APPL1 promotes the migration of gastric cancer cells by regulating Akt2 phosphorylation

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Abstract. As a multifunctional adaptor protein, APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and a leucine zipper motif 1) is overexpressed in many cancers, and has been implicated in tumorigenesis and tumor progression. The present study investigated the expression of APPL1 in gastric carcinoma and the function in regulating cell migration. We investigated the expression of APPL1 in gastric carcinoma based upon The Cancer Genome Atlas (TCGA) database. The expression of APPL1 in collected gastric carcinoma tissues and cultured cells was measured by qRT-PCR and western blot analysis. Transwell assay and wound healing assay were used to analyze the effects of APPL1 on tumor cell migration. The statistical results based upon TCGA database showed significantly higher expression of APPL1 in gastric carcinoma compared to adjacent normal tissues, and we confirmed these findings by measuring APPL1 expression in collected gastric carcinoma tissues and cultured cells. The results of Transwell assay and wound healing assay showed that when APPL1 was silenced by siRNA, cell migration was inhibited and overexpression of APPL1 promoted migration. Western blot results demonstrated that changes in several mesenchymal markers were consistent with the observed reduction or enhancement of cell migration. Importantly, the expression of APPL1 significantly affected the phosphorylation of Akt2. In addition, MMP2 and MMP9, downstream effectors of Akt2 changed accordingly, which is a critical requirement for Akt2-mediated cell migration. The results demonstrate an important new function of APPL1 in regulating cell migration through a mechanism that depends on Akt2 phosphorylation.

Introduction

Gastric cancer (GC) is one of the most common human malignant diseases and the second leading cause of cancer mortality worldwide (1). The reason for the high fatality rate associated with this disease is that most cases of GC are clinically detected only at an advanced stage with distant metastasis. Metastasis is the spread of cancer cells from their primary location to other parts of the body. Unfortunately, once cancer becomes metastatic it cannot be treated effectively by surgical and radiation therapies (2). Tumor metastasis is a complex process that requires the integration of signaling events that occur in distinct locations within the cell. However, little is known about molecular mechanisms underlying tumor metastasis (3,4). Therefore, a highly critical issue is to explore the molecular mechanisms and to identify 'key' molecular markers related to metastasis in gastric cancer, which will provide new targets for intervention in the metastatic recurrence of gastric cancer (5).

The recent discovery of altered adaptor proteins in cancer has identified a fundamental change involved in cell migration and invasion (6-8). Adaptor proteins are composed exclusively of domains and motifs that mediate molecular interactions, and can thereby link signaling proteins such as activated cell-surface receptors to downstream effectors. Adaptor proteins are emerging as important regulators of key signaling events that control cellular behavior underlying many biological and pathological processes (6). Adaptor proteins accomplish this through their multiple functional domains by bringing together and targeting protein-binding partners to specific locations within cells. For example, APPL1 is a 709 amino acid, endosomal protein, containing a pleckstrin-homology (PH) domain, phosphotyrosine-binding (PTB) domain and leucine zipper motif. APPL1 interacts with 14 proteins, including follicle-stimulating hormone receptor, deleted in colorectal carcinoma (DCC), Rab5a and Akt2 (9). These membrane receptors and signaling molecules take part in various signaling pathways to

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mediate apoptosis, development (10), cell proliferation, chromatin remodeling (11), DNA repair (12) and cell survival (13). Recently, emerging data suggest that APPL1 also plays a key role in the regulation of cell migration (14). However, the molecular mechanism is not well understood.

APPL1 was originally identified as an Akt2-binding protein in a yeast two-hybrid screening system and Akt2 is a known regulator of cell migration (15). Akt is a serine/threonine kinase that is activated downstream of phosphatidylinositol 3-kinase. Subsequently, active Akt phosphorylates its downstream effectors to regulate several cellular processes, including cell growth, survival and proliferation (16). Moreover, there has recently been growing interest in the function of Akt in the regulation of cell migration. Akt has been shown to stimulate the migration of epithelial cells, fibroblasts and fibrosarcomas (17,18).

Recent studies have indicated that APPL1 gene amplification is common in breast (20), prostate cancer (7) and several cell lines, including pancreatic carcinoma cells (12), HCT116 and SW480 colorectal cancer cell lines (9). Importantly, the expression of APPL1 protein and mRNA were highly upregulated in gastric cancer. It was also reported that the expression of APPL1 in GC was statistically associated with depth of infiltration and lymph node metastasis (19). The observation suggested that expression of APPL1 had a role in tumor infiltration and metastasis. However, little is known about the molecular mechanism of APPL1 in tumor metastasis in gastric cancer. In the present study, we investigated the expression of APPL1 protein in gastric cancer and its direct effect on cell migration. We also showed that APPL1 promoted cell migration via the Akt2 pathway using loss of function assays. Our results suggest that APPL1 promotes invasion and metastasis of gastric cancer cells and the underlying molecular mechanism may facilitate Akt2 phosphorylation and activation of downstream effectors.

Materials and methods

Retrieval of TCGA public data. Based on The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/) public datasets, we evaluated and analyzed APPL1 expression in gastric carcinoma. Clinical information and gene expression profile data were downloaded at the website of the UCSC cancer browser (http://xena.ucsc.edu/). The APPL1 mRNA expression levels were measured experimentally using the Illumina HiSeq 2000 RNA Sequencing platform of the British Columbia Cancer Agency TCGA genome characterization center. The results was shown as in $\log_2$ RPKM (reads per kilobase of exon model per million mapped reads), which approximates the relative abundance of APPL1 transcripts in different samples. The value of APPL1 expression of the gastric carcinoma was compared with the value of adjacent non-cancerous stomach tissues; the clinical data were then used to analyze the association between the APPL1 expression and selected clinical characteristics.

The expression of miR-145 in gastric carcinoma was also downloaded from TCGA datasets, shown as in $\log_2$ RPM (reads per million mapped reads). The correlation between miR-145 and APPL1 expression in GC tissues was analyzed by using the Pearson's correlation coefficient.

Human gastric carcinoma tissue collection and cell culture. Human gastric carcinoma and adjacent normal tissues were consecutively collected between April 2013 and November 2015 at the Pathology Department of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University. No local or systemic treatment was conducted prior to operation. Clinicopathological data such as age and gender, as well as lymph node metastasis status, lymphatic and venous invasion status, tumor stage, and pTNM stage were obtained by reviewing their pathology records. Tumor stage was determined according to the American Joint Committee on Cancer (AJCC) staging criteria. A total of 32 paired samples were performed to examine APPL1 expression, including 18 (56.3%) men and 14 (43.7%) women. The age distribution of the patients examined