Calcium/calmodulin-dependent protein kinase II enhances metastasis of human gastric cancer by upregulating nuclear factor-κB and Akt-mediated matrix metalloproteinase-9 production

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Abstract. Calcium/calmodulin-dependent protein kinase II (CaMKII) is a multi-functional serine/threonine protein kinase, involved in processes that cause tumor progression, including cell cycle regulation, apoptosis and differentiation. However, the role of CaMKII in cancer cell metastasis has not been fully elucidated. In the present study, the function of CaMKII in gastric cancer cell metastasis is reported. Firstly, it was demonstrated that the overexpression of H282R (constitutively active CaMKII) enhanced gastric cancer cell migration and invasion, and the inhibition of CaMKII activity by KN-62 decreased gastric cancer cell metastasis. Furthermore, H282R upregulated matrix metalloproteinase-9 (MMP-9) expression and production, which were dependent on CaMKII-mediated increase in nuclear factor (NF)-κB and Akt activation. Finally, CaMKII activation, through phosphorylation of the Thr 286 site, was significantly increased in the metastatic gastric cancer tissues compared with non-metastatic tissues, suggesting that CaMKII has an important function in the regulation of gastric cancer cell metastasis. Collectively, the present study demonstrated that CaMKII promotes gastric cancer cell metastasis by NF-κB and Akt-mediated-MMP-9 production. These findings suggest a novel function of CaMKII in the control of gastric cancer metastasis, offering a promising target for future therapeutics to treat and prevent gastric cancer metastases via the inhibition of CaMKII activity.

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Abbreviations: CaMKII, calcium/calmodulin-dependent protein kinase II; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Key words: calcium/calmodulin-dependent protein kinase I, gastric cancer metastasis, matrix metalloproteinase-9, nuclear factor-κB, Akt

Introduction

Gastric cancer is the fourth most common type of cancer and the second leading cause of cancer-associated mortality worldwide (1). Cancer metastasis is the leading cause of cancer treatment failure and is chiefly responsible for the poor prognosis of patients with gastric cancer (2). The biological mechanisms of metastasis appear to involve a complex array of genetic alterations, including changes in the expression of adhesion molecules (such as, integrins and cadherins), proteolytic enzymes (such as, matrix metalloproteinases) and signaling pathway components [such as, mitogen-activated protein kinase (MAPK) and Akt] (3). However, the molecular mechanisms involved in gastric cancer remain unclear. Therefore, examining the biological mechanisms underlying gastric cancer metastasis and developing a novel effective therapeutic target for gastric cancer treatment remains critical.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine protein kinase with wide distribution and multiple substrates in mammalian cells (4,5). CaMKII is composed of a combination of four enzyme isoforms (α, β, γ or δ), and each subunit contains a catalytic domain, a regulatory domain (including an auto-inhibitory region) and a calmodulin-binding region (6). In resting cells, CaMKII is a dodecameric holoenzyme that auto-inhibits catalytic activity via the auto-inhibitory domain. Binding of calcium/calmodulin to CaMKII relieves its auto-inhibition, resulting in Ca^{2+}-independent CaMKII activation and phosphorylation of the Thr 286 site to sustain CaMKII activity (7). CaMKII inhibitors have been used to investigate CaMKII function, specifically several CaMKII inhibitors (including chemically synthesized KN-62 and KN-93) and endogenous inhibitory proteins (e.g. CaMKIINα and CaMKIINβ derived from humans and mice). KN-62 and KN-93 inhibit CaMKII phosphorylation by interfering with calcium/calmodulin binding (8,9), thereby suppressing CaMKII activity. Endogenous inhibitory proteins CaMKIINα

and CaMKIINβ interact with activated CaMKII and directly inhibit CaMKII activity (10,11).

