from chronic diseases, such as cardiovascular disease [8,9]. Quercetin is the major flavonol found in our dietary food, especially in onions [1]. It has been expected that the people with high quercetin intake show a reduced risk of type 2 diabetes, lower mortality from ischemic heart disease, and a lower incidence of bronchial asthma [10]. Some human intervention studies conducted by supplementary intake of quercetin demonstrated the reduction of the risk of cardiovascular diseases and the lowering of the blood pressure of hypertension patients [9]. Quercetin also reportedly shows anti-carcinogenic activity against cancer cells that have been derived from various tissues, including the liver [6]. Myricetin that is the structural similar to quercetin, is the second most abundant flavonol in dietary plants [3]. However, the exact mechanism of quercetin and myricetin remains still unclear.

Liver cancer is the second leading cause of cancer-related deaths, and 90% of primary liver cancer cases are hepatocellular carcinoma (HCC) [11]. HCC is known to be developed by chronic hepatic inflammation and tissue damages caused by hepatitis virus B or C infections, alcohol intake, or metabolic syndromes due to diabetes and obesity [11–13]. Although treatments for HCC, such as surgery and liver transplantation, are improved in recent years, recurrence of HCC and intrahepatic or extrahepatic metastases of tumor cells are highly occurred in HCC patients, and the survival rate of the patients is still poor [11,13–15]. Circulating HCC cell is a cause of the recurrence and metastases of HCC [16,17]. However, the details of how HCC cells migrate have not been fully investigated.

Hepatocyte growth factor (HGF) is secreted by stellate cells and known to act as a potent mitogen of hepatocyte [18,19]. Accumulating

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evidence indicates that aberrant activation of HGF and its unique receptor tyrosine kinase c-mesenchymal-epithelial transition factor receptor (c-MET) signaling pathway is involved in HCC cell invasion and metastasis [18–20]. Activation of the cellular signaling pathways by transforming growth factor-α (TGF-α) and its receptor, epidermal growth factor receptor (EGFR), is also known to be involved in metastatic recurrence of HCC [19,21–23]. Activation of mitogen-activated protein kinases (MAPKs) and AKT signaling pathways induced by HGF/c-MET and TGF-α/EGFR systems are involved in the migration and invasion of HCC cells [18,19,21–24]. We have previously demonstrated that HGF [25,26] and TGF-α [25,27,28] induce the migration of the human HCC-derived HuH7 cells, and p38 MAPK and AKT signaling pathways are involved in those growth factors-induced cell migration.

Although anti-proliferative activation of quercetin [6] and myricetin [29] on HCC cells has been demonstrated, the study of the flavonol for effect on HCC migration is still poor. Therefore, this study aimed to clarify the influence of the flavonol, especially quercetin, on the growth factor-induced migration of HCC cells. Herein, we demonstrated that quercetin and myricetin suppressed HGF- and TGF-α-induced migration of the HuH7 cells by inhibition of the AKT signaling pathway.

2. Materials and methods

2.1. Antibodies and chemicals

Recombinant human HGF and recombinant human TGF-α were obtained from R&D Systems Inc. (Minneapolis, MN, USA). Quercetin and myricetin were purchased from EMD Millipore Co. (Darmstadt, Germany). Phospho-specific c-MET (Y1234/1235) antibodies (#3126), phospho-specific EGFR (Y1068) antibodies (#2234), phosphoinositide 3-kinase (PI3K) p85 antibodies (#4292), phospho-specific AKT (T308) antibodies (#13038), AKT antibodies (#9272), phospho-specific p38 MAPK antibodies (#4511), p38 MAPK antibodies (#9212), and E-cadherin antibodies (#3195) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Phospho-specific PI3K p85 (Y607) antibodies (ab182651) were purchased from Abcam (Cambrige, UK). GAPDH antibodies (sc47724) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). Isokaempferide was obtained from Sigma-Aldrich Co Ltd. (St. Louis, MO). Other chemicals were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). All other materials were obtained from commercial sources. Quercetin and myricetin were dissolved in dimethyl sulfoxide (Sigma-Aldrich Co., St. Louis, MO). The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the cell migration assay or Western blot analyses.

2.2. Cell culture

Human HCC-derived HuH7 cells (JCRB0403) [30] were obtained from the JCRB Cell Bank (Tokyo, Japan). The cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich Co.) containing 10% fetal calf serum (FCS; Hyclone Laboratories Inc., Logan, UT) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. For cell migration assay, the cells were seeded into 100-mm diameter dishes (4×10^5 cells/dish) in RPMI1640 medium containing 10% FCS for 4 days and then used for experiments. For Western blot analyses, the cells were seeded into 100-mm diameter dishes (6×10^5 cells/dish) in RPMI1640 medium containing 10% FCS. After 3 days, the medium was exchanged for serum-free RPMI1640 medium. The cells were then used for the experiments after 24 h.

2.3. Cell migration assay

A transwell cell migration assay was performed using a Boyden chamber (polycarbonate membrane with 8-μm pores, Transwell; Costar; Corning, Inc., Corning, NY) as described previously [25–28]. In briefly, the cultured cells were seeded (1×10^5 cells/well) onto the upper chamber in serum-free RPMI1640 medium. The cells were pretreated with quercetin, myricetin or isokaempferide in the upper chamber for 60 min at 37 °C. HGF or TGF-α was then added to the lower chamber for 23 h at 37 °C. After incubation, the cells on the upper-side of the membrane were mechanically removed by cotton swabs. The migrated cells adherent to the underside of the membrane were fixed with 4% paraformaldehyde (Alfa Aesar, Thermo Fisher Scientific Co., Lancashire, UK) and stained with 4',6-diamidino-2-phenylindole (DAPI) solution for 30 min at room temperature. The migrated cells were then photographed and counted using fluorescent microscopy at a magnification of 20×.

2.4. Western blot analyses

To examine the effects of quercetin or myricetin on the receptor tyrosine kinases, p38 MAPK, and AKT signaling pathways stimulated by HGF- or TGF-α, the cultured cells were pretreated with the indicated doses of quercetin or myricetin for 60 min. After incubation, the cells were stimulated with 30 ng/ml HGF or vehicle for 3 min for c-MET, AKT and PI3K, and 5 min for p38 MAPK or stimulated with 30 ng/ml TGF-α or vehicle for 1 min for EGFR, AKT and PI3K, and 5 min for p38 MAPK. To examine the effects of isokaempferide on AKT, the cells were stimulated with the 30 ng/ml of HGF, 30 ng/ml TGF-α or vehicle for 3min after 60 min pretreatment of 30 μM isokaempferide. To examine the E-cadherin levels, the cultured cells were pretreated with 7 μM quercetin for 60 min, and then, stimulated with 30 ng/ml HGF, 10 ng/ml TGF-α or vehicle for 23 h. The cells in each dish were washed with ice-cold phosphate-buffered saline (PBS) and then lysed in the lysis buffer [62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol]. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [31]. Western blot analyses were performed as described previously [26–28] using phospho-specific c-MET (Y1234/1235) antibodies, phospho-specific EGFR (Y1068) antibodies, GAPDH antibodies, phospho-specific p38 MAPK antibodies, p38 MAPK antibodies, phospho-specific AKT (T308) antibodies, AKT antibodies, phospho-specific PI3K p85 (Y607) antibodies, PI3K p85 antibodies and E-cadherin antibodies as primary antibodies. Peroxidase-labeled anti-rabbit IgG antibodies (Seracare Life Sciences, Milford, MA) were used for antibodies other than GAPDH as secondary antibodies. Peroxidase-labeled anti-mouse IgG antibodies (Cell Signaling Technology, Inc.) were used for GAPDH antibodies as secondary antibodies. The peroxidase activity on a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA) was visualized on X-ray film using the ECL Western blotting detection system (GE Healthcare Life Sciences Ltd., Little Chalfont, UK). A densitometric analysis was performed using an image analysis software program (image J version 1.48; National Institutes of Health, Bethesda, MD). The background-subtracted signal intensity of each phosphorylation signal was normalized to GAPDH or the respective total protein signals and then plotted.

