Caveolin-1 enhances RANKL-induced gastric cancer cell migration

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Abstract. The classical pathway involving receptor activator of nuclear factor-κB (RANK) and its ligand (RANKL) induces the activation of osteoclasts and the migration of a variety of tumor cells, including breast and lung cancer. In our previous study, the expression of RANK was identified on the surface of gastric cancer cells, however, whether the RANKL/RANK pathway is involved in the regulation of gastric cancer cell migration remains to be fully elucidated. Lipid rafts represent a major platform for the regulation of cancer signaling; however, their involvement in RANKL-induced migration remains to be elucidated. To investigate the potential roles and mechanism of RANKL/RANK in gastric cancer migration and metastasis, the present study examined the expression of RANK by western blot analysis and the expression of caveolin-1 (Cav-1) in gastric cancer tissues by immunohistochemistry, in addition to cell migration which is measured by Transwell migration assay. The aggregation of lipid reft was observed by fluorescence microscopy and western blotting was used to measure signaling changes in associated pathways. The results showed that RANKL induced gastric cancer cell migration, accompanied by the activation of Cav-1 and aggregation of lipid rafts. Nystatin, a lipid raft inhibitor, inhibited the activation of Cav-1 and markedly reversed RANKL-induced gastric cancer cell migration. The RANKL-induced activation of Cav-1 has been shown to occur with the activation of proto-oncogene tyrosine-protein kinase Src (c-Src). The c-Src inhibitor, PP2, inhibited the activation of Cav-1 and lipid raft aggregation, and reversed RANKL-induced gastric cancer cell migration. Furthermore, it was demonstrated that Cav-1 was involved in RANKL-induced cell migration in lung, renal and breast cancer cells. These results suggested that RANKL induced gastric cancer cell migration, likely through mechanisms involving the c-Src/Cav-1 pathway and lipid raft aggregation.

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Introduction

Tumor metastasis significantly affects the prognosis of patients with gastric cancer, and is the primary cause of treatment failure (1). Mechanisms of tumor metastasis are complex and the tumor microenvironment, enriched in cytokines, growth factors and tumor cell-derived vesicles, is key in its pathophysiology. Receptor activator of nuclear factor-κB ligand (RANKL), an important cytokine belonging to the tumor necrosis factor (TNF) family, promotes osteoclast maturation and migration. In addition to being secreted by osteoclast cells, previous studies have revealed that RANKL is secreted by infiltrating T cells; whereas RANK is expressed on the surface of various cancer cells, including breast, renal and lung cancer cells (2-6). According to our previous study, RANK is also expressed in gastric cancer cells (7), and infiltrating T cells have been found to be abundant in gastric cancer tissues (8,9). Collectively, these studies indicate that RANKL may also promote gastric cancer cell migration, although there is no supporting data at present.

Lipid rafts, comprised of assemblies of cholesterol, sphingolipids and certain types of proteins, form sorting platforms for targeted proteins (10) and are essential in a variety of signaling processes, including cell migration, through the regulation of proteins located in the cell membrane (11,12). Lipid rafts are reported to be able to control human melanoma cell migration by regulating focal adhesion disassembly (13), and promote breast cancer cell migration by restricting interactions between CD44 and ezrin (14). A previous study showed lipid rafts to be critical for RANK functions in osteoclasts (15). Based on this, it was hypothesized that lipid rafts may be involved in RANKL-induced cancer cell migration.

Caveolin-1 (Cav-1), a pivotal component of lipid rafts, is a membrane-bound scaffolding protein that regulates signal transduction (16). The role of Cav-1 in cancer remains controversial; it can regulate a number of metastatic cancer cells, either negatively or positively. Cav-1 reportedly inhibits cell migration and invasion via the suppression of epithelial-mesenchymal transition in pancreatic cancer cells (17), and has been shown to reduce the metastatic capacity of colon cancer cells (18). By contrast, the expression of Cav-1 appears to be increased in prostate tumors, lung cancer, melanoma cells and renal cell carcinoma (18-21), thereby favoring tumor progression and migration (22). RANKL induces the expression of Cav-1, which is immediately conveyed to lipid rafts to promote osteoclastogenesis (23).

As there has been no previous study reporting the effect of Cav-1 on RANKL-induced cell migration, the present study aimed to identify the potential roles and mechanisms of RANKL/RANK in gastric cancer cell migration and metastasis. The results indicated that the proto-oncogene tyrosine-protein kinase Src (c-Src)/Cav-1 pathway and lipid raft aggregation may be the primary mechanisms involved in RANKL-induced gastric cancer cell migration.

Materials and methods

Cell culture. The MGC803, BGC823 and SGC7901 (gastric cancer), H460 (lung cancer), ACHN (renal cancer) and MDA-MB-231 (breast cancer) cells were purchased from the Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MGC803, BGC823 and SGC7901, H460 and ACHN cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA). MDA-MB-231 cells were cultured in L15 medium (Gibco; Thermo Fisher Scientific, Inc.) RPMI-1640 and L15 media were supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml) in an atmosphere of 95% air and 5% CO_2 at 37˚C.

Cell treatment. We added sRANKL (PeproTech, Inc., Rocky Hill, NJ, USA) to cancer cells to final concentration of 10 μg/ml for 0, 5, 10, 30 or 60 min. We added 10 μM PP2 (Sigma-Aldrich St. Louis, MO, USA) or Nystatin (50 μg/ml; cat. no. N3503; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany and/or its affiliates) 1 h prior to sRANKL. To detect the lipid raft aggregation, we used CTXB (1 mg/ml; cat. no. SAE0069-500UG; Sigma-Aldrich; Merck KGaA).

