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Myristoylated alanine-rich C kinase substrate phosphorylation promotes cholangiocarcinoma cell migration and metastasis via the protein kinase C-dependent pathway

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Myristoylated alanine-rich C kinase substrate (MARCKS), a substrate of protein kinase C (PKC) has been suggested to be implicated in cell adhesion, secretion, and motility through the regulation of the actin cytoskeletal structure. The quantitative real-time–polymerase chain reaction analysis revealed that MARCKS is significantly overexpressed in *Opisthorchis viverrini*-associated cholangiocarcinoma (CCA) ($P = 0.001$) in a hamster model, which correlated with the results of mRNA *in situ* hybridization. An immunohistochemical analysis of 60 CCA patients revealed a significant increase of MARCKS expression. Moreover, the log–rank analysis indicated that CCA patients with a high MARCKS expression have significantly shorter survival times than those with a low MARCKS expression ($P = 0.02$). This study investigated whether MARCKS overexpression is associated with CCA metastasis. Using a confocal microscopic analysis of CCA cell lines that had been stimulated with the PKC activator, 12-*0*-tetradecanoyl phorbol-13-acetate (TPA), MARCKS was found to be translocated from the plasma membrane to the perinuclear area. In addition, phosphorylated MARCKS (pMARCKS) became highly concentrated in the perinuclear area. Moreover, an adhesion assay demonstrated that the exogenous overexpression of MARCKS remarkably promoted cell attachment. Interestingly, after TPA stimulation, the CCA cell line-depleted MARCKS showed a decrease in migration and invasion activity. It can be concluded that in non-stimulation, MARCKS promotes cell attachment to the extracellular matrix. After TPA stimulation, PKC phosphorylates MARCKS leading to cell migration or invasion. Taken together, the results of this study reveal a prominent role for MARCKS as one of the key players in the migration of CCA cells and suggest that cycling between MARCKS and pMARCKS can regulate the metastasis of biliary cancer cells. (*Cancer Sci* 2010; 101: 658–665)

Cholangiocarcinoma (CCA), the malignant tumor arising from the bile duct epithelium, is the most common cancer in Thailand, especially in the northeast. There is strong epidemiological evidence[1–3] and experimental studies[4–6] that support an important etiological role for liver fluke (*Opisthorchis viverrini* [Ov]) infection in the development of CCA in humans. There is accumulating evidence that Ov infection enhances the risk of CCA via chronic inflammation.[7,8] Recently, Pinlaor *et al.*[9] suggested that oxidative and nitrosative damage to DNA in the liver of Ov-infected hamsters can play a key role in the modulation of gene expression, which enhances the effect of the DNA adduct and can drive a normal cell undergo tumor development. Additionally, Thanan *et al.*[10] reported that the highest 8-oxodG level, which is the biomarker of DNA damage, was observed for CCA patients, and a higher level was found in the urine and leukocytes of Ov-infected patients than that in healthy patients.

The gene expression profiles of Ov-associated CCA tumors, as investigated by Loilome *et al.*[11], indicated that myristoylated alanine-rich protein kinase C substrate (MARCKS) is significantly upregulated in tumor tissues, suggesting a role for this protein in cholangiocarcinogenesis. MARCKS is a ubiquitous heat-stable protein[12–14] that shuttles between the plasma membrane and cytoplasm in a protein kinase C (PKC) phosphorylation-dependent manner.[15] The binding of MARCKS to the membrane is regulated by two determinants, including the insertion of its myristoylated N terminus into the lipid bilayer, and the electrostatic interaction of basic residues in the effector domain with the acidic lipids of the membrane.[16–18] The phosphorylation of MARCKS by PKC induces the translocation of MARCKS from the membrane to cytosol by introducing negative charges into the basic cluster.[19–22] MARCKS is also responsible for the binding and cross-linking of actin filaments (F-actin) directly to the membrane,[13] suggesting its role in cytoskeletal organization.[23] MARCKS has been implicated in the coordination of membrane–cytoskeletal events, including the regulation of integrin mobility underlying cell adhesion and migration, cytokine secretion, and phagocytosis of inflammatory cells.[16,24–27] In humans, the MARCKS protein is detectable in normal tissues, such as the brain, lung, and kidney, whereas the expression level is low in the liver, heart, and skeletal muscles.[28] The overexpression of MARCKS was found in hepatocellular carcinoma,[29] as well as in the pancreatic cancer cell line.[30] The ectopic overexpression of MARCKS found in a melanoma cell line led to increased spreading and the formation of membrane processes, indicating that MARCKS might play role in cell spreading and focal contact formation.[31]

Therefore, the aims of the present study are to determine the MARCKS expression during Ov-induced CCA development in the hamster model, as well as to assess a prognostic value of MARCKS in CCA patients. Moreover, the role(s) of MARCKS and its signaling pathway in CCA cell adhesion, migration, and invasion is investigated.

Materials and Methods

Animals and tumor induction. The animal and tumor induction was as previously described.[11]

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Human CCA specimens. The 60 paraffin-embedded liver tissues collected from primary tumors of CCA patients were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. The research protocols were approved by the Human Research Ethics Committee, Khon Kaen University (no. HE471214), and informed consent was obtained from each patient before surgery.

Cell culture and chemicals. The CCA cells were cultured in Ham's F-12 medium supplemented with 44 mM $NaHCO_3$ and 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2. The medium was supplemented with penicillin (100 units/mL) and streptomycin (100 mg/mL). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma–Aldrich (St Louis, MO, USA) and GF109203x was from Invitrogen (Carlsbad, CA, USA). All other chemicals used were of analytical grade.