2.5. Statistical analyses

The data are expressed as the means ± standard deviation (SD) of triplicate determinations from three independent cell preparations. The statistical significance of the data from the cell culture experiments was analyzed by two-way analysis of variance (ANOVA) followed by Tukey test for multiple comparisons between pairs. The values of $p < 0.05$ were considered to be statistically significant.

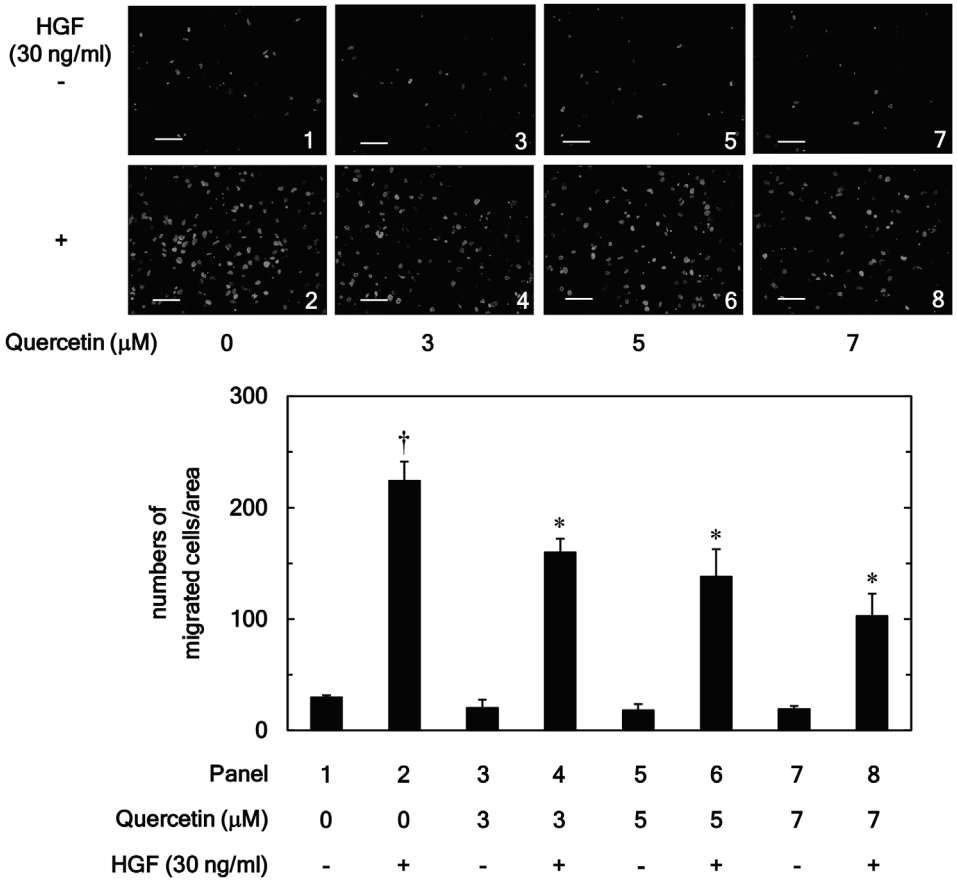

Fig. 1. Effect of quercetin on the HGF-induced HuH7 cell migration. The cells were pretreated with the indicated concentrations of quercetin for 1 h, and then stimulated with 30 ng/ml of HGF or vehicle for 23 h. The migrated cells were stained with DAPI for the nuclei. The cells were photographed by fluorescent microscopy at a magnification of 20× (upper panel) and counted (bar graph). Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.001$, compared to the value of the control cells without HGF and quercetin. *$p < 0.05$, compared to the value of the cells with HGF alone. Scale bar: 100 μm.

3. Results

3.1. Effects of quercetin or myricetin on the HGF- and TGF-α-induced migration of HuH7 cells

We have previously shown that both HGF and TGF-α stimulate the migration of human HCC-derived HuH7 cells assessed by a transwell cell migration assay [25–28]. In this study, we first examined whether quercetin, a flavonol [1–3], affects the migration of the HuH7 cells induced by these growth factors. As shown in Fig. 1, quercetin significantly suppressed the HGF (30 ng/ml)-induced migration of HuH7 cells. The effect of quercetin was dose-dependent in the range between 3 and 7 μM. Quercetin also reduced 10 ng/ml TGF-α-induced cell migration over the range of 5–7 μM (Fig. 2). To investigate whether the suppressive effect of quercetin on the HuH7 cell migration is the common effect of flavonols, we additionally examined the effect of myricetin, another flavonol [2,3], on the cell migration induced by HGF or TGF-α. Myricetin (7 μM), as well as quercetin, significantly inhibited the cell migration by HGF (Fig. 3A) and TGF-α (Fig. 3B). Furthermore, we examined the effects of quercetin on the HuH7 cell migration by HGF or TGF-α compared with the effect of an inactive flavonol, isokaempferide [32]. As shown in Fig. 4, isokaempferide failed to inhibit HGF- (Fig. 4A) or TGF-α- (Fig. 4B) induced cell migration, whereas we confirmed that the migration was inhibited by quercetin.

3.2. Effects of quercetin or myricetin on the HGF-stimulated phosphorylation of c-MET or TGF-α-stimulated phosphorylation of EGFR in HuH7 cells

HGF and TGF-α bind as ligands to the receptor tyrosine kinases, c-MET and EGFR, respectively [24,27,33]. These growth factors then induce autophosphorylation of own receptors and subsequently stimulate various intracellular signaling pathways [24,27,33]. Therefore, we next examined whether quercetin and myricetin affect the autophosphorylation of c-MET and EGFR. HGF-stimulated autophosphorylation of c-MET was not affected by quercetin (30 μM) (Fig. 5A) or myricetin (10 μM) (Fig. 5B). Furthermore, both quercetin (Fig. 5C) and myricetin (Fig. 5D) failed to reduce the TGF-α-stimulated phosphorylation of EGFR.