Western blot analysis. Western blot analysis was performed as previously described (24). The following antibodies were used: Anti-phospho-Scr (1:250; rabbit monoclonal; cat. no. 6943S; Cell Signaling Technology, Danvers, MA, USA), anti-Scr (1:1,000; mouse monoclonal; cat. no. 2110s; Cell Signaling Technology), anti-phospho-Cav-1 (1:250; rabbit polyclonal; cat. no. 3251s; Cell Signaling Technology), anti-Cav-1 (1:1,000; rabbit polyclonal; cat. no. sc-894; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-phospho-Akt (1:500; rabbit polyclonal; cat. no. 9271L; Cell Signaling Technology), anti-Akt (1:1,000; rabbit polyclonal; cat. no. 9272S; Cell Signaling Technology), anti-phospho-ERK1/2 (1:500; rabbit polyclonal; cat. no. sc-16982; Santa Cruz Biotechnology), anti-ERK1/2 (1:1,000; rabbit polyclonal; cat. no. 9102S; Santa Cruz Biotechnology), anti-RANK (1:500; rabbit polyclonal; cat. no. A303-897A; Bethyl Laboratories, Inc., Montgomery, TX, USA), anti-β-actin (1:1,000; rabbit polyclonal; cat. no. sc-1616-R; Santa Cruz Biotechnology), followed by incubation with appropriate secondary antibodies. Secondary goat anti-rabbit (1:1,000) and goat anti-mouse antibodies were purchased from Santa Cruz Biotechnology, Inc.

Transwell assay. The cells were pretreated with appropriate solvent control (dimethyl sulfoxide) or various concentrations of inhibitors (PP2: 10 μM; Nystatin: 50 μg/ml) for 60 min in serum-free media. The treated cells were plated in the upper insert of a 24-well chemotaxis chamber ($2x10^4$ cells/well; 8-μm pore size; Corning Inc., Corning, NY, USA) in serum-free medium. Medium containing 2.5% serum (0.5 ml) and recombinant RANKL (1 μl), with DMSO or inhibitors, was added to the bottom well and incubated for 24 h. The porous inserts were carefully removed, and the cells was stained and counted at x200 magnification (Olympus Corp., Tokyo, Japan) in at least five different fields of each filter.

Fluorescence microscopy. The MGC803 cells were first treated with PP2 or nystatin for 1 h, and then RANKL was added at a final concentration of 1 μg/ml for 10 min. The cells were fixed in 4.4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 15 min, and then blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich, Merck KGaA) for 1 h. The slides were incubated with CTXB antibody or anti-RANK antibody for 1 h and then with FITC-conjugated goat anti-mouse or anti-rabbit IgG were added for 1 h. Images were captured with a fluorescence microscope (Olympus Corp.).

Surface RANK expression analysis. Surface RANK expression was determined by flow cytometry as previously described (24). The following antibodies were used: Mouse anti-RANK (1:500; mouse monoclonal; cat. no. MAB683; R&D Systems, Minneapolis, MN, USA) or isotype control (R&D Systems), FITC-conjugated anti-mouse secondary antibody (1:200; mouse monoclonal; cat. no. sc-2356; Santa Cruz Biotechnology).

Transfection with small interfering (si)RNA. The cells were cultivated at a density of $2x10^5$/well in 6-well plates. After 24 h, the cells were transfected with siRNA using Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The CAV-1 siRNAs were designed to target the sequence 5'-AAC CAGAAGGGACACACAGTT-3'. The cells were treated with or without RANKL at 48 h post-transfection. The gene silencing effect was evaluated by western blot analysis.

Patients and tissue samples. Specimens of gastric adenocarcinoma tissue were collected from 228 patients who underwent surgical resection at the First Hospital of China Medical University (Shenyang, China) from March 2006 to October 2011. None of the patients had received operative radiotherapy, chemotherapy or immunotherapy previously. Age, sex, pathological tumor-node-metastasis (pTNM) stage and Lauren

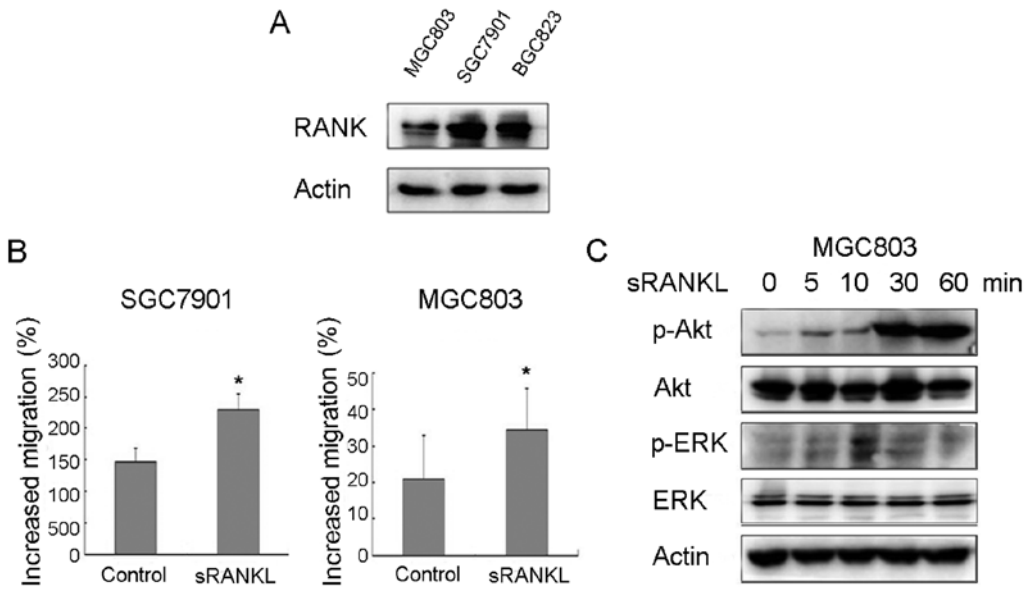

Figure 1. RANKL induces gastric cancer cell migration. (A) Expression of RANK in gastric cancer cells was assessed by western blot analysis. (B) MGC803 and SGC7901 cells were incubated with or without 1 μg/ml recombinant RANKL for 24 h. Then migration ability was measured with a Transwell assay. Error bars represent the standard deviation of three biological replicates. *P<0.05. (C) MGC803 cells were treated with 1 μg/ml recombinant RANKL for the indicated time, and p-Akt/Akt, p-erk/ERK1/2 and β-actin were analyzed by western blot analysis. RANK, receptor activator of nuclear factor-κB; sRANKL, soluble RANK ligand; ERK, extracellular signal-regulated kinase; p-, phosphorylated.