CaMKII is involved in numerous different physiological and pathological cellular processes (12,13). Multiple studies have identified CaMKII to be important for cancer cell control by regulating cell cycle progression, cellular apoptosis and proliferation (8-11). For example, KN-62 and KN-93 induce cancer cell cycle arrest (8,9). The endogenous inhibitors hCaMKIINα and hCaMKIINβ suppress tumor cell growth by inducing cell cycle arrest and apoptosis (10,11). In addition, CaMKII has been reported to be required for prostate cancer cell survival (14). Accumulating evidence suggests that CaMKII is involved in the regulation of cell migration (15-19); specifically, it enhances vascular smooth muscle migration after injury and stress (15-17). CaMKII is also required for ghrelin-induced glioma cell migration (18) and ClC-3-induced glioma invasion (19), suggesting that CaMKII is associated with cancer cell migration and invasion. However, the function of CaMKII in gastric cancer cell metastasis remains to be elucidated.

The present study investigated the function of CaMKII in gastric cancer cell metastasis and the underlying molecular mechanisms.

Materials and methods

Reagents and cell culture. KN-62 and PDTC were purchased from Calbiochem (San Diego, CA, USA). Dimethylsulfoxide was purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies against β-actin (sc-8432; mouse monoclonal IgG1), MMP-9 (sc-12759; mouse monoclonal IgG1), IKBα (sc-203; rabbit polyclonal IgG), phospho (p)-IKBα (sc-8404; mouse monoclonal IgG2b), CaMKII (sc-9035; rabbit polyclonal IgG) and p-CaMKIIα (Thr 286) (sc-12886-R; rabbit polyclonal IgG) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Four human gastric cancer cell lines (MKN28, GBC-SD, BGC-803 and SGC-7901) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) at 37˚C in a 5% CO_2 atmosphere.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of MMP9 mRNA expression. Total cellular RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) according to manufacturer instructions. Single strand cDNA was synthesized from 2 mg total RNA with the Superscript II system (Life Technologies, Rockville, MD, USA) using an oligo(dT)12-18 primer (Invitrogen Life Technologies). The primer sequences were as follows: 5'-TGTACCGCTATGGTTACACTCG-3' and 5'-GGCAGGGACAGTTGCTTCT-3' for human MMP9; and 5'-TGGAGAAAATCTGGCACCACACC-3' and 5'-GATGGGCACAGTGTGGGTGACCC-3' for actin (as a control gene). Primers were synthesized by Sangon Corporation (Shanghai, China). The synthesis of cDNA was checked by qRT-PCR using the actin primers. PCR consisted of denaturing (95˚C for 15 sec), annealing (56˚C for 30 sec), and extension (72˚C for 30 sec) for 30 cycles using a PCR system (Applied Biosystems, Foster City, CA, USA).

Tissue processing and protein extraction. The surgically resected gastric cancer tissues were collected from 20 gastric cancer patients (including, 10 non-metastatic cancer tissues and 10 metastatic cancer tissues with lymph nodes or vessels) from the Department of Oncology at Changzheng Hospital, the Second Military Medical University (Shanghai, China). The samples were acquired following obtaining informed consent from the patients. All of the experimental procedures were approved by the Institute Research Ethics Committee at the Second Military Medical University. All of the fresh specimens were washed three times with phosphate-buffered saline (PBS) and dissected, and the protein was extracted from cell lysates with cell lysis buffer (Cell Signaling Technology, Inc., Boston, MA, USA) according to the manufacturer's instructions.

Western blot analysis. Protein concentration was measured by the Bicinchoninic Acid protein assay reagent kit (Pierce, Rockford, IL, USA). The protein (50 μg) from gastric cancer cells and tissue samples were resolved with 10% SDS-PAGE and subjected to western blot analysis. The blots were probed with the specific primary antibodies (1:1,000) followed by the appropriate horseradish peroxidase-conjugated secondary antibodies (1:2,000; Cell Signaling Technology Inc., Beverly, MA, USA). The bands were visualized with SuperSignal West Femto chemiluminescent reagents (Pierce Biotechnology, Inc., Rockford, IL, USA).