3.3. Effects of quercetin on the HGF- or TGF-α-stimulated phosphorylation of p38 MAPK in HuH7 cells

We have previously reported that both HGF- and TGF-α-induced migrations of HuH7 cells are mediated through the p38 MAPK signaling pathway [26,28]. Therefore, we examined the effect of quercetin on the activation of p38 MAPK by HGF and TGF-α in HuH7 cells. However, quercetin (30 μM) attenuated neither HGF (Fig. 6A)- nor TGF-α (Fig. 6B)-stimulated phosphorylation of p38 MAPK.

3.4. Effects of quercetin on the HGF- or TGF-α-stimulated phosphorylation of AKT in HuH7 cells

We demonstrated that the AKT signaling pathway, in addition to the p38 MAPK pathway, acts as a potent regulator in both HGF- and TGF-α-induced migrations of HuH7 cells [25,26]. Therefore, we investigated whether quercetin affects the activation of AKT induced by HGF and TGF-α. The HGF-stimulated phosphorylation of AKT was significantly suppressed by quercetin (30 μM) (Fig. 7). In addition, quercetin (30 μM) significantly downregulated the TGF-α-stimulated phosphorylation of AKT (Fig. 8). We examined the effect of myricetin on the phosphorylation of AKT by HGF and TGF-α. HGF- and TGF-α-stimulated phosphorylation of AKT were significantly attenuated by myricetin (10 μM) (Fig. 9). Furthermore, we found that isokaempferide did not suppress

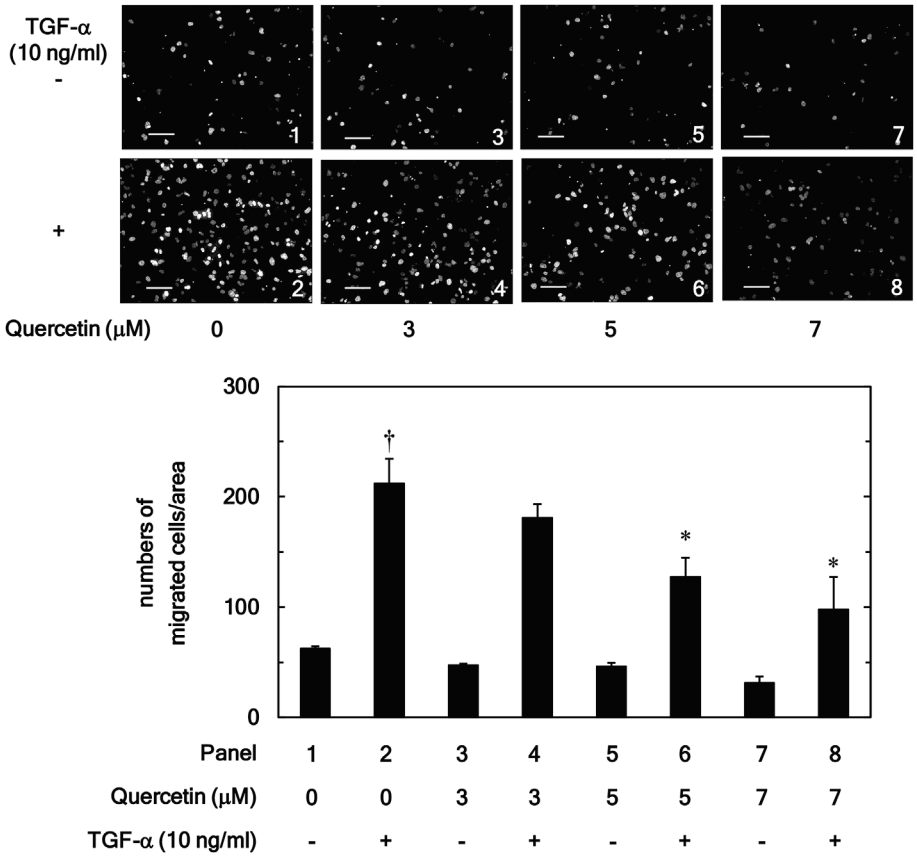

Fig. 2. Effect of quercetin on the TGF-α-induced HuH7 cell migration. The cells were pretreated with the indicated concentrations of quercetin for 1 h, and then stimulated with 10 ng/ml of TGF-α or vehicle for 23 h. The migrated cells were stained with DAPI for the nuclei. The cells were photographed by fluorescent microscopy at a magnification of 20× (upper panel) and counted (bar graph). Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.001$, compared to the value of the control cells without TGF-α and quercetin. *$p < 0.05$, compared to the value of the cells with TGF-α alone. Scale bar: 100 μm.

HGF- or TGF-α-stimulated phosphorylation of AKT (Fig. 10).

3.5. Effects of quercetin on the HGF- or TGF-α-stimulated phosphorylation of PI3K in HuH7 cells

We have previously shown that LY294002, a PI3K inhibitor [34], significantly suppresses TGF-α-stimulated AKT phosphorylation in HuH7 cells [25]. Additionally, we found that HGF-induced AKT phosphorylation was also inhibited by LY294002 in HuH7 cells (data not shown). Since PI3K is activated by the phosphorylation of p85 subunit (Y607) [35,36], we examined whether the suppressive effect of quercetin on HGF- and TGF-α-induced AKT activation relates to the inhibition of PI3K by Western blotting analyses using phospho-specific PI3K p85 (Y607) antibodies. As shown in Fig. 11, quercetin significantly attenuated the phosphorylation of PI3K.