grade were evaluated following medical charts and pathological records. The pTNM stage was examined according to the seventh edition of the AJCC Cancer Staging Manual (25). The Lauren grade was assigned according to the classification of the World Health Organization. The First Hospital of China Medical University Ethical Committee approved the study, and no consent was required due to the retrospective nature of the study.

Immunohistochemistry. Formalin-fixed paraffin-embedded tumor specimens were collected from the Department of Pathology at the First Hospital of China Medical University. The immunohistochemical staining observed with Olympus microscope (Olympus Corp.) was performed using the biotinstreptavidin method (UltraSensitive S-P kit; MaixinBio, Shanghai, China) as previously described (26). Two observers, who had no prior information of the clinical or pathological parameters, performed the evaluation of results independently. The immunoreactivity was scored based on the intensity of staining (negative, 0; weak, 1; moderate, 2; strong, 3).

Statistical analysis. The experimental data are summarized and presented as the mean ± standard deviation. The significance of differences was analyzed statistically using Student's two-tailed t-test, P<0.05 was considered to indicate a statistically significant difference. Each experiment was repeated at least three times. Statistical analyses were performed using the SPSS statistical package software (SPSS for Windows, version 20.0; IBM Corp., Armonk, NY, USA).

Results

RANKL induces the migration of gastric cancer cells via phosphoinositide 3-kinase (PI3K)/Akt and ERK pathways. The western blot analysis revealed the expression of RANK in MGC803, BGC823 and SGC7901 cell lines. Stimulation of the MGC803 and SGC7901 cells with 1.0 μg/ml RANKL significantly increased cell migration by 63.8 and 56.3%, respectively (Fig. 1B). As RANKL had no effect on the proliferation of MGC803 or SGC7901 cells (data not shown), the increased number of MGC803 and SGC7901 cells traversing the filter may have resulted from increased migratory abilities. The downstream signaling of RANKL/RANK was also examined in BGC803 cells; Akt and ERK were markedly increased in response to RANKL treatment (Fig. 1C). Therefore, the RANKL/RANK pathway appeared to be significantly involved in the migration of gastric cancer cells.

Lipid rafts are involved in RANKL-induced migration. Lipid rafts represent a major platform for signaling regulation in cancer. To examine the involvement of lipid rafts in RANKL-induced gastric cancer cell migration, the MGC803 cells were pretreated with nystatin, a lipid raft inhibitor, for 1 h, followed by RANKL treatment for 10 min. The immunofluorescence indicated that RANKL significantly induced lipid raft aggregation, which was reversed by nystatin (Fig. 2A). Downstream signals, including the activation of Akt, were also markedly promoted by RANKL, but were decreased by pretreatment with nystatin (Fig. 2B). Nystatin also decreased RANKL-induced gastric cancer cell migration from 168.8 to 75.6% (Fig. 2C). These results suggested that the aggregation of lipid rafts was associated with RANKL-induced gastric cancer cell migration.

Cav-1 promotes the migration of RANKL-induced gastric cancer cells via interactions with RANK. To investigate the effect of Cav-1 on gastric cancer cell migration, the activation of Cav-1 was examined. The results showed that RANKL not