Isolation of total RNA and quantitative real-time-polymerase chain reaction. The isolation of total RNA and quantitative real-time–polymerase chain reaction (qRT–PCR) was as described previously[11] with MARCKS-specific primers.

mRNA *in situ* hybridization. A non-radioactive *in situ* hybridization (ISH) method for the detection of hamster MARCKS mRNA in paraffin sections was performed according to Yamada *et al.*[32] The immunoreactive products were visualized using diaminobenzidine (DAB) as the chromogen, with hematoxylin counterstaining. The stained sections were viewed under a standard light microscope.

Immunohistochemistry. MARCKS or phosphorylated MARCKS (pMARCKS) was detected on the paraffin-embedded sections using standard immunohistochemistry protocols. The sections were incubated with the 1:1000 anti-MARCKS or 1:200 anti-pMARCKS antibody (Santa Cruz, Santa Cruz, CA, USA) at 4°C overnight. After that, the sections were incubated with peroxidase-conjugated Envision secondary antibody (DAKO, Glostrup, Denmark). Peroxidase activity was observed using the DAB solution as the substrate, with hematoxylin counterstaining. The staining frequency of MARCKS was semiquantitatively scored based on the percentage of positive cells as: 0% = negative; 1%–25% = +1; 26%–50% = +2; and >50% = +3. The intensity of the MARCKS expression was scored as weak = 1, moderate = 2, and strong = 3.

Constructing the retroviral vector encoding MARCKS and the retroviral transduction of MARCKS in CCA cells. MARCKS cDNA (a kind gift from Professor Naoaki Saito, Kobe University, Kobe, Japan) with the *Eco*RI site at both the 5′ and 3′ termini was produced by polymerase chain reaction (PCR). After that, the PCR products were subcloned into a pMXs-IG retroviral vector (kindly provided by Professor Toshio Kitamura, University of Tokyo, Tokyo, Japan). pMXs-MARCKS was transfected into Plat-A packaging cells using FuGene6 (Roche, Mannheim, Germany). KKU-M156 CCA cells that express endogenous MARCKS at a low level were incubated in the virus-containing supernatants with 8 μg/mL polybrene (Sigma–Aldrich). The green fluorescent protein-expressing infected cells were sorted by Cell Sorter (FACS Vantage SE; Beckton Dickinson, San Jose, CA, USA).

PKC activity. PKC activity was detected by using a non-radioactive PKC activity assay kit according to the manufacturer's protocol (Promega, Madison, WI, USA). In brief, the protein extract was incubated with the PKC reaction mixture, which contains a PKC-specific peptide substrate (PepTag C1). The migration of the phosphorylated peptide toward the cathode (+) was then detected using agarose gel electrophoresis, and the gel image was analyzed and quantified using an ImageQuant Imager (GE Healthcare, Buckinghamshire, UK).

Transient knocking down of MARCKS using siRNA. KKU-M214 CCA cells (with a high endogenous MARCKS expression; 5×10^4 cells) were seeded into a 12-well plate for 24 h before transfection. The siRNA specific for human MARCKS mRNA (5′-CTACACTTGGGCTCCTTTT-3′) was obtained from JbioS (Saitama, Japan). The cells were transfected either with 20 pM siMARCKS or with a scrambled siRNA or with no siRNA sequence at all. Transfections were carried out using the oligofectamine system (Invitrogen) according to the manufacturer's instructions. After siRNA transfection, the plates were incubated at 37°C for 24–72 h for further analysis.

Western blot analysis. The cells were treated with 10 ng/mL TPA in serum-free medium for 12 h, or 5 μM GF109203x for 30 min before TPA treatment. The cell lysate was electrophoresed and transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies at 4°C overnight and secondary antibodies at room temperature for 1 h. Peroxidase activity was detected by Western lightning chemiluminescence reagents (Perkin Elmer, Boston, MA, USA). The apparent density of the bands on the membranes was estimated using densitometry.

Confocal microscopic analysis. The cells were cultured on glass microscope slides, then immunostained using primary antibodies specific to MARCKS or pMARCKS (Santa Cruz). Either anti-goat immunoglobulin G (IgG) Alexa 488 or anti-rabbit IgG Alexa 555 (Molecular Probes, Eugene, OR, USA) was used as the secondary antibody. For pMARCKS and F-actin colocalization, either donkey anti-rabbit IgG Alexa 488 or pholloidin-actin staining (Molecular Probes) was used as the secondary antibody. The stained slides were visualized using a fluoview Olympus confocal laser microscope (Tokyo, Japan) with a ×40 objective lens.

Re-attachment assay. In total, 4×10^4 CCA cells in serum-free medium were plated onto fibronectin-coated 24 well plates (BD Bioscience, San Jose, CA, USA) and allowed to attach overnight. All assays were performed in triplicate in each of two independent experiments. After incubation, the wells were washed three times with phosphate-buffered saline. The attached cells were then fixed and stained with Diff-Quick staining solution (Sysmex, Japan) and counted under a microscope.

Migration and invasion assay. In total, 4×10^4 siMARCKS transfected cells were incubated with 10 ng/mL TPA or 5 μM GF109203x in serum-free medium for 30 min before TPA treatment. After that, the cells were seeded into the upper chamber of migration or invasion Transwell (8 μm pore size; Becton Dickinson, San Jose, CA, USA) and aliquots of Ham's F 12 medium supplemented with 10% w/v fetal bovine serum were placed in the lower chamber. After incubation at 37°C for 18 h, the cells in the upper surface of the filter were scraped off. Cells that had migrated to the underside of the filter were fixed and stained with Diff-Quick staining solution and then counted under a microscope. The mean values of 10 low-power fields (×100 magnification) were determined. Assays were done in triplicate, and two independent experiments were repeated.