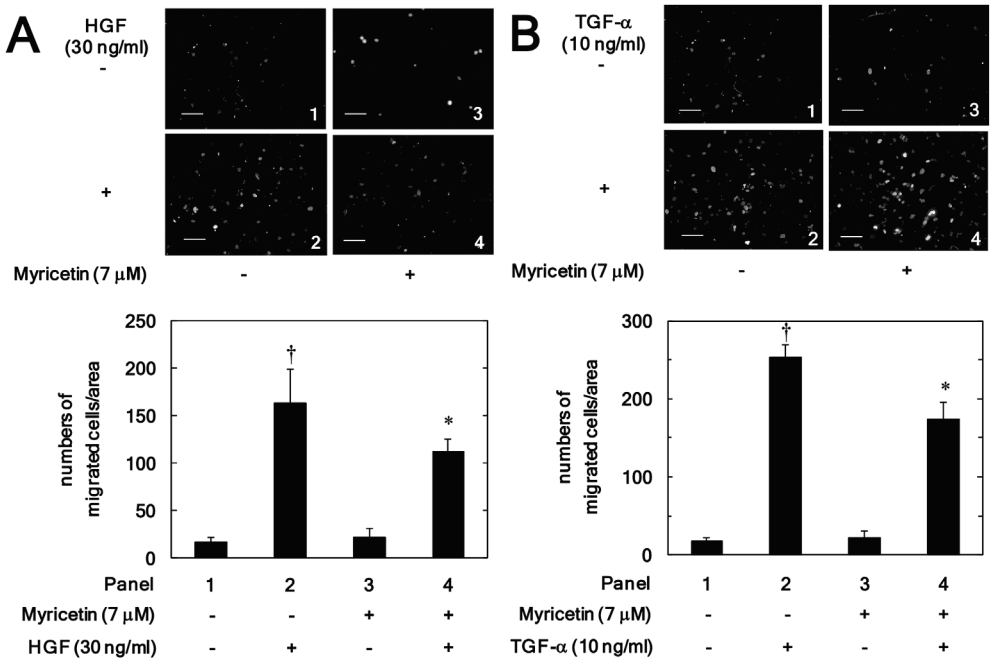

Fig. 3. Effects of myricetin on the HGF (A)- and the TGF-α (B)-induced HuH7 cell migration. (A) The cells were pretreated with 7 μM of myricetin or vehicle for 1 h, and then stimulated with 30 ng/ml of HGF or vehicle for 23 h. (B) The cells were pretreated with 7 μM of myricetin or vehicle for 1 h, and then stimulated with 10 ng/ml of TGF-α or vehicle for 23 h. The migrated cells were stained with DAPI for the nuclei. The cells were photographed by fluorescent microscopy at a magnification of 20× (upper panel) and counted (bar graph). Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.001$, compared to the value of the control cells without myricetin and HGF (A) or without myricetin and TGF-α (B). *$p < 0.05$, compared to the value of the cells with HGF alone (A) or TGF-α alone (B). Scale bar: 100 μm.

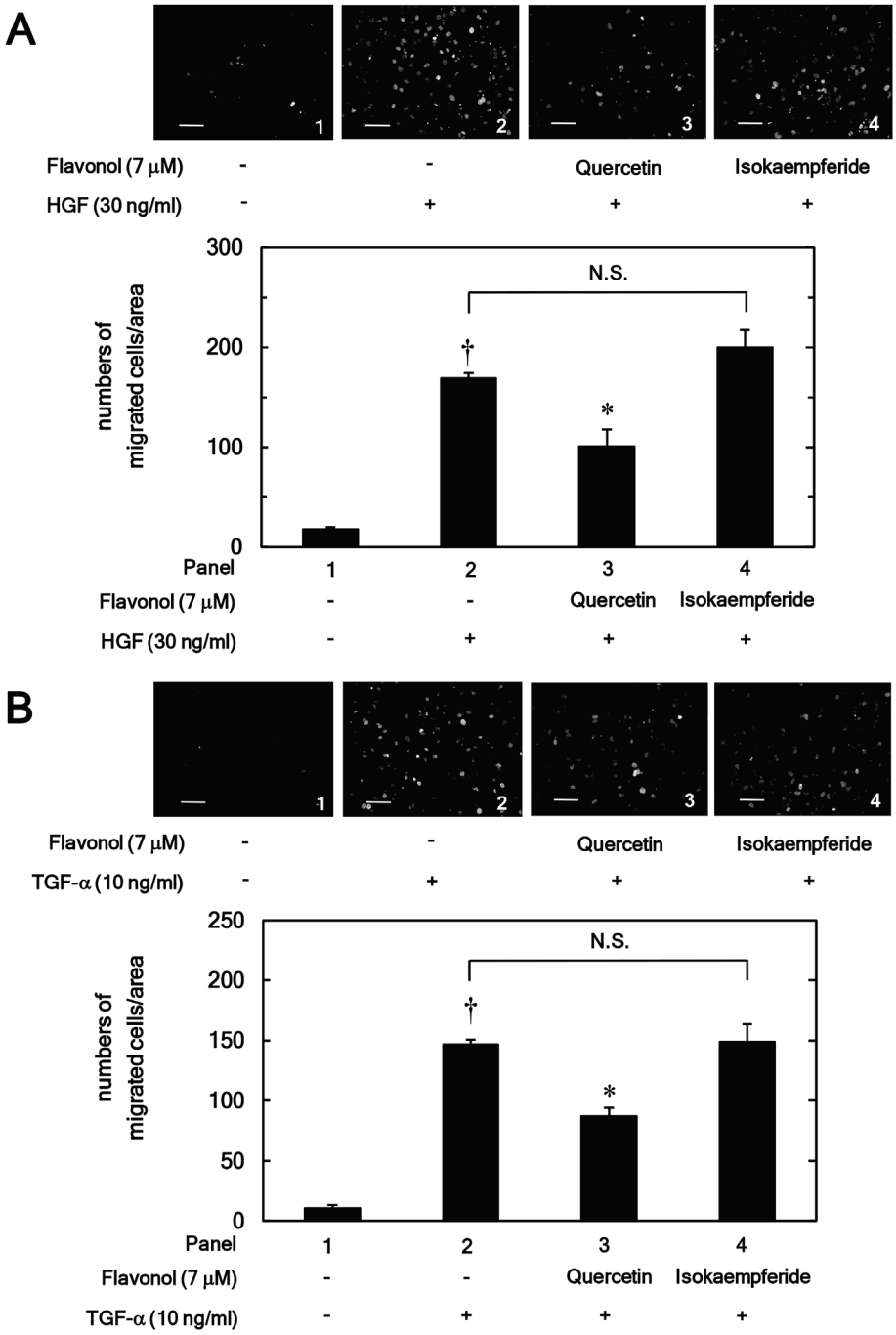

Fig. 4. Effects of isokaempferide on the HGF (A)- and the TGF-α (B)-induced HuH7 cell migration. (A) The cells were pretreated with 7 μM of isokaempferide, quercetin or vehicle for 1 h, and then stimulated with 30 ng/ml of HGF or vehicle for 23 h. (B) The cells were pretreated with 7 μM of isokaempferide, quercetin or vehicle for 1 h, and then stimulated with 10 ng/ml of TGF-α or vehicle for 23 h. The migrated cells were stained with DAPI for the nuclei. The cells were photographed by fluorescent microscopy at a magnification of 20× (upper panel) and counted (bar graph). Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.001$, compared to the value of the control cells without flavonols and HGF (A) or without flavonols and TGF-α (B). *$p < 0.05$, compared to the value of the cells with HGF alone (A) or TGF-α alone (B). Scale bar: 100 μm.

3.6. Effects of quercetin on E-cadherin expression in HGF- or TGF-α-stimulated HuH7 cells

It has been known that epithelial-mesenchymal transition (EMT) drives a more invasive phenotype of HCC through the PI3K/AKT signaling pathway, [37]. EMT is characterized as suppression of E-cadherin, an epithelial marker [37–39]. Therefore, we examined whether quercetin affects E-cadherin protein levels in HuH7cells. We confirmed that HGF and TGF-α down-regulated E-cadherin protein levels in HuH7 cells. In addition, we found that quercetin markedly increased the E-cadherin protein levels in HuH7 cells both in the absence and in the presence of HGF- (Fig. 12A) or TGF-α- (Fig. 12B) stimulation. Quercetin might play a potent role to regulate the E-cadherin expression in HCC cells.