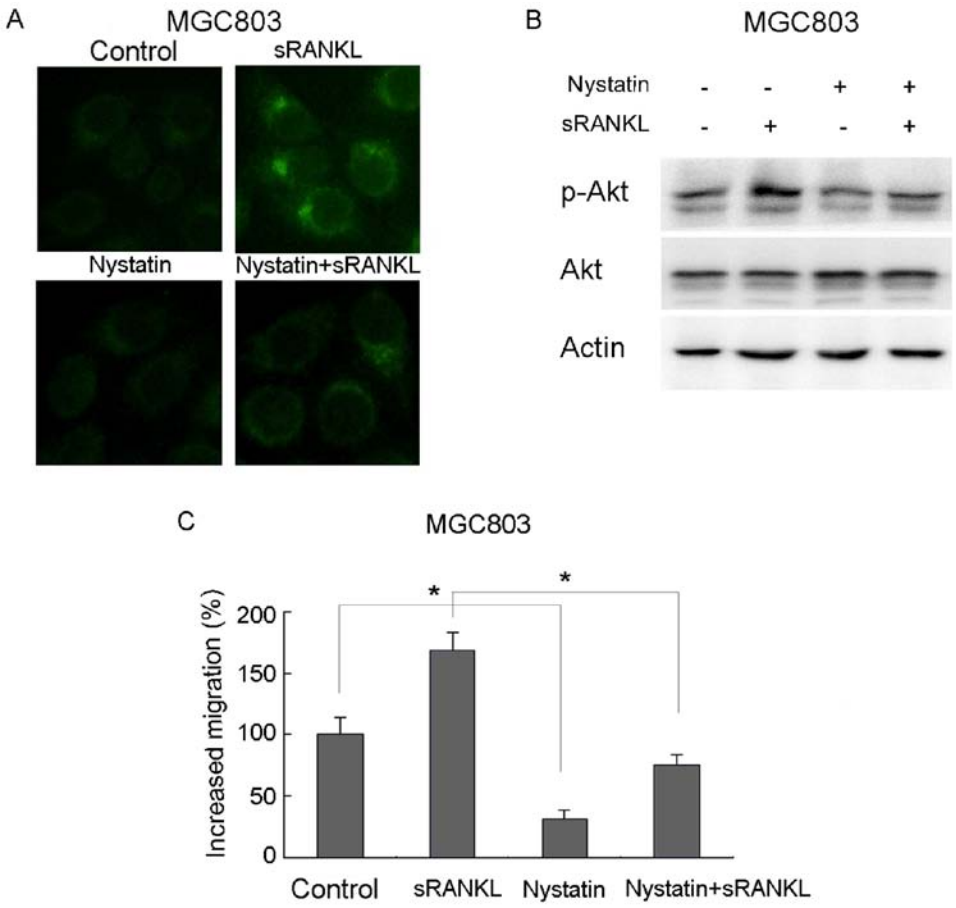

Figure 2. Lipid rafts are involved in RANKL-induced migration. (A) MGC803 cells were pretreated with or without 50 μM nystatin for 1 h, and then with 1 μg/ml recombinant RANKL for 10 min. The lipid raft status was assayed by immunofluorescence following incubation with CTXB (magnification, x40.) (B) MGC803 cells were pretreated with or without 50 μg/ml nystatin for 1 h, and then with 1 μg/ml recombinant RANKL for 10 min. Western blot analysis was used to determine the expression level of p-Akt, Akt and β-actin. (C) Cell migration ability was investigated by Transwell assays. Error bars represent standard deviation of three independent experiments. *P<0.05, vs. corresponding control cells. RANK, receptor activator of nuclear factor-κB; sRANKL, soluble RANK ligand; p-, phosphorylated.

only activated Cav-1 in a time-dependent manner (Fig. 3A), but also triggered an interaction between RANK and Cav-1 (Fig. 3B). The knockdown of Cav-1 by siRNA suppressed RANKL-induced lipid raft aggregation, accompanied by a decrease in the activation of Akt and ERK in MGC803 cells (Fig. 3C and D). Cav-1 knockdown also significantly reduced RANKL-induced gastric cancer cell migration from 176.2 to 18.5% (Fig. 3E). These results suggested that Cav-1 promoted RANKL-induced gastric cancer cell migration via interactions with RANK.

RANKL induces the activity of caveolin-1 via c-Src. To characterize the downstream mechanisms occurring due to the activation of Cav-1, the cells were incubated with RANKL over different periods of time and examined for the activation of c-Src. As shown in Fig. 4A, c-Src was rapidly activated and reached a peak at 10 min. The c-Src inhibitor PP2 inhibited the activation of Cav-1 and Akt/ERK (Fig. 4A). The immunofluorescence and Transwell experiments revealed that PP2 significantly suppressed lipid raft aggregation and RANKL-induced migration (Fig. 4B and C). Collectively, these results suggested that the c-Src-mediated activation of Cav-1 promoted RANKL-induced gastric cancer cell migration.

RANKL-induced migration is suppressed by Cav-1 knockdown. The expression of RANK was examined in a variety of cancer cells by flow cytometry. The results showed that H460 (lung cancer), ACHN (renal cancer) and MDA-MB-231 (breast cancer) cells expressed RANK on their surface (Fig. 5A). The knockdown of Cav-1 by siRNA significantly suppressed RANKL-induced migration of the cancer cells (Fig. 5B and C).

Cav-1 is independently a poor predictive factor for the overall survival rate of patients with gastric cancer. To examine the association between RANK and Cav-1, 228 histologically confirmed gastric cancer samples were selected for investigation. The follow-up time ranged between 3 and 83 months, with a mean follow-up time of 38 months. The immunostaining confirmed that Cav-1 was expressed in 56.5% of patients (Table I), whereas 47.4% were positive for RANK. The correlation between the expression of RANK or Cav-1 and patient characteristics is shown in Table I. The expression of RANK, observed in 58.3% of the diffuse patients, was correlated with Lauren classification. The prognostic value of Cav-1 in patients with RANK-positive cells was also analyzed. Within this population, a higher expression of Cav-1 was correlated with poor survival rate (P=0.025), as the mean overall