Statistical analysis. Data were expressed as mean ± SD and statistically analyzed using the Student's *t*-test. The association of MARCKS expression in CCA tissue and patients' histopathological data was analyzed and patient survival was calculated according to the Kaplan–Meier log–rank test. A P-value <0.05 was considered statistically significant.

Results

Histopathological changes. Hamster liver tissues were collected at different times following treatment with either Ov or *N*-nitrosodimethylamine (NDMA) or both. No histopathological changes in bile duct epithelial cells were observed in the control group. Carcinomas were seen at week 26 in all Ov plus NDMA-treated hamsters.

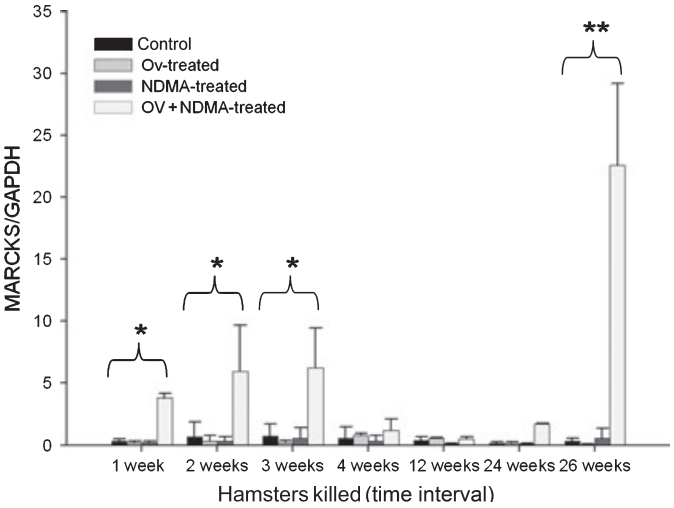

Fig. 1. Quantitative real-time–polymerase chain reaction assay was performed to determine the myristoylated alanine-rich C kinase substrate (MARCKS) mRNA level in the tissues at different times after treatment with either *Opisthorchis viverrini*, *N*-nitrosodimethylamine (NDMA) or both. Data are shown as mean ± SD. Asterisks denote significant increases in the MARCKS expression compared with the control. $*P < 0.05$, $**P = 0.001$.

MARCKS mRNA upregulation in liver tissue during CCA development. QRT–PCR and mRNA ISH were performed to determine the MARCKS mRNA level and localization in the tissues at different time points after treatment with either Ov or NDMA or both. The MARCKS mRNA level was found to be increased after Ov plus NDMA administration from weeks 1 to 3 and was strongly upregulated again at week 26 when the CCA developed (Fig. 1). mRNA ISH showed little or no staining in the normal bile duct at all time points of the control group. When Ov was administered together with NDMA, a strong positive expression was found in cancer cells at 26 weeks, whereas only faint to moderate staining was observed in the bile duct at 4, 12, and 24 weeks. At 1, 2, or 3 weeks of Ov plus NDMA treatment, moderate to strong expression was observed in inflammatory cells (Fig. 2).

Immunohistochemical analysis of MARCKS and pMARCKS in human CCA tissues. Of the 60 CCA patients studied, 19 were males and 41 were females. The mean age was 54.95 ± 9.1 years (range: 33–74 years). Most of the patients were at an advanced stage, with 61% metastasis. The certain immunoreaction of MARCKS and pMARCKS was observed in tumor cells (Fig. 3a,c), whereas it was only weakly expressed in normal bile duct epithelia and hepatocytes. Positive staining for the MARCKS protein was illustrated in the cell membrane, whereas pMARCKS was localized in the cytoplasm of the tumor biliary cells. Immunohistochemical staining for MARCKS showed positive staining in 53 cases (88%), whereas positive staining for pMARCKS was seen in 49 (81%) CCA patients. A percentage of the 53 CCA patients exhibited a high expression of both forms with statistical significance ($P = 0.032$). Neither an increased level of MARCKS nor pMARCKS was associated with age, sex, or histological grading. Of the 53 cases with MARCKS positive staining, a high level of this protein was significantly correlated with a positive metastasis status ($P = 0.039$). Moreover, of the 49 cases of pMARCKS positive staining, a high level of pMARCKS was significantly correlated with a positive metastasis status ($P = 0.009$). The cumulative survival analysis revealed that a high level of MARCKS, but not pMARCKS, showed a significant correlation with 5-year survival ($P = 0.02$) (Fig. 3b,d). To prove whether the correlation between a high expression of MARCKS and poor prognosis of CCA patients was due, at least in part, to metastasis induction, we investigated in depth the mechanisms by which MARCKS influences the adhesion, migration, and invasion of CCA cells.