Construct of constitutively active CaMKII and cell transfection. Constitutively active CaMKII expression plasmid was cloned with PCR cloning and mutation (H282 is mutated H282R). Flag-tagged expression vectors of H282R were constructed and transfected into BGC-803 using Jetpei (Polyplus Transfection, Illkirch, France) according to the manufacturer's instructions. cDNA encoding constitutively active human CaMKIIα (His282 mutated to Arg, H282R) was amplified by PCR. Primer were: 5'-GGCATCCCTGCATGGGCAGAC-3' and 5'-ACGGTGGAGCGGTGCGAGAT-3'. PCR was performed by denaturing the reaction mixture at 94˚C for 2 min, followed by 32 cycles (1 min at 94˚C, 1 min at 58˚C and 1 min at 72˚C). The amplification product was inserted into the Flag-tagged expression vectors (Invitrogen Life Technologies), and transfected into BGC 803 using Jetpei (Polyplus Transfection) according to the manufacturer's instructions.

Migration and invasion assay. Transwell polycarbonate membranes and Matrigel-coated invasion chambers (BD Biosciences, San Jose, CA, USA) were used to determine the effect of CaMKII on cell migration and invasion, respectively. The BGC-803 and SGC-7901 cells were starved with free medium, and $5x10^4$ cells were suspended in RPMI-1640 medium with KN-62 (10 μm) and added to the upper chamber. The lower chamber was filled with 500 μl of media containing 10% FBS. Following 24 h culture, non-migrated or non-invasive cells were scraped from the upper side of the Transwell membrane filter inserts with a cotton-tipped swab. Migrated/invasive cells on the lower side were stained with crystal violet and counted. The number of migrated/invaded cells were counted in three independent high powered fields (x20) readings with a light microscope (DFC420C; Leica Microsystems, Wetzlar, Germany).