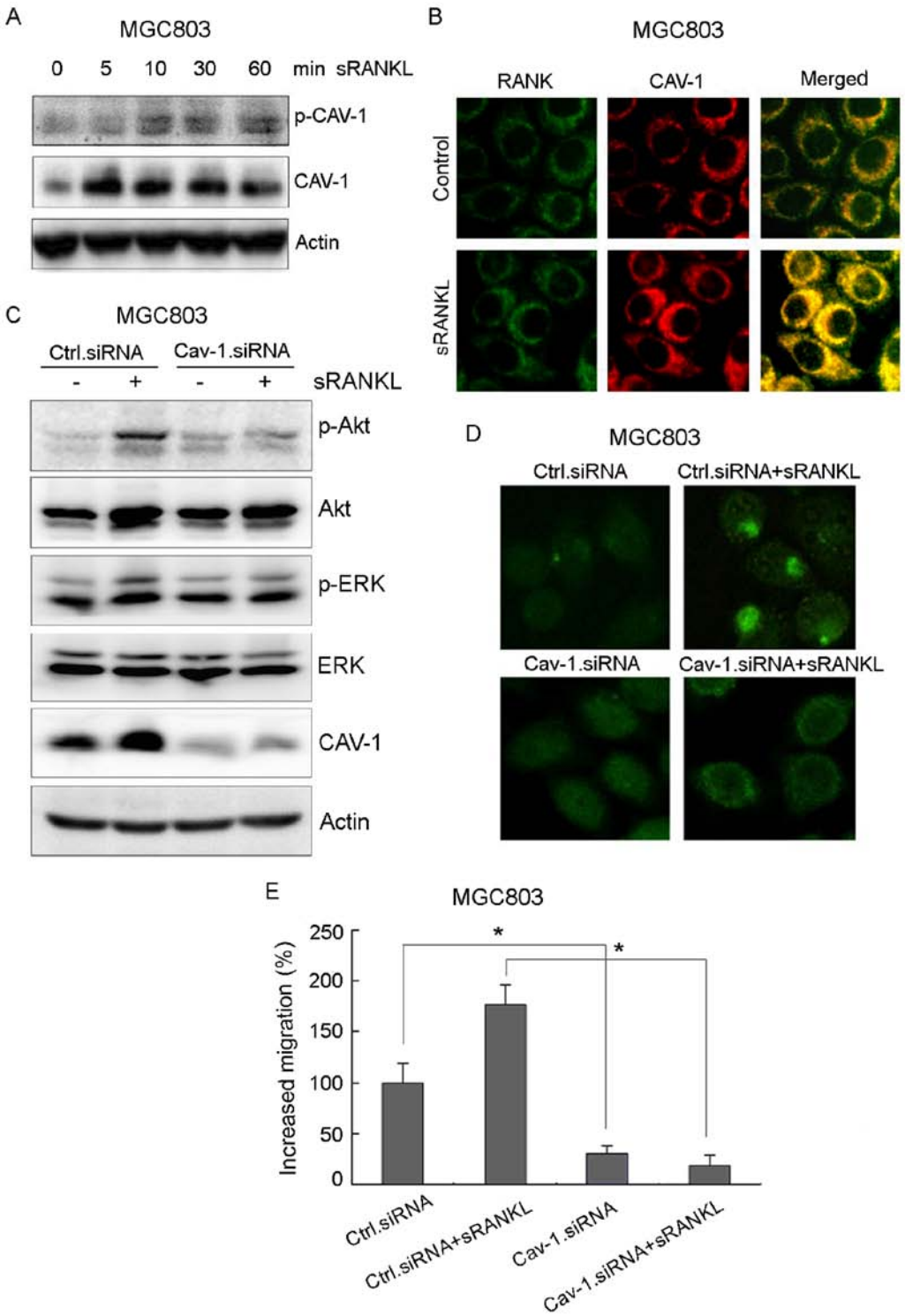

Figure 3. Cav-1 promotes RANKL-induced gastric cancer cell migration via interaction with RANK. (A) MGC803 cells were treated with 1 μg/ml recombinant RANKL at indicated times, and the activation of Cav-1, Akt and ERK was examined by western blot analysis. (B) MGC803 cells were treated with 1 μg/ml recombinant RANKL for 10 min, and the interaction between Cav-1 and RANK was analyzed by immunofluorescence at high magnification (x40). RANK and Cav-1 were indicated as green and red respectively. (C) Cav-1 siRNA or control siRNA were transfected into MGC803 cells. Lipid raft status was analyzed by immunofluorescence following incubation with CTXB (magnification, x40). (D) Cav-1 siRNA or control siRNA transfected cells were treated with 1 μg/ml recombinant RANKL for 10 min, and the activation of Cav-1, Akt and ERK was examined by western blot analysis. (E) Migration activity of MGC803 cells was measured with a Transwell assay following treatment with 1 μg/ml recombinant RANKL for 24 h. Error bars represent the standard deviation of three independent experiments. *P<0.05, vs. corresponding control cells (Student's t-test). RANK, receptor activator of nuclear factor-κB; sRANKL, soluble RANK ligand; Cav-1, caveolin-1; ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA; p-, phosphorylated; Ctrl, control.

survival rate of patients was 45 months in the Cav-1-positive arm, compared with 64 months in the Cav-1-negative arm (Fig. 6). In patients with RANK-positive cells, univariate analysis revealed that the positive expression of Cav-1, T stage, N stage and pTNM stage indicated poor prognosis. The multivariate analysis indicated that Cav-1, T stage and N stage