4. Discussion

Growth factors and their receptor tyrosine kinases are closely implicated in HCC development, and recognized as potent molecular targets for HCC therapies [11]. HGF/c-MET plays a major role in HCC cell migration during invasion and metastasis of HCC [18–20,33]. In addition, TGF-α/EGFR is also involved in the metastasis of HCC [19,21–23]. It has been shown that the expression of TGF-α is increased in metastatic liver tumors [23] and the EGFR signaling is involved in the promotion of HCC cells to have the high metastatic potential [22]. Therefore, it is currently established that HGF/c-MET and TGF-α/EGFR play important roles in HCC cell metastasis and the recurrence. In the present study, we examined the effects of flavonols on HGF- and TGF-α-stimulated migration of human HCC cells. We showed that quercetin at 3 μM and 5 μM significantly suppressed both HGF- and TGF-α-induced

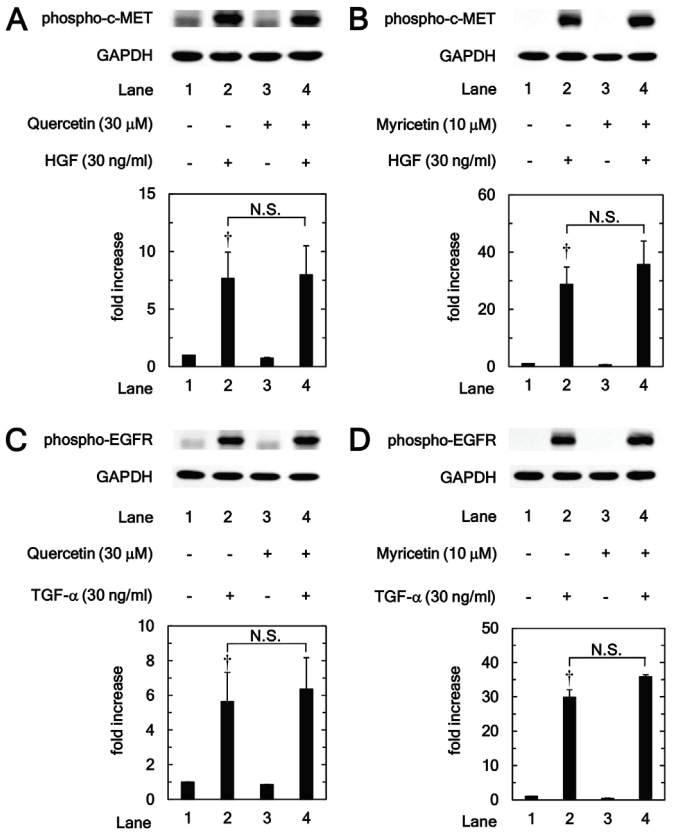

Fig. 5. Effects of quercetin (A) and myricetin (B) on the HGF-induced phosphorylation of c-MET, and effects of quercetin (C) and myricetin (D) on the TGF-α-induced phosphorylation of EGFR in HuH7 cells. The cells were pretreated with the indicated doses of quercetin (A, C) or myricetin (B, D) for 1 h, and then stimulated with 30 ng/ml of HGF or vehicle for 3 min (A, B) or 30 ng/ml of TGF-α or vehicle for 1 min (C, D). A Western blot analyses using antibodies against phospho-specific c-MET (A, B) or phospho-specific EGFR (C, D) and GAPDH were performed. The histogram represents the relative levels of phospho-c-MET or phospho-EGFR. The phospho-c-MET and the phospho-EGFR levels were corrected by the GAPDH levels, and then expressed as the fold increase compared with the basal levels presented in lanes 1. Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.05$, compared to the value of the control cells without quercetin and HGF (A), without myricetin and HGF (B), without quercetin and TGF-α (C) or without myricetin and TGF-α (D). N.S., indicates no significant difference between the indicated pairs.

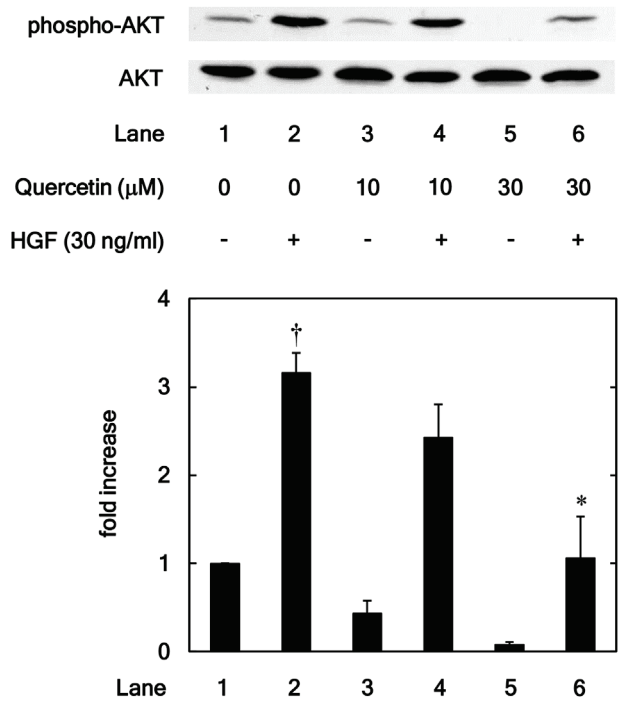

Fig. 7. Effect of quercetin on the HGF-induced phosphorylation of AKT in HuH7 cells. The cells were pretreated with the indicated doses of quercetin for 1 h, and then stimulated with 30 ng/ml of HGF or vehicle for 3 min. A Western blot analyses using antibodies against phospho-specific AKT and AKT were performed. The histogram represents the relative levels of AKT phosphorylation. The phosphorylation levels were corrected by the total protein levels, and then expressed as the fold increase compared with the basal level presented in lane 1. Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.05$, compared to the value of the control cells without HGF and quercetin. *$p < 0.05$, compared to the value of the cells with HGF alone.

migration of human HCC-derived HuH7 cells. In addition, myricetin at 7 μM, another flavonol, also reduced these growth factors-induced HuH7 cell migrations. Quercetin and myricetin reportedly induce apoptosis of HuH7 cells [29,40]. However, it has been shown that 48 h incubation with 5 μM quercetin fails to affect the cell viability of HuH7 cells [40] and that treatment with 100 μM myricetin for 24 h does not change the cell number of HuH7 cells [29]. Based on these findings, it seems unlikely that the suppressive effects of quercetin and myricetin