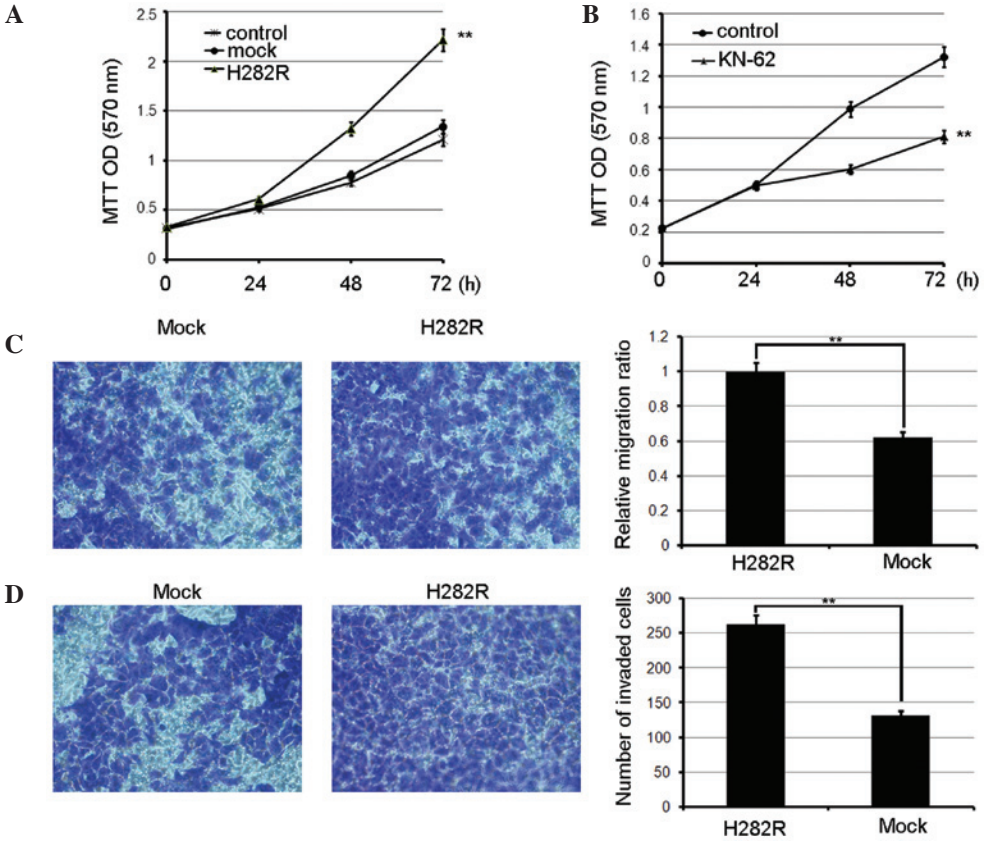

Figure 1. CaMKII promotes cell migration and invasion in gastric cancer cells. (A) BGC-803 cells were transfected with H282R expression vectors or mock vectors for 48 h and (B) BGC-803 cells were treated with KN-62 (10 μm) for the indicated times. *In vitro* cell growth was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data from the (C) transwell migration assays and (D) invasion assays in BGC-803 cells stably expressing H282R or the mock expression vector. Magnification, x20. The cells were quantified with crystal violet staining. Data are expressed as the mean ± standard deviation (n=4). Similar results were obtained in three independent experiments. **P<0.01, compared with mock. CaMKII, calcium/calmodulin-dependent protein kinase II; OD, optical density.

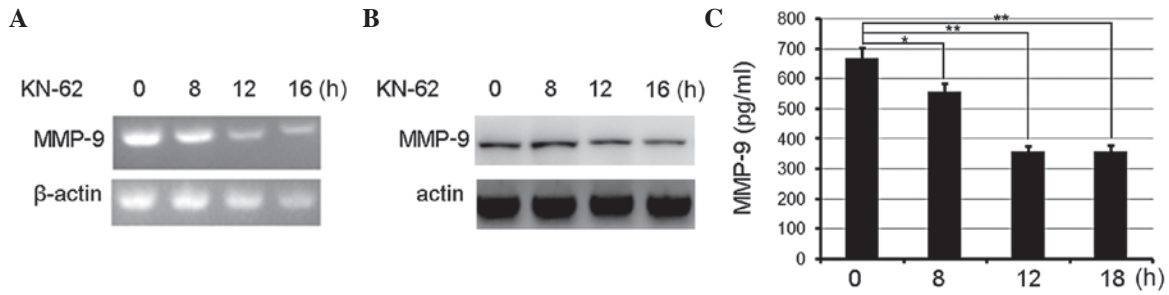

Figure 2. Inhibition of CaMKII activity suppresses MMP-9 expression and production. BGC-803 cells were treated with KN-62 (10 μm) for the indicated time and (A) polymerase chain reaction analysis was utilized for detecting the MMP-9 mRNA expression. (B) Western blot analysis was utilized for identifying MMP-9 protein expression measured with the indicated antibodies. (C) ELISA was used to measure the MMP-9 production. Data are expressed as the mean ± standard deviation (n=6). Similar results were obtained in three independent experiments. *P<0.05 and **P<0.01. CaMKII, calcium/calmodulin-dependent protein kinase II; MMP-9, matrix metalloproteinase-9.

Measurement of MMP-9 production by ELISA. The production of MMP-9 in the culture supernatants was quantified with ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, as described previously (20).

Nuclear factor (NF)-κB activation assay. The cells were co-transfected with a mixture of NF-κB luciferase reporter plasmid, pRL-TK-*Renilla*-luciferase plasmid and the appropriate additional constructs or KN-62 for 24 h. Total DNA was equalized with an empty control vector. Luciferase activity was measured with a dual-luciferase reporter assay system (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The data were normalized for transfection efficiency by measuring firefly luciferase activity and comparing it with that of *Renilla* luciferase.