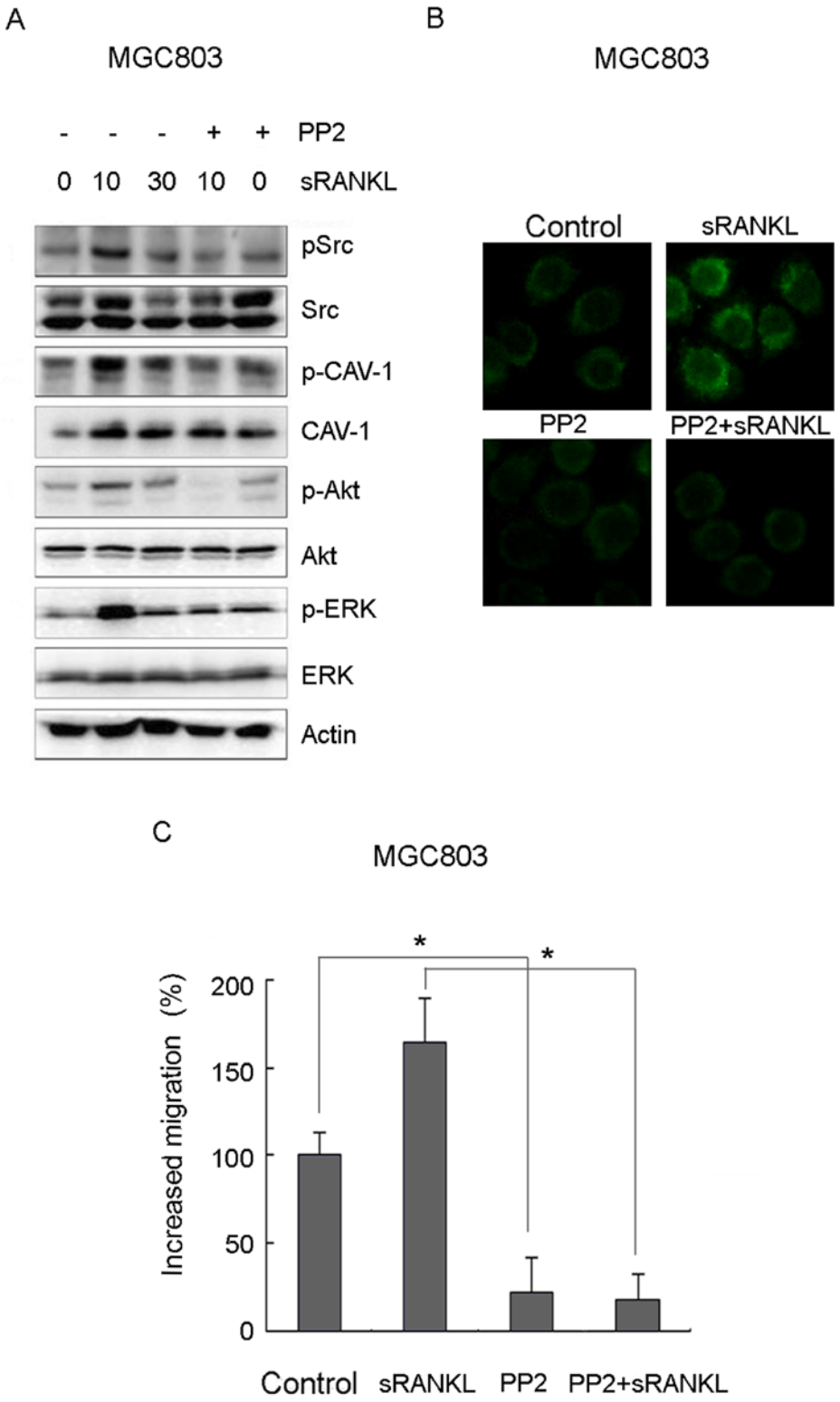

Figure 4. Src-mediated activation of Cav-1 promotes RANKL-induced gastric cancer cell migration. (A) MGC803 cells were pretreated with 10 μM PP2 or control for 1 h, following incubation with 1 μg/ml recombinant RANKL for the indicated times. The expression levels of pSrc/Src, pCav-1/Cav-1, pAkt/Akt, pERK/ERK were examined by western blot analysis. (B) MGC803 cells were pretreated with or without 10 μM PP2 for 1 h, and then treated with or without 1 μg/ml recombinant RANKL for 10 min. Lipid raft status was observed by immunofluorescence at high magnification (x40). (C) Cell migration was examined by Transwell assays. Error bars represent the standard deviation. Data are representative of three independent experiments. *P<0.05, vs. corresponding control cells (Student's t-test). RANK, receptor activator of nuclear factor-κB; sRANKL, soluble RANK ligand; Cav-1, caveolin-1; ERK, extracellular signal-regulated kinase; p-, phosphorylated.

were independent predictors for patients with RANK-positive cells (Table II). These results demonstrated that the expression of Cav-1 was predictive of poor prognosis in patients with RANK-positive gastric cancer cells.