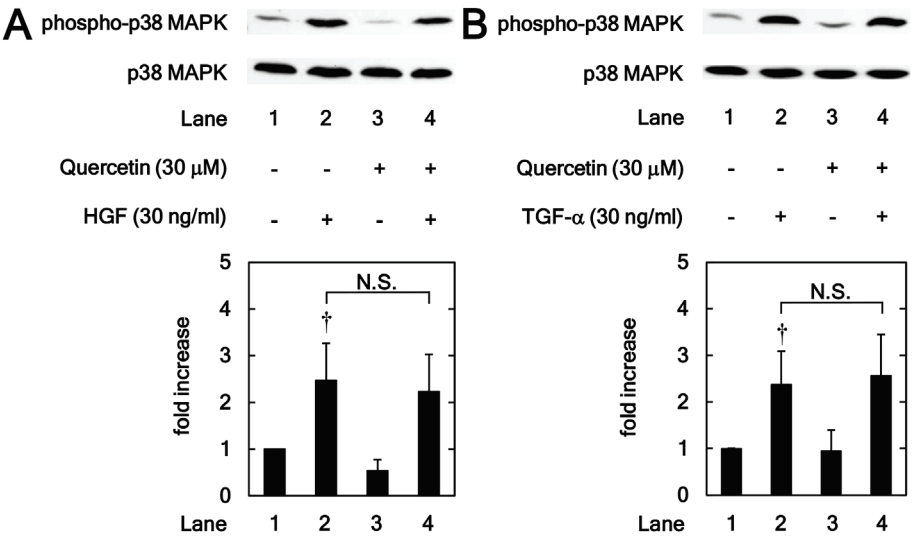

Fig. 6. Effects of quercetin on HGF (A)- and TGF-α (B)-induced phosphorylation of p38 MAPK in HuH7 cells. The cells were pretreated with the 30 μM of quercetin or vehicle for 1 h, and then stimulated by 30 ng/ml of HGF or vehicle (A) or 30 ng/ml of TGF-α or vehicle (B) for 5 min. A Western blot analyses using antibodies against phospho-specific p38 MAPK and p38 MAPK were performed. The histogram represents the relative levels of p38 MAPK phosphorylation. The phosphorylation levels were corrected by the total protein levels, and then expressed as the fold increase compared with the basal levels presented in lanes 1. Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.05$, compared to the value of the control cells without quercetin and HGF (A) or without quercetin and TGF-α (B). N.S., indicates no significant difference between the indicated pairs.

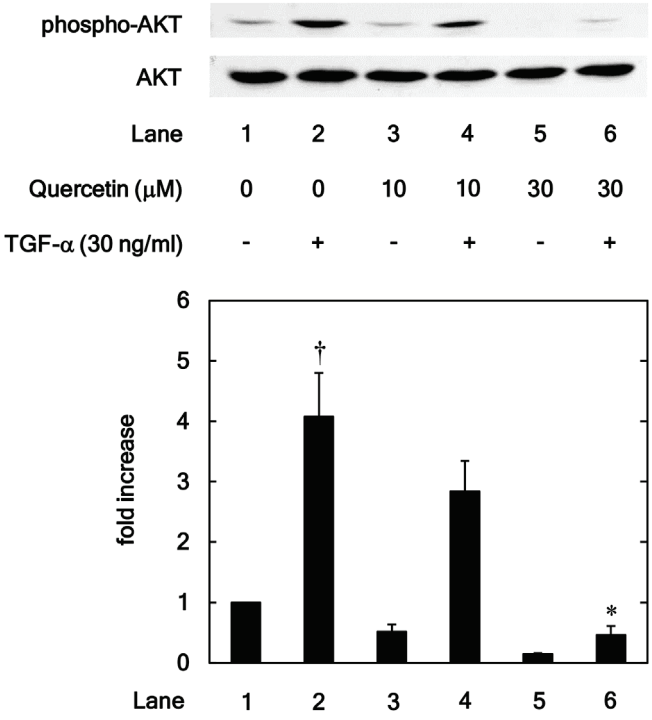

Fig. 8. Effect of quercetin on the TGF-α-induced phosphorylation of AKT in HuH7 cells. The cells were pretreated with the indicated doses of quercetin for 1 h, and then stimulated with 30 ng/ml of TGF-α or vehicle for 1 min. A Western blot analyses using antibodies against phospho-specific AKT and AKT were performed. The histogram represents the relative levels of AKT phosphorylation. The phosphorylation levels were corrected by the total protein levels, and then expressed as the fold increase compared with the basal level presented in lane 1. Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.05$, compared to the value of the control cells without TGF-α and quercetin. *$p < 0.05$, compared to the value of the cells with TGF-α alone.

on the migration are due to the apoptotic effects of these flavonols.

Antioxidant, anti-inflammatory and anti-apoptotic effects of quercetin against alcohol-induced liver damage are reportedly mediated through the PI3K/AKT/nuclear factor κB/signal transducers and activators of transcription (STAT)3 pathway [8]. In addition, it has been shown that quercetin suppresses the proliferation of SMMC7221 cells, a human-derived hepatoma cell line, by inhibiting the AKT signaling pathway [41]. While, quercetin reduces lipopolysaccharide-induced oxidation markers in the acute injured liver by inhibition of the MAPK signaling pathways, including p38 MAPK [8]. Intraperitoneally injection of quercetin also reportedly suppresses p38 MAPK activity in the fibrotic liver of rats affected with carbon tetrachloride [42]. We have previously reported that HGF- [26] and TGF-α- [25,28] induced-HuH7 cell migration is mediated by activation of PI3K/AKT and p38 MAPK. Therefore, we next investigated whether suppressive effects of quercetin and myricetin against HGF- and TGF-α-induced HuH7 cell migration are mediated by the AKT and/or p38 MAPK signaling pathways. Both quercetin and myricetin failed to affect the autophosphorylations of HGF- and TGF-α-stimulated their receptor tyrosine kinases, c-MET and EGFR. In addition, quercetin inhibited neither HGF- nor TGF-α-induced activation of p38 MAPK. On the contrary, quercetin and myricetin significantly reduced HGF- and TGF-α-induced activation of AKT in HuH7 cells. We have previously shown that LY294002 [34] significantly suppresses TGF-α-stimulated AKT phosphorylation in HuH7 cells [25]. Additionally, in the present study, we found that HGF-induced AKT phosphorylation was also inhibited by LY294002 in HuH7 cells (data not shown). Thus, our findings suggest that PI3K plays at a point upstream of AKT in the intracellular signaling of HGF or TGF-α in HuH7 cells. We showed that quercetin significantly attenuated the phosphorylation of PI3K. On the other hand, we found that the phosphorylation of p38 MAPK stimulated by HGF or TGF-α were not suppressed by LY294002 in HuH7 cells (data not sown). Therefore, it seems unlikely that PI3K plays at a point upstream of p38 MAPK in the signaling of HGF or TGF-α in HuH7 cells. Taking our findings into account, it is most likely that quercetin reduces the growth factor-stimulated migration of HCC cells by suppression of the PI3K/AKT signaling pathway but not the p38 MAPK signaling pathway. Regarding downstream of PI3K/AKT signaling pathway in migration, it is well known that EMT plays an important role in cell migration and invasiveness, leading the enhancement of metastasis of HCC cells [37,43–46]. On the other hand, it has been shown that the p38 MAPK signaling pathway promotes the secretion of matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, which is also closely related to HCC cell migration [47,48]. We have previously reported that c-*jun* N-terminal kinase (JNK) and Rho kinase, in addition to AKT and p38 MAPK, are involved in TGF-α-induced migration of HuH7 cells [28]. However, we have already found that quercetin (30 μM) did not show suppressive effects on TGF-α-induced phosphorylation of JNK or myosin phosphatase targeting subunit 1 (MYPT-1), a substrate of Rho kinase (data not shown). The potential mechanism of suppression of HGF- and TGF-α-induced HCC cell migration by the quercetin and myricetin shown here is summarized in Fig. 13.