Statistical analysis. Data are expressed as the mean ± standard deviation and compared using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

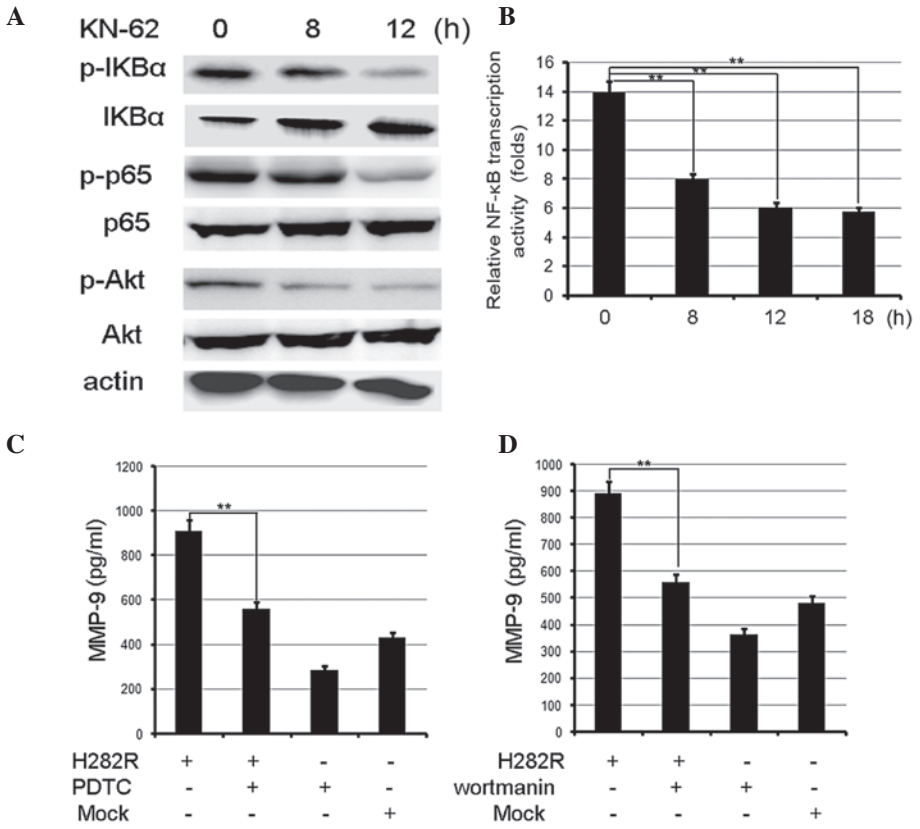

Figure 3. CaMKII-mediated NF-κB and Akt activation enhanced MMP-9 production in gastric cancer cells. (A) BGC-803 and SGC-7901 cells were treated with KN-62 (10 μm) for 24 h and then analyzed with western blot analysis with the indicated antibodies. (B) BGC-803 cells were transfected with pGL3.5xNF-κB luciferase plasmid or pRL-TK-*Renilla* plasmid as a control plasmid, and KN-62 was added (10 μm). Following (24 h) transfection, the luciferase activity was measured using a luciferase reporter assay kit. (C) BGC-803 cells were transfected with H282R plasmid for 24 h, and then treated with PDTC (10 μm) and (D) wortmanin for 24 h. MMP-9 production was measured with ELISA. Data are expressed as the mean ± standard deviation (n=6). Similar results were obtained in three independent experiments. **P<0.01. CaMKII, calcium/calmodulin-dependent protein kinase II; NF-κB, nuclear factor κB, MMP-9, matrix metalloproteinase-9.