PKC induces the translocation and phosphorylation of MARCKS in CCA cell lines upon TPA stimulation. The subcellular localization of MARCKS upon stimulation with TPA was investigated using a PKC activator. In the M156 CCA cell line, MARCKS was overexpressed; in the M214 CCA cell line, high endogenous MARCKS expression was either exposed to 10 ng/mL TPA or to 5 μM GF109203x (PKC inhibitor) for 30 min prior to TPA treatment. Confocal laser microscopy was performed to visualize the intracellular localization of MARCKS and pMARCKS. In non-stimulated cells, MARCKS was mostly located at the plasma membrane, and pMARCKS was evenly distributed throughout the cytoplasm. After stimulation with TPA, MARCKS was translocated to the cytoplasm and perinuclear

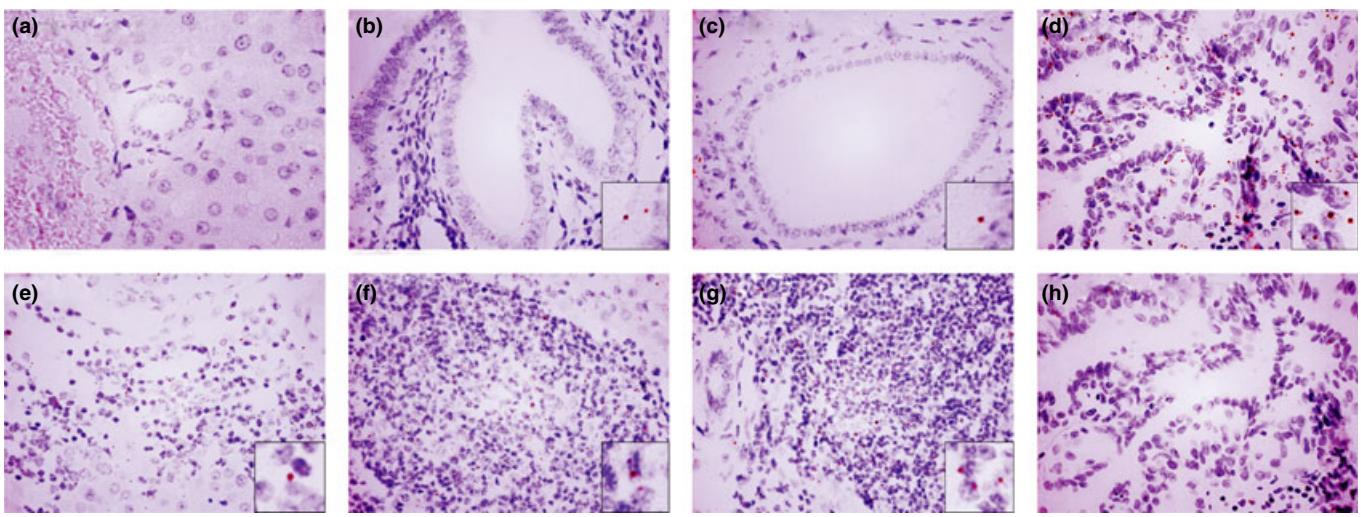

Fig. 2. Non-radioactive *in situ* hybridization was used to determine myristoylated alanine-rich C kinase substrate mRNA localization in the liver tissue. To verify the specificity of the cRNA probe, a hybridization mixture that contained a non-digoxiginin (DIG)-labeled antisense probe and a DIG-labeled probe at a ratio of 30:1 was used as the negative control. (a) Non-treated; (b) *Opisthorchis viverrini* treated; (c) *N*-nitrosodimethylamine (NDMA) treated; (d) cholangiocarcinoma (CCA); Ov plus NDMA-treated groups at (e) week 1; (f) week 2; (g) week 3; (h) negative control.

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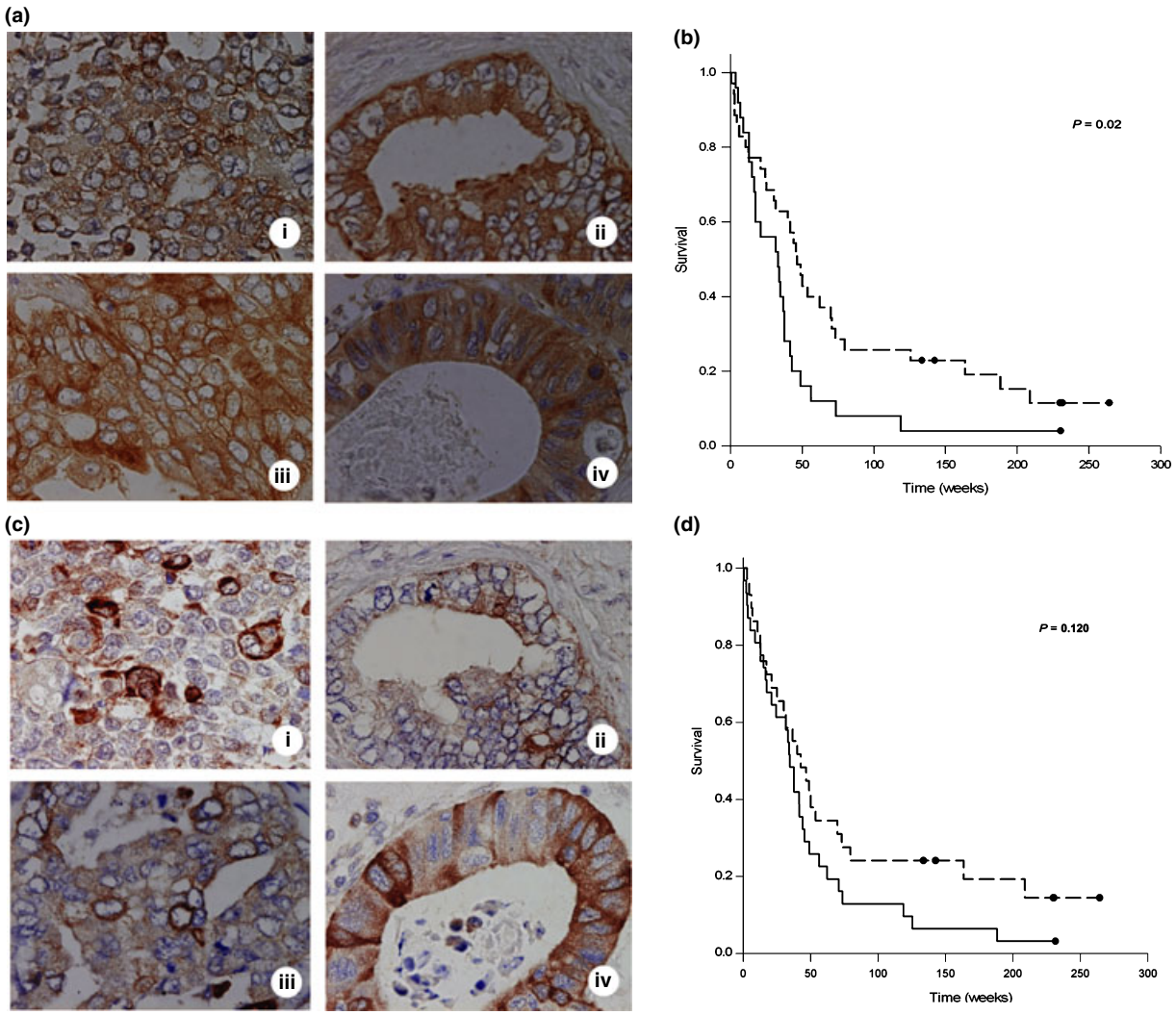