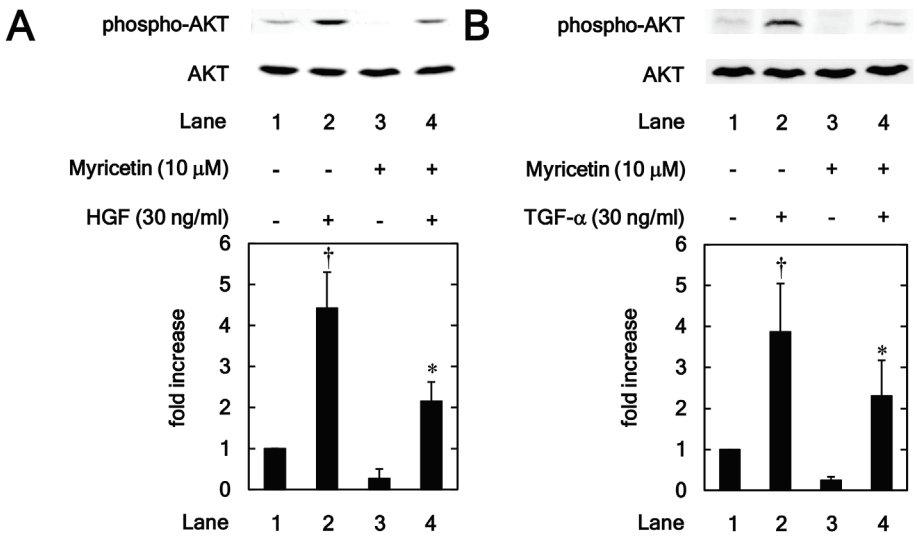

Fig. 9. Effects of myricetin on HGF (A)- and TGF-α (B)-induced phosphorylation of AKT in HuH7 cells. The cells were pretreated with the 10 μM of myricetin or vehicle for 1 h, and then stimulated by 30 ng/ml of HGF or vehicle for 3 min (A) or 30 ng/ml of TGF-α or vehicle for 1min (B). A Western blot analyses using antibodies against phospho-specific AKT and AKT were performed. The histogram represents the relative levels of AKT phosphorylation. The phosphorylation levels were corrected by the total protein levels, and then expressed as the fold increase compared with the basal levels presented in lanes 1. Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.05$, compared to the value of the control cells without myricetin and HGF (A) or without myricetin and TGF-α (B). *$p < 0.05$, compared to the value of the cells with HGF alone (A) or TGF-α alone (B).

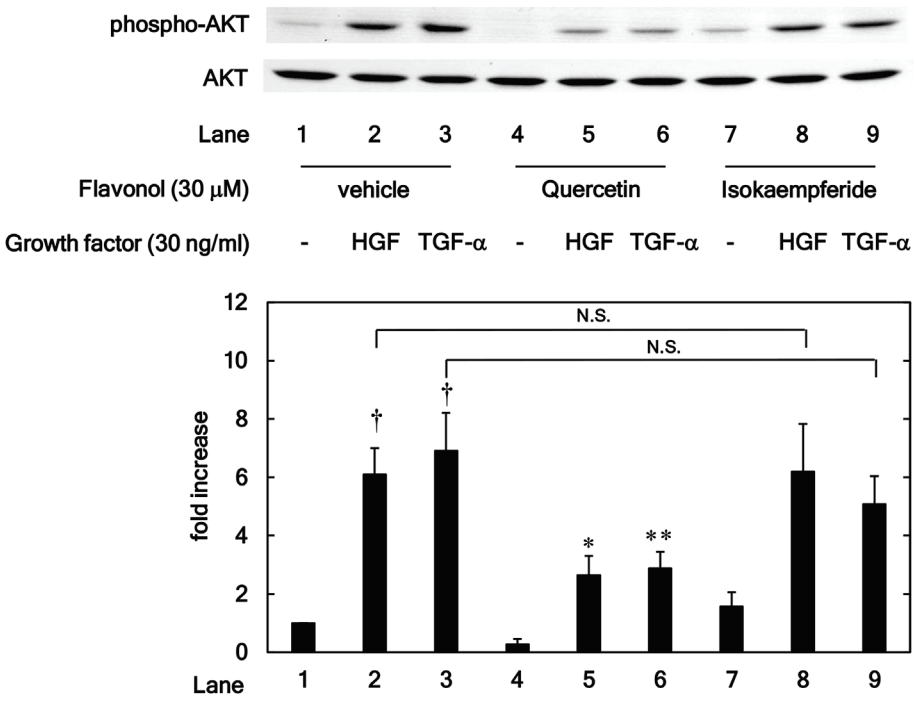

Fig. 10. Effects of isokaempferide on the HGF- and TGF-α induced phosphorylation of AKT in HuH7 cells. The cells were pretreated with 30 μM of isokaempferide, 30 μM of quercetin or vehicle for 1 h, and then stimulated with 30 ng/ml of HGF or 30 ng/ml of TGF-α or vehicle for 3 min. A Western blot analyses using antibodies against phospho-specific AKT and AKT were performed. The histogram represents the relative levels of AKT phosphorylation. The phosphorylation levels were corrected by the total protein levels, and then expressed as the fold increase compared with the basal level presented in lane 1. Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.05$, compared to the value of the control cells without flavonols and the growth factors (HGF and TGF-α). *$p < 0.05$, compared to the value of the cells with HGF alone. **$p < 0.05$, compared to the value of the cells with TGF-α alone. N.S., indicates no significant difference between the indicated pairs.

Flavonols are widely contained in dietary foods, especially vegetables and fruits [3], are absorbed from small intestine, and then distributed to liver [1,4]. It has been reported that the absorption of flavonol is relatively high, and the plasma concentration reached 1.34 μM after consumption of 139 mg onion-derived quercetin [1]. As for the suppressive activity of quercetin on the migration of HCC cells, Wu L. et al. recently reported that 80 μM quercetin inhibits spontaneous migration of human HCC-derived HCC-LM3 cells by reduction of janus kinase (JAK)2/STAT3 [49]. It seems likely that the suppressive effect of high concentration flavonols on HCC cell migration might be caused by apoptosis or their toxicity. On the other hand, we demonstrated that quercetin or myricetin, which alone had no effect on the spontaneous migration of HuH7 cells, probably not apoptotic or toxic, reduced the growth factor-induced migration. It is probable that the concentrations of quercetin and myricetin in the present study *in vitro* is achievable *in vivo*. Therefore, it seems likely that the intake of flavonol containing foods could prevent HCC metastasis.

A case-control study demonstrates that a decreasing tendency of HCC risk is dependent on flavonols intake [50]. AKT has been known as a predictor of HCC recurrence after surgical resection [18]. In this study, we found that quercetin and myricetin, flavonols, suppressed the HGF- and TGF-α-induced migration of HuH7 cells due to the attenuating of the AKT signaling pathway. There are many reports showing that quercetin possesses anticancer activities via AKT pathway including HCC [8,41]. In addition, it has been reported that quercetin suppresses the mobility of breast cancer through AKT pathway [51], and reverses EGF-induced EMT and invasiveness in prostate cancer by down-regulation of EGFR and its down-stream PI3K/AKT pathway [52]. However, to the best of our knowledge, our findings showing that quercetin, which alone fails to affect HCC cell migration, inhibits the growth factor-induced migration via PI3K/AKT signaling pathway may provide new insights. Although further investigations, using another HCC cell line as well as HuH7 cells, are required to clarify the exact mechanism behind the effects of flavonols against HCC cell migration, our findings indicate that flavonols are good candidates for preventive medicine against HCC metastasis and the recurrence.