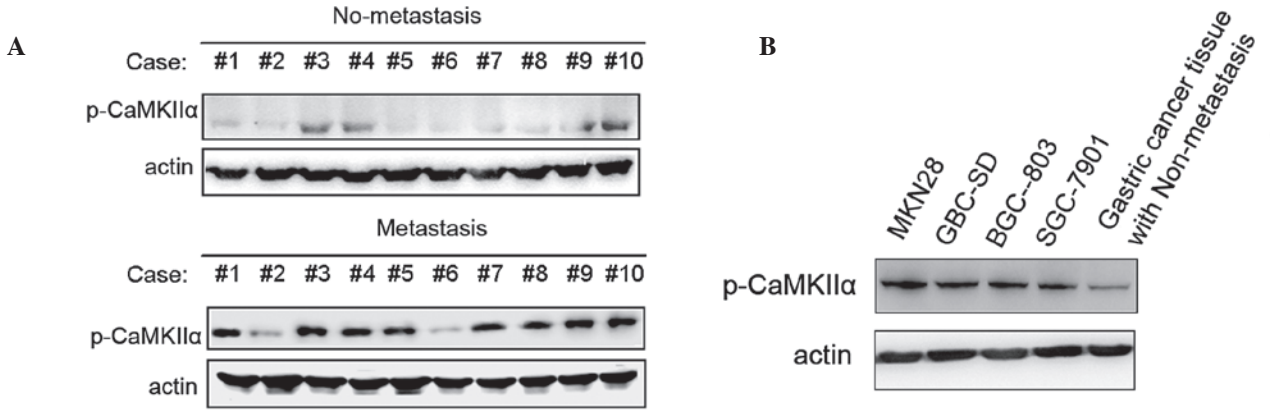

Figure 4. CaMKII activity in gastric cancer tissue and cell lines. CaMKII (Thr 286) phosphorylation in (A) gastric cancer tissues and (B) gastric cancer cell lines. Gastric cancer tissue protein and cells were extracted and quantified using a bicinchoninic aicd kit, and 50 μg protein was separated with SDS-PAGE and the proteins were detected using antibodies. CaMKII, calcium/calmodulin-dependent protein kinase II.

Results

CaMKII promotes gastric cancer cell migration and invasion. The mechanisms underlying cancer metastasis involve multiple factors and the alteration of various genes. To investigate the role of CaMKII on the regulation of gastric cancer cells, constitutively active CaMKII (H282 mutated to R) expression plasmid with Flag-tagged H282R, was constructed by PCR. Firstly, when observing the effects of CaMKII on gastric cancer growth via a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, it was noted that H282R overexpression accelerated BGC-803 cell

proliferation compared with the mock transfected cells. Also, the inhibition of CaMKII activity by KN-62 significantly suppressed cell growth compared with the control (Fig. 1A and B). Next, to assess the role of CaMKII in gastric cancer cell invasion and metastasis, it was observed that greater numbers of BGC-803 cells stably expressing H282R migrated to the opposite sides of the filters compared with the control cells treated with KN-62 (Fig. 1C). Similarly, greater numbers of BGC-803 cells stably expressing H282R invaded the matrigel compared with the control cells (Fig. 1D). Therefore, CaMKII was able to promote gastric cancer metastasis.

CaMKII enhances MMP-9 production of gastric cancer cells. MMP-9 is known to have a crucial role in cancer cell metastasis (21,22), thus, it was investigated whether MMP-9 was involved in CaMKII-mediated promotion of migration and invasiveness. Firstly, the MMP-9 expression in KN-62-treated gastric cancer cells was measured and it was observed that the inhibition of CaMKII activity by KN-62 decreased MMP-9 expression in a time-dependent manner and peaked following treatment for 16 h as measured by PCR (Fig. 2A). Immunoblotting assays confirmed that KN-62 decreased the MMP-9 expression at the protein level (Fig. 2B). In addition, MMP-9 was released from KN-62-treated cells according to ELISA. MMP-9 production was decreased in KN-62-treated BGC-803 cells compared with the controls (Fig. 2C). Therefore, CaMKII inhibition suppressed MMP-9 expression and production.

CaMKII enhances NF-κB transcription and Akt activation, thereby promoting MMP-9 production. Several signaling pathways participate in cancer metastasis, including MAPK, phosphoinositide 3-phospate (PI3K)-Akt and NF-κB. CaMKII was recently demonstrated to be a central regulator of NF-κB activation in lymphocytes (23). To examine whether CaMKII affects NF-κB transcription activity in gastric cancer cells, phosphorylated IKBα, total IKBα and active NF-κB (p-p65) levels were measured by western blot analysis. As demonstrated in Fig. 3A, KN-62 decreased IKBα and p65 phosphorylation, and increased IKBα in BGC-803 cells. Using a luciferase reporter assay, it was identified that NF-κB transcription activation was inhibited by KN-62 (Fig. 3B). In addition, Akt activation was suppressed in KN-62-treated BGC-803 cells (Fig. 3A). Therefore, CaMKII increased NF-κB transcription and Akt activation in gastric cancer cells.