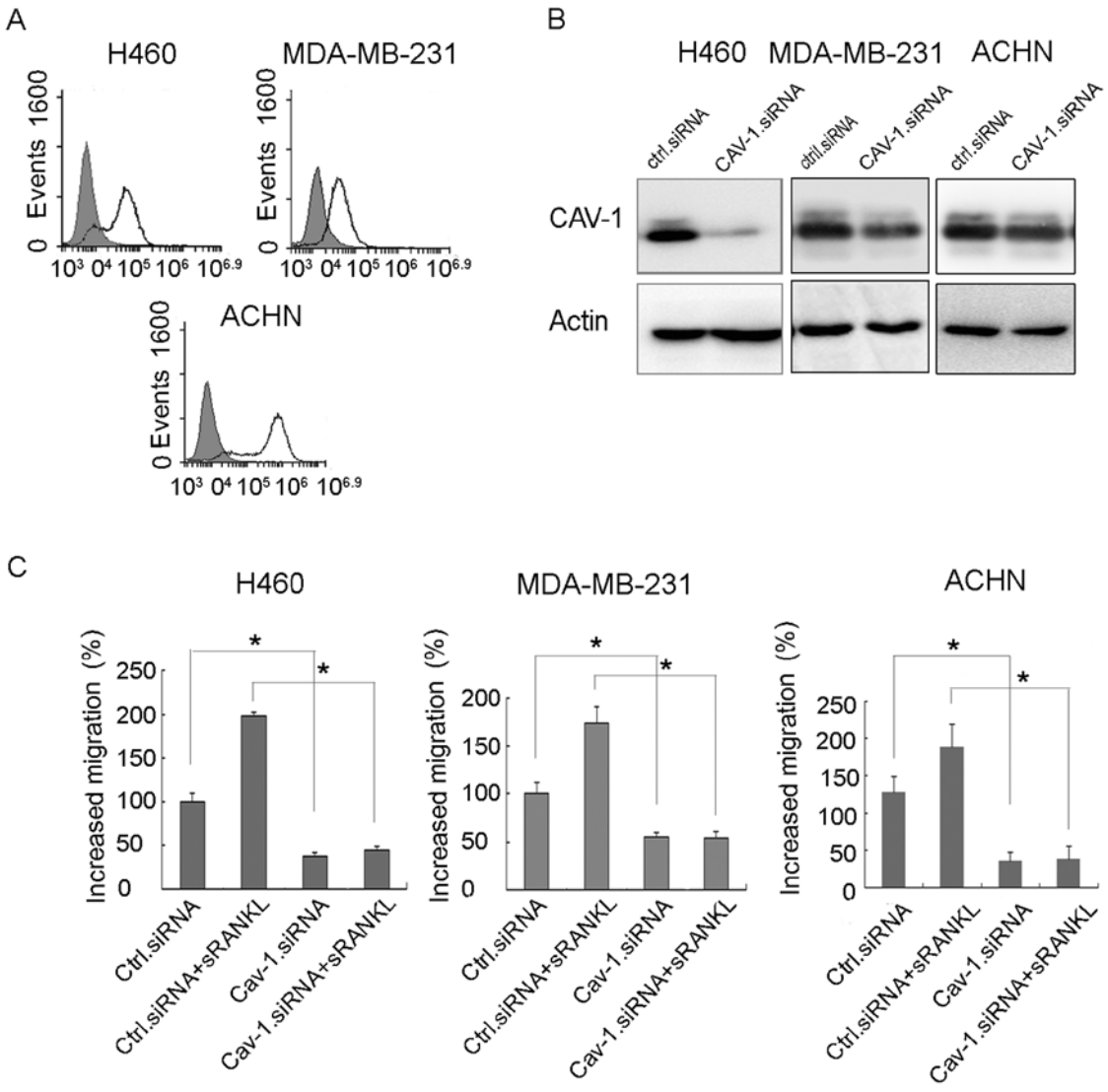

Figure 5. RANKL-induced cancer cell migration is promoted by activation of Src and Cav-1. (A) Expression of RANK was examined by flow cytometry. (B) Expression of RANK in cancer cells was assessed by western blot analysis. (C) Cav-1 siRNA or control siRNA/control was transfected into H460, ACHN and MDA-MB-231 cells, and migration activities of these cells were measured with the Transwell assay following treatment with 2 μg/ml recombinant RANKL for 24 h. Error bars represent the standard deviation of three independent experiments. *P<0.05, vs. corresponding control cells (Student's t-test). RANK, receptor activator of nuclear factor-κB; sRANKL, soluble RANK ligand; Cav-1, caveolin-1; siRNA, small interfering RNA; Ctrl, control.

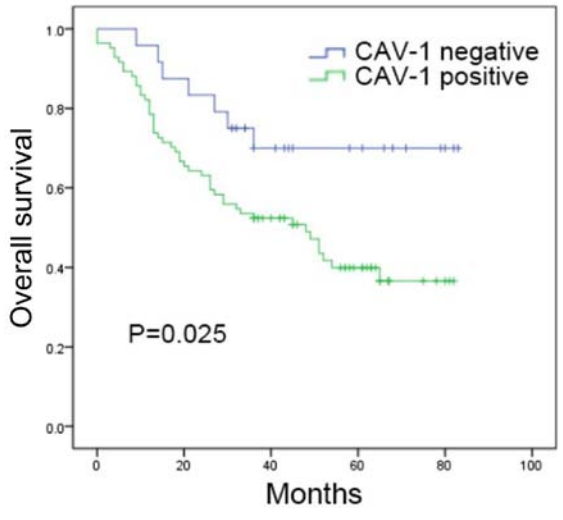

Figure 6. A. Kaplan-Meier survival curves for overall survival rate in patients with RANKL-positive gastric cancer. Cav-1, caveolin-1.

Discussion

The RANKL/RANK pathway is a classical pathway for osteoclast maturation and activation, whereby RANKL interacts with RANK to recruit TNF-receptor associated factor, resulting in the activation of nuclear factor-FB, c-Jun N-terminal kinase, p38, ERK and Akt (27-29). In breast, lung and prostate cancer cells, the inhibition of PI3K and mitogen-activated protein kinase kinase 1/2 can reduce RANKL-induced migration (30-32). According to the results of the present study, RANK was expressed in gastric cancer cells. Furthermore, RANKL significantly increased the migration ability of gastric cancer cells, accompanied by the activation of Akt and ERK. As gastric cancer tissues are enriched in infiltrating T cells capable of secreting RANKL, RANKL-induced migration may represent a pivotal mechanism for gastric cancer metastasis. Drugs, including denosumab, which target the RANKL/RANK pathway, likely inhibit this process and

Table I. Correlation of the expression of RANK and Cav-1 with clinic-pathological parameters in 228 patients with gastric cancer.

		RANK			Cav-1		
Factor	n	Negative (%)	Positive (%)	P-value	Negative (%)	Positive (%)	P-value
Number	228	120 (52.6)	108 (47.4)		74 (43.5)	154 (56.5)	
Age (years)				0.111			0.089
≤60	107	50 (46.7)	57 (53.3)		41 (38.3)	66 (61.7)	
>60	121	70 (57.9)	51 (42.1)		33 (27.3)	88 (72.7)	
Sex				0.307			0.439
Male	162	89 (54.9)	73 (45.1)		50 (30.9)	112 (69.1)	
Female	66	31 (47.0)	35 (53.0)		24 (36.4)	42 (63.6)	
T stage				0.500			0.714
T1	2	0 (0)	2 (100)		1 (50.0)	1 (50.0)	
T2	18	10 (55.6)	8 (44.4)		4 (22.2)	14 (77.8)	
T3	36	17 (47.2)	19 (52.8)		12 (33.3)	24 (66.7)	
T4	172	93 (54.1)	79 (45.9)		57 (33.1)	115 (66.9)	
N stage				0.869			0.149
N1	51	27 (52.9)	24 (47.1)		19 (37.3)	32 (62.7)	
N2	36	21 (58.3)	15 (41.7)		14 (38.9)	22 (61.1)	
N3	47	25 (53.2)	22 (46.8)		9 (19.1)	38 (80.9)	
N4	94	47 (50.0)	47 (50.0)		32 (34.0)	62 (66.0)	
pTNM stage				0.540			0.323
I+II	55	31 (56.4)	24 (43.6)		21 (38.2)	34 (61.8)	
III+IV	173	89 (51.4)	84 (48.6)		53 (30.6)	120 (69.4)	
Lauren grade				**<0.001**			0.059
Intestinal	89	62 (69.7)	27 (30.3)		21 (23.6)	68 (76.4)	
Diffuse	98	35 (35.7)	63 (64.3)		39 (39.8)	59 (60.2)	
Mixed	41	23 (56.1)	18 (43.9)		14 (34.1)	27 (65.9)	
Location				0.672			
Cardia	28	15 (53.6)	13 (46.4)		10 (35.7)	18 (64.3)	
Body	20	13 (65.0)	7 (35.0)		6 (30.0)	14 (70.0)	
Antrum	147	74 (50.3)	73 (49.7)		46 (31.3)	101 (68.7)	
Other	33	18 (54.5)	15 (45.5)		12 (36.4)	21 (63.6)	
Histological classification				**<0.001**			**0.023**
Well	12	8 (66.7)	4 (33.3)		3 (25.0)	9 (75.0)	
Moderate	75	53 (70.7)	22 (29.3)		16 (21.3)	59 (78.7)	
Poor	141	59 (41.8)	82 (58.2)		55 (39.0)	86 (61.0)	

P-values shown in bold are statistically significant (two-sided, P<0.05). RANKL, receptor activator of nuclear factor-κB; Cav-1, caveolin-1; pTNM, pathological tumor-node-metastasis.

Table II. Cox univariate and multivariate analyses of overall survival in patients with receptor activator of nuclear factor-κB-positive gastric cancer (n=228).

	Univariate			Multivariate		
Biomarker	Hazard	95% CI	P-value	Hazard	95% CI	P-value
Age	1.489	0.876-2.530	0.142			
T stage	2.812	1.410-5.609	**0.003**	2.559	1.292-5.065	**0.007**
N stage	1.518	1.176-1.960	**0.001**	1.496	1.156-1.936	**0.002**
pTNM stage	3.688	1.468-9.263	**0.005**			
Lauren	1.102	0.738-1.645	0.635			
Caveolin-1	2.392	1.082-5.289	0.031	2.603	1.174-5.773	**0.019**

P-values shown in bold are statistically significant (two-sided, P<0.05).

can be potentially used as novel therapeutic intervention for treating metastatic gastric cancer.

Previous studies have provided evidence in support of the involvement of lipid rafts in cancer cell invasion and metastasis (33-35). Yamaguchi *et al* reported the requirement of lipid rafts for invadopodia formation and extracellular matrix degradation in human breast cancer cells (36). Chinni *et al* showed that C-X-C motif chemokine ligand 12/C-X-C chemokine receptor type 4 transactivates human epidermal growth factor receptor 2 in lipid rafts to promote prostate cancer cell migration (37). In the present study, the finding that RANKL triggered lipid raft aggregation, which was reversed by nystatin, and reduced RANKL-induced migration in gastric cancer cells indicated the importance of lipid rafts in gastric cancer cell migration. Lipid rafts are known to be regulated by other important factors, including Cav-1. Cav-1 can also result in further clustering of lipid rafts mediated by the activation of several downstream signaling pathways (36,38). In the present study, Cav-1 was shown to be involved in RANKL-induced lipid raft aggregation and cell migration. It was confirmed that certain RANK-expressing gastric cancer cells also express Cav-1, which was significantly correlated with the poor prognosis in individuals with RANK-positive cells. Univariate and multivariate analyses demonstrated that the expression of Cav-1 was an independent predictor of poor overall survival rate in these patients. Furthermore, the involvement of Cav-1 in RANKL-induced cell migration was confirmed in several cancer cell lines. These findings indicated that Cav-1 is essential not only for appropriate RANK-localization within the lipid raft, but also for RANKL-induced lipid raft aggregation and cancer cell migration.

Although the data obtained in the present study revealed that Cav-1 was rapidly activated by RANKL, the question regarding the key mediator remains unanswered. The tyrosine protein kinase c-Src is known to be involved in the regulation of cellular metabolism, survival and proliferation. In cancer cells, the activation of c-Src results in increased tumor progression, invasion and metastasis (39-42). Furthermore, RANKL has shown potential in activating c-Src in breast cancer cells (30). Previous reports have suggested that the interaction between Cav-1 and Rho-GTPases promotes metastasis by controlling the activation of c-Src, Ras and Erk (43). In the present study, the activation of Cav-1 accompanied that of c-Src. In addition, the activation of Cav-1, lipid raft aggregation and cell migration were almost completely reversed by the PP2-mediated inhibition of c-Src function, which is an important regulator in several signaling pathways (44). These results suggested that the c-Src-mediated activation of Cav-1 promoted RANKL-induced gastric cancer cell migration.

In conclusion, RANKL-induced gastric cancer cell migration is at least partially dependent on lipid rafts and its main component, Cav-1, and is promoted by the activation of c-Src and Cav-1. These findings demonstrate a detailed mechanism underlying the effect of RANK on gastric cancer cell migration. This may shed light on the potential drug targets for novel treatment of metastatic gastric cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YW, YL and XQ conceived and designed the study. YW, QW, XZ, LZ, JQ, ZL, LX, YZ, KH, YF and XC performed the experiments. YS provided the samples and collected the patient information. XC and YW contributed in the statistical analysis. YW wrote the manuscript. XQ, YL and XC reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The First Hospital of China Medical University Ethical Committee approved the study. No consent was required due to the retrospective nature of the study.

Patient consent for publication

No consent was required due to the retrospective nature of the study.

Competing interests

The authors declare that they have no competing interests.

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