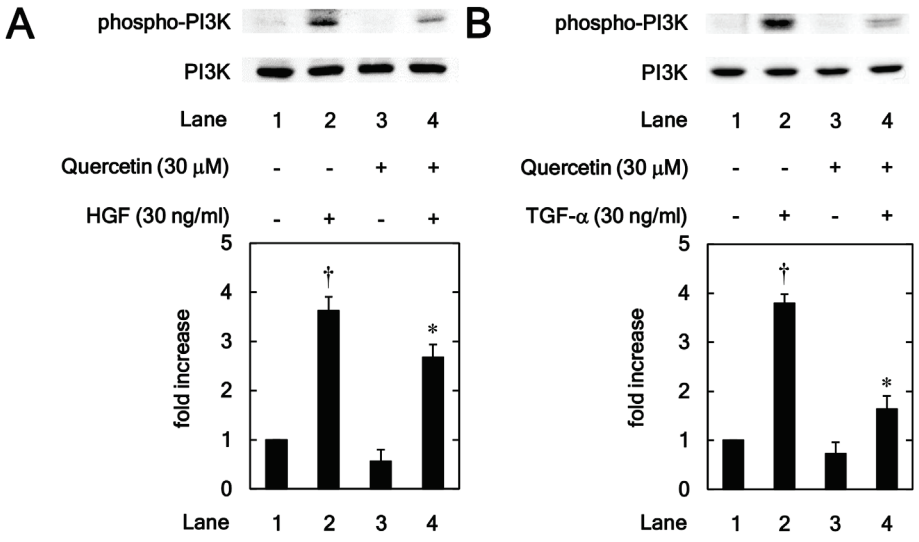

Fig. 11. Effects of quercetin on HGF (A)- and TGF-α (B)-induced phosphorylation of PI3K in HuH7 cells. The cells were pretreated with 30 μM of quercetin or vehicle for 1 h and then stimulated with 30 ng/ml of HGF or vehicle for 3 min (A) or 30 ng/ml of TGF-α or vehicle for 1 min (B). A Western blot analyses using antibodies against phospho-specific PI3K p85 and PI3K p85 were performed. The histogram represents the relative levels of PI3K phosphorylation. The phosphorylation levels were corrected by the total protein levels, and then expressed as the fold increase compared with the basal levels presented in lanes 1. Each value represents the mean ± SD of triplicate determinations from three independent cell preparations. †$p < 0.001$, compared to the value of the control cells without quercetin and HGF (A) or without quercetin and TGF-α (B). *$p < 0.05$, compared to the value of cells with HGF alone (A) or TGF-α alone (B).

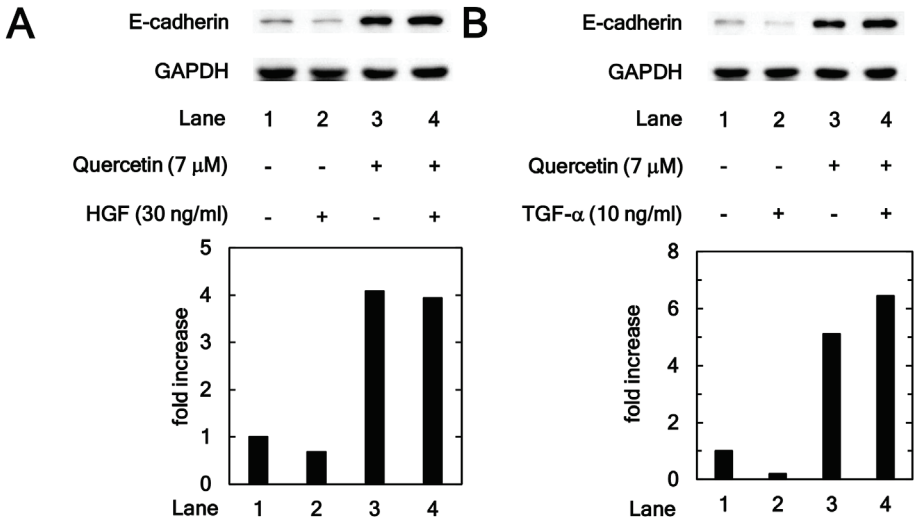

Fig. 12. Effects of quercetin on E-cadherin expression in HGF (A)- and TGF-α (B)-stimulated HuH7 cells. The cells were pretreated with 7 μM of quercetin or vehicle for 1 h, and then stimulated by 30 ng/ml of HGF or vehicle (A) or 10 ng/ml of TGF-α or vehicle (B) for 23 h. A Western blot analyses using antibodies against E-cadherin and GAPDH were performed. The histogram represents the relative levels of E-cadherin. The E-cadherin levels were corrected by GAPDH levels, and then expressed as the fold increase compared with the basal levels presented in lanes 1.

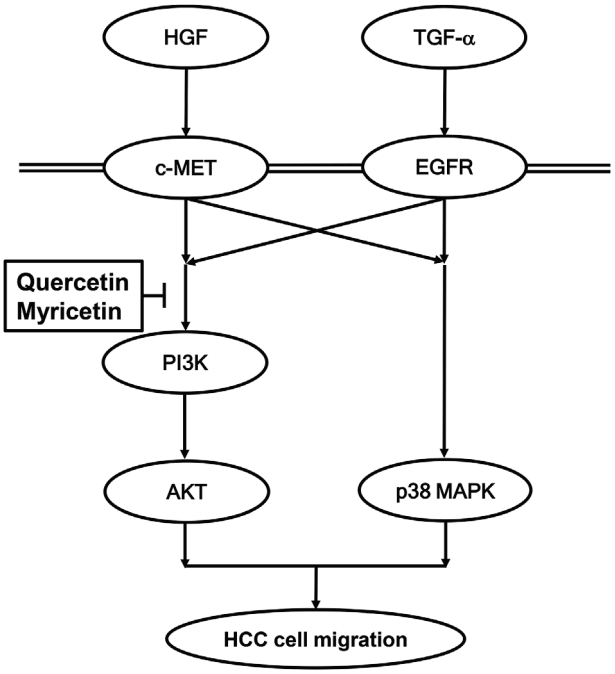

Fig. 13. A schematic illustration of the regulatory mechanism of quercetin and myricetin in HGF- and TGF-α-induced HCC cell migration.

In conclusion, our present findings strongly suggest that quercetin, a flavonol, suppresses the HGF- and TGF-α-induced HCC cell migration due to inhibiting the signaling pathway of AKT but not p38 MAPK.

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CRediT authorship contribution statement

Noriko Yamada: Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Rie Matsushima-Nishiwaki:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Project administration, Funding acquisition. **Osamu Kozawa:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

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