NF-κB is a key transcription factor for MMP-9 expression in physiological and pathological conditions (24), it was therefore important to examine whether NF-κB is involved in CaMKII-mediated upregulation of MMP-9 expression. It was identified that the overexpression of H282R increased MMP-9 production, and that PDTC, a specific NF-κB inhibitor, partly inhibited the H282R-mediated upregulation of MPP-9 production (Fig. 3C). It was also investigated whether Akt is involved in CaMKII-mediated upregulation of MMP-9 expression, it was identified that wortmanin, a specific Akt inhibitor, decreased H282R-mediated upregulation of MPP-9 production (Fig. 3D). Therefore, CaMKII upregulated MMP-9 in gastric cancer cells and this was dependent on NF-κB and Akt activation.

CaMKII activity was enhanced in metastatic gastric cancer. Next, the activation of CaMKII in gastric cancer tissues was observed. It was noted that 70% of the cases of gastric cancer with metastasis, including lymph node and/or lymphatic vessel metastasis, had higher CaMKII phosphorylation (at Thr286) compared with 20% of the cases of non-metastatic cancer (Fig. 4A). Next, CaMKII phosphorylation in diverse gastric cancer cell lines, which have greater capability of invasiveness was examined. The CaMKII phosphorylation was increased in four higher metastatic gastric cancer cell lines (MNK28, GBC-SD, BGC-803 and SGC-7901; Fig. 4B). These findings further confirm that CaMKII may be involved in gastric cancer metastasis and therefore targeting this kinase may represent a strategy for preventing gastric cancer metastasis.

Discussion

Cancer metastasis is an obstacle to cancer therapy and a leading cause of mortality for gastric cancer patients (2). To overcome and improve treatment outcomes, understanding the biological mechanisms of gastric cancer metastasis is required. To the best of our knowledge, the present study demonstrates for the first time that CaMKII promotes gastric cancer metastasis by upregulating NF-κB/Akt-dependent MMP-9 production.

To investigate the molecular mechanisms underlying pro-gastric cancer metastatic properties of CaMKII, MMP-9, a classic metastatic-prompting gene implicated in numerous types of human cancer for its ability to cleave various extracellular matrix molecules, was examined (25,26). CaMKII has been reported to regulate MMP-9 activity, as conferred by the evidence that CaMKII promotes MMP-9 expression in cardiomyocytes (27) and promotes vascular smooth muscle migration via the regulation of MMP-9 activity (15). In the present study, inhibition of CaMKII decreased MMP-9 expression and production, and the overexpression of H282R or constitutively activate CaMKII, increased MMP-9 production, which was consistent with the results of previous studies (15,27). Furthermore, CaMKII activity was observed to be increased in samples of metastatic gastric cancer, which also confirms the pro-metastatic biological function of CaMKII. Therefore, CaMKII enhances gastric cancer cell metastasis by increasing MMP-9 expression and production.

MMP-9 production is regulated by multiple signaling pathways (28-30). Previous studies have demonstrated that NF-κB is a key regulator of MMP-9 (31). The present data indicate that CaMKII upregulated MMP-9 expression, and inhibition of NF-κB activation eliminated CaMKII-mediated MMP-9 production. This suggests that CaMKII increased MMP-9 expression by enhancing NF-κB activation. A binding site for NF-κB has been reported to be the promoter of the MMP-9 gene, contributing to MMP-9 expression (32), a concept that supports the present findings that NF-κB-dependent CaMKII-mediated MMP-9 expression patterns. In addition, these data demonstrate that CaMKII-mediated MMP-9 expression was partly dependent on Akt activation. However, Further studies are required to elucidate the details of this regulatory mechanism

In conclusion, CaMKII enhanced gastric cancer cell metastasis by upregulating NF-κB-/Akt-dependent MMP-9 production. To the best of our knowledge, this is the first study

that demonstrates that CaMKII is able to regulate gastric cancer metastasis. These data provide the theoretical basis for the development of CaMKII inhibitors to treat gastric cancer metastasis.

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