Fig. 3. Immunohistochemistry of myristoylated alanine-rich C kinase substrate (MARCKS) (a) and phosphorylated MARCKS (pMARCKS) (c) in cholangiocarcinoma (CCA) tissues. Poorly (i), moderately (ii), papillary (iii), and well-differentiated (iv) CCA showed a strong expression of MARCKS. Some CCA cells in the bile duct showed a strong intensity of pMARCKS. Original magnification ×40. Survival curves calculated for MARCKS (b) and pMARCKS (d) according to the Kaplan–Meier log–rank test ($P = 0.02$ and $P = 0.12$, respectively). (—), high level of MARCKS or pMARCKS, (- - -), low level of MARCKS or pMARCKS.

area, and pMARCKS became highly concentrated at the perinuclear area. With prior PKC inhibitor treatment, MARCKS and pMARCKS distribution and abundance were similar to those observed in non-stimulated cells (Fig. 4a). In addition, TPA induced the pMARCKS protein (Fig. 4b,c) and PKC activity up to 1.8-fold (Fig. 4d) over untreated cells.

MARCKS is associated with the re-attachment of CCA cell lines. To address the functional importance of MARCKS, gain and loss of function experiments were carried out using M156 and M214 CCA cell lines. M156 cells were infected with the retroviral vector encoding a human MARCKS cDNA to construct the stable overexpressing MARCKS cell line. The overexpression of MARCKS was clearly observed in infected cells, as determined by western blot analysis (Fig. 5a). As shown in Figure 5(c,d), MARCKS-overexpressing M156 showed a 2.5-fold increase in cell re-attachment over the untransfected M156 cell line. Suppressing MARCKS mRNA function in the M214 CCA cell line by siRNA decreasing cell re-attachment by up to twofold. A similar effect was observed in the knocking down of MARCKS-overexpressing M156 cells (data not shown).

TPA-induced MARCKS phosphorylation promotes cell migration and invasion in CCA cell lines. To investigate the function of pMARCKS on CCA cell migration and invasion upon TPA stimulation, the M214 cell line was transiently transfected either with MARCKS siRNA to suppress MARCKS protein translation or with the scrambled siRNA as a control. After 48 h of transfection, the western blot analysis revealed that the level of the MARCKS protein was diminished (Fig. 5b). In comparison, modified Boyden chamber migration and invasion assays were performed, as shown in Figure 5(e–h). After TPA stimulation, siRNA against MARCKS decreased cell motility and invasion approximately five-fold and two-fold, respectively. After the PKC inhibitor was added, fewer numbers of cell migration and invasion were observed. The same result was observed in the knocking down of MARCKS-overexpressing M156 cells (data not shown).

Phosphorylation of MARCKS affects F-actin disassembly upon TPA stimulation. This study investigated the subcellular localization of MARCKS and F-actin upon stimulation with TPA. M214 cells were exposed to 10 ng/mL TPA or 5 μM GF109203x. The immunofluorescent staining of MARCKS and

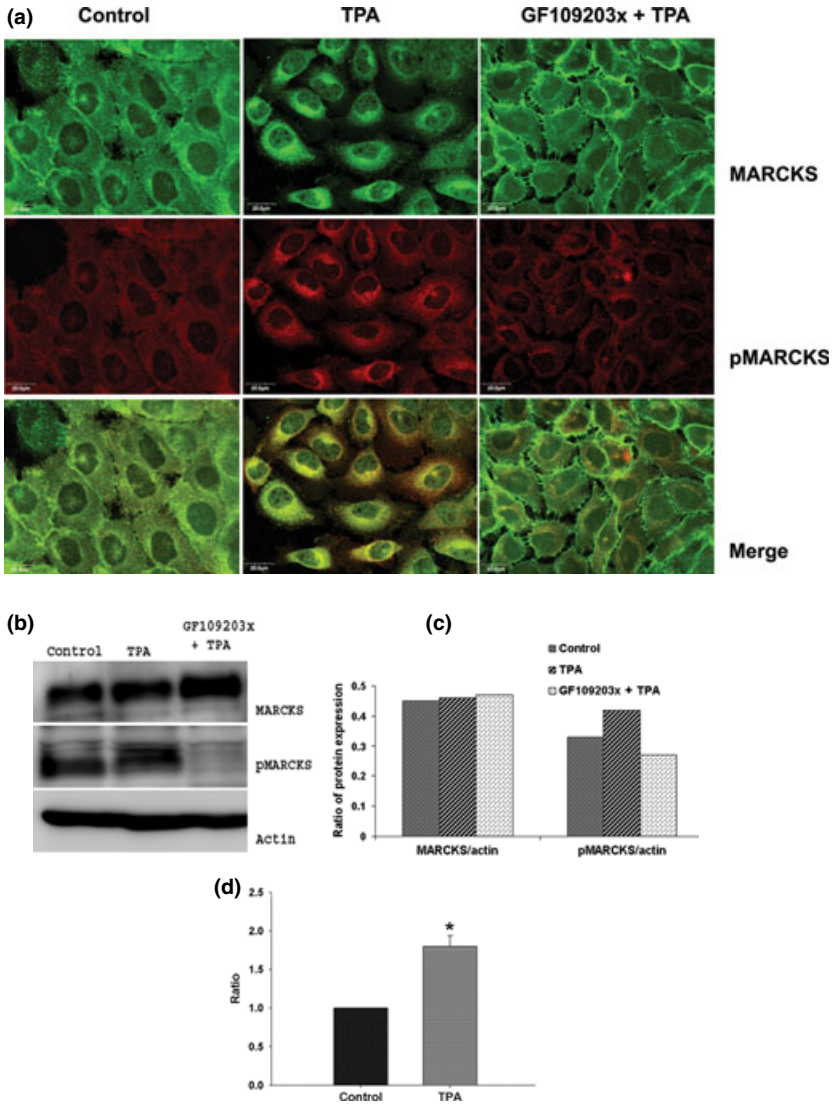

Fig. 4. Intracellular localization of myristoylated alanine-rich C kinase substrate (MARCKS) and phosphorylated MARCKS (pMARCKS) (a). Cholangiocarcinoma (CCA) cells were stimulated with 10 ng/mL 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) or 5 μM GF109203x before TPA treatment. Confocal laser microscopy with an objective lens of ×40 was employed. In the control group, MARCKS was mostly located at the plasma membrane and pMARCKS was evenly distributed throughout the cytoplasm. Upon TPA treatment, MARCKS was translocated to the cytoplasm and the perinuclear area, and pMARCKS became highly detectable at the perinuclear level. Upon GF109203x + TPA treatment, MARCKS and pMARCKS distribution and abundance were similar to those observed in non-stimulated CCA cells. Western blotting was used for protein checking (b). Apparent intensity of bands on the membranes was estimated using a densitometer (c). Protein kinase C (PKC) activities were compared between the control and TPA-treated cells (d). Asterisk denotes a significant increase of PKC activity when compared with the control. $*P < 0.05$.

F-actin was performed to visualize the intracellular localization of MARCKS and F-actin. In non-stimulated cells, MARCKS was found in cell membranes and cytoplasms, whereas F-actin formation was seen in the plasma membranes. After stimulation with TPA, MARCKS became highly expressed around the perinuclear area, and F-actin disassembly was rearranged. Prior to PKC inhibitor treatment, MARCKS and F-actin distribution and abundance were similar to that observed in the control cells (Fig. 6).

Discussion

This study is the first description of MARCKS expression and localization patterns during CCA carcinogenesis. In Ov, NDMA, or Ov plus NDMA-treated hamsters, MARCKS mRNA levels at 1, 2, and 3 weeks were predominantly positive in inflammatory cells, whereas strong expression was found differentially in cancer cells at 26 weeks. No obvious expression was found in normal tissues of either the control or treated group. This finding suggests that MARCKS can play a role in both the early and end stage of cholangiocarcinogenesis. Further study on the molecular mechanism of MARCKS in the early stage of carcinogenesis associated with inflammation is underway.

To clarify the impact of MARCKS and pMARCKS in humans, CCA tissues taken from patients who were assessed for MARCKS and pMARCKS localization patterns. An immunohistochemical analysis revealed that MARCKS and pMARCKS were rarely localized in normal bile duct epithelia. In contrast, significant increases of MARCKS and pMARCKS protein levels were found in cancer cells. Moreover, the strong intensity of the pMARCKS protein was found in some tumor cells of the biliary duct, as shown in Figure 3(b). One explanation is that CCA cells that are certainly activated by certain stimuli or proteins can increase the phosphorylation of MARCKS. These results demonstrate a statistically significant association between the positive metastasis status with high MARCKS and pMARCKS protein levels in CCA tissues. The cumulative survival analysis demonstrated that CCA patients with a high MARCKS level had a significantly shorter survival time than those with a low MARCKS level ($P = 0.02$). In the case of pMARCKS, a high level tends to be associated with a shorter survival time, but the statistical value is not significant ($P = 0.12$). This indicates that MARCKS in tumor cells could serve as a prognostic marker for CCA.

To elucidate the mechanism by which MARCKS functions in cellular metastasis, the regulation of MARCKS in response to the PKC activator was determined in this study. The results revealed that PKC via TPA stimulation has the ability to activate the intracellular cycling of MARCKS between the plasma membrane and the cytoplasm and also to induce a burst of MARCKS

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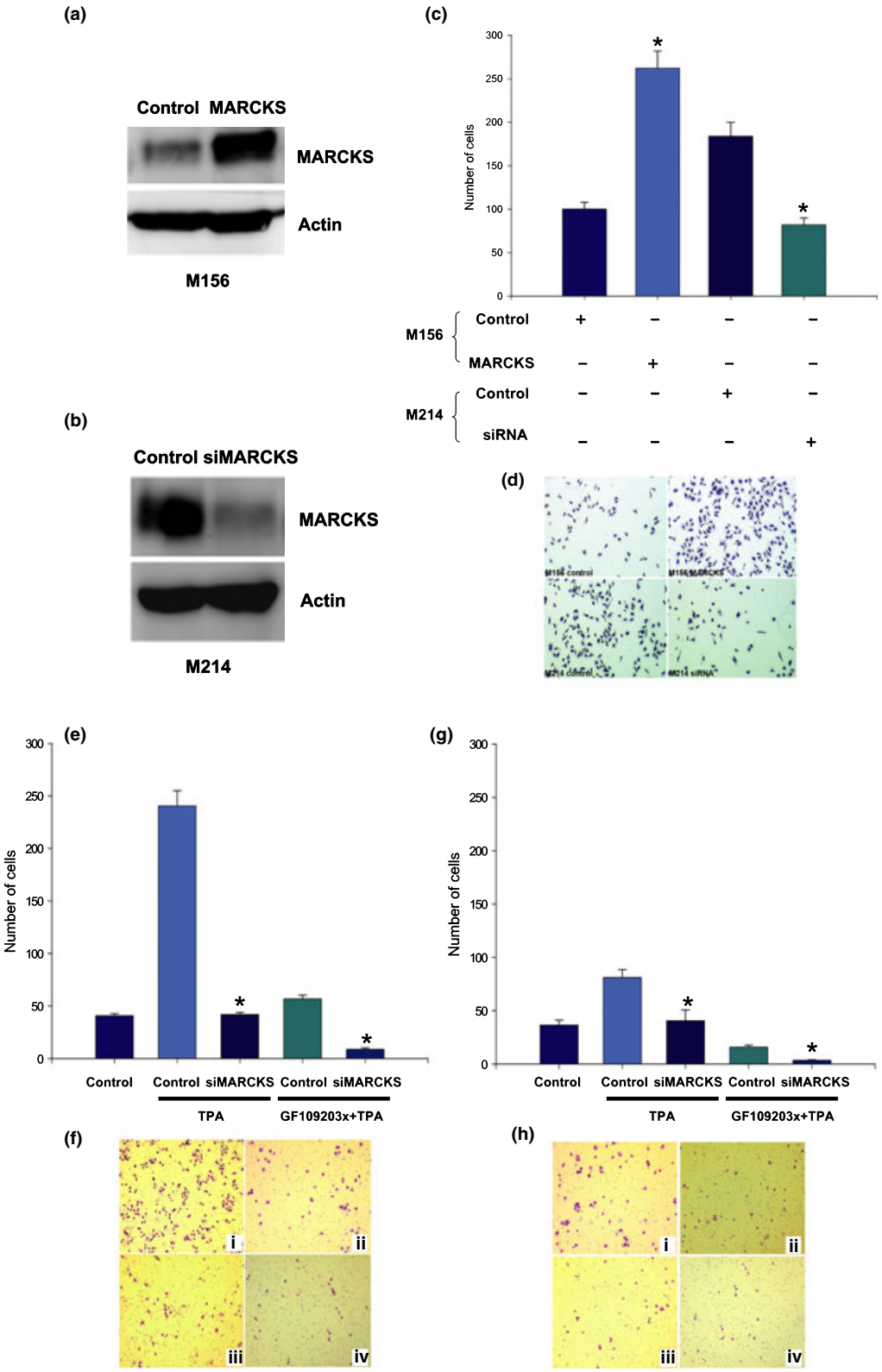

Fig. 5. Gain-on and gain-off function of myristoylated alanine-rich C kinase substrate (MARCKS). Western blot analysis was performed to confirm the overexpression of MARCKS in the M156 cholangiocarcinoma (CCA) cell line in comparison to the empty vector control cells (a) and RNAi against MARCKS in the M214 CCA cell line (b). MARCKS promotes cell re-attachment in CCA cell lines (c,d). Re-attachment assay was employed. Values are expressed as mean ± SD. Asterisk denotes a significant increase. *$P < 0.05$. In contrast, the knocking down of endogenous MARCKS in the M214 CCA cell line leads to a decrease in cell migration or invasion after 12-*0*-tetradecanoyl phorbol-13-acetate (TPA) stimulation. Migration (e,f) or invasion (g,h) assays were performed in TPA-treated control cells (i), under TPA-treated siRNA conditions (ii), GF109203x with TPA-treated control cells (iii), and GF109203x under TPA-treated siRNA conditions (iv). Values are expressed as mean ± SD. Asterisk denotes a significant decrease. *$P < 0.05$.

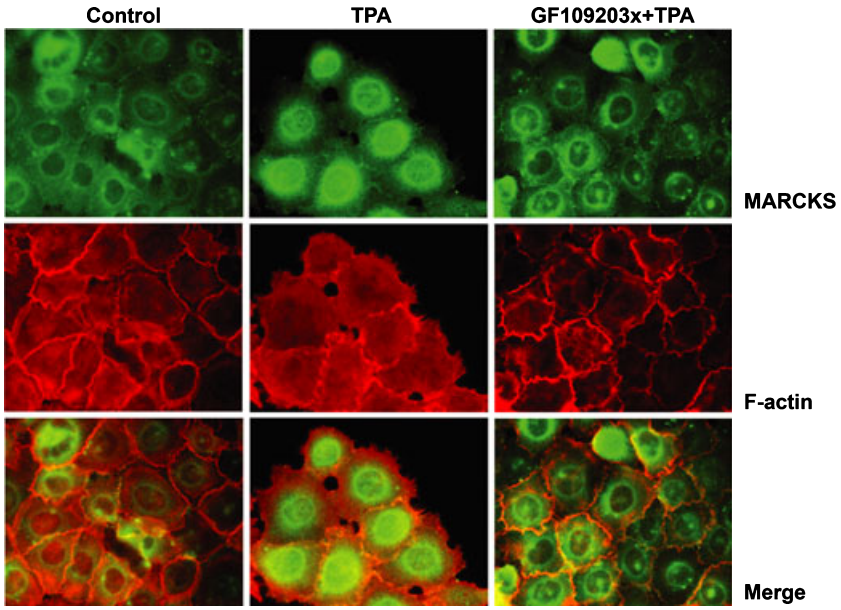

Fig. 6. Intracellular localization of myristoylated alanine-rich C kinase substrate (MARCKS) and actin filaments (F-actin). Cholangiocarcinoma cells were stimulated with 10 ng/mL 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) or 5 μM GF109203x before TPA treatment. Immunofluorescence microscopy with an objective lens of ×40 was employed. In the control group, MARCKS and F-actin were colocalized at the plasma membrane. Upon TPA treatment, MARCKS was translocated to cytoplasm at the perinuclear area, and F-actin disassembly was found. In GF109203x + TPA treatment, MARCKS and F-actin distribution was similar to that observed in the control cells.

phosphorylation in CCA cell lines. In addition, the induction of MARCKS phosphorylation appears to be necessary for mediating cell motility and invasion. In contrast, the TPA-induced phosphorylation of MARCKS was inhibited when cells were pretreated with the PKC inhibitor. These data suggest that PKC is involved in the phosphorylation of MARCKS upon TPA stimulation. In addition, the localization of PKC and pMARCKS was observed in both human tissues and CCA cells (data not shown). In cholangiocarcinogenesis with an increase in MARCKS expression, PKC stimulation might be the common pathway to activate MARCKS phosphorylation. Thuwajit *et al.*[33] showed that PKC is upregulated in NIH-3T3 cells cocultured with Ov. In addition, Jittimanee *et al.*[34] demonstrated that the mRNA expression of transforming growth factor-β, which is the upstream signal of the PKC pathway, is significantly upregulated in the liver of Ov-infected hamsters. These findings indicate that Ov activates the PKC signaling pathway. Thereafter, activated PKC can control multiple downstream signals in cholangiocarcinogenesis, including the MARCKS signaling pathway.

The involvement of MARCKS in the proliferation, re-attachment, migration, and invasion of CCA cells was analyzed by the overexpression and suppression of MARCKS mRNA. The results of the present study revealed that the knocking down or overexpression of MARCKS did not affect the cell proliferation rate (data not shown). Without PKC stimulation, MARCKS-overexpressing CCA cells promote cell re-attachment. In contrast, suppression by siRNA decreased cell re-attachment. When the PKC signaling pathway was stimulated by TPA, the MARCKS protein was phosphorylated and less cell re-attachment was observed (data not shown). Cell attachment can result from the stabilization of integrin-mediated adhesions controlled by non-pMARCKS.[35]

We then sought to clarify whether the PKC-mediated phosphorylation of MARCKS is necessary for the induction of cell motility or invasion induced by TPA stimulation. When MARCKS mRNA was suppressed in the presence of TPA, cell motility and invasion were significantly reduced in comparison to the control cells. In addition, a decrease in cell migration and invasion was observed following treatment with the PKC inhibitor. These data correspond to a previous study by Stensman *et al.*[36] who demonstrated that MARCKS phosphorylation by TPA induced neuroblastoma cell motility. The present results indicate that MARCKS and F-actin were colocalized at the membrane of CCA cells. Upon PKC activation, MARCKS was translocated to the perinuclear area and F-actin disassembly was found. These data suggested that PKC induces MARCKS translocation and leads to F-actin rearrangement, resulting in a cellular shape change and easy moving. Taking these results together, the phosphorylation of MARCKS induced by PKC activation can alter the actin rearrangement, resulting in enhancement of cell motility and invasion.

Metastasis formation involves several steps. Initially, single tumor cells migrate from the primary tumor, then re-attach and invade the extracellular matrix, intravasate, and transmigrate through the endothelium, before finally re-attaching to form a secondary tumor in the targeted tissue.[37,38] Based on the findings of this study, a description is provided of the possible mechanism of MARCKS and pMARCKS involvement in cell re-attachment and metastasis. In the absence of stimulation by PKC, MARCKS induces cross-linking of actin at the plasma membrane causing cell re-attachment to the extracellular matrix. After TPA stimulation, PKC phosphorylates MARCKS, and then pMARCKS translocates from the plasma membrane to be highly concentrated at the perinuclear area. The translocation of pMARCKS is associated with actin reorganization, resulting in cell migration or invasion. Therefore, the cycling between MARCKS and pMARCKS could be one of the key factors in the metastatic process of CCA cells by controlling the re-attachment, migration, and invasion of biliary cancer cells.

In conclusion, the data from the present study demonstrate that the overexpression of MARCKS is found in Ov-associated cholangiocarcinogenesis. Additionally, the high expression of MARCKS correlates with a poor prognosis of CCA patients, which might be due to the involvement of non-phosphorylated and pMARCKS via the PKC-dependent pathway in controlling cell re-attachment, migration, and invasion in the metastasis process of CCA cells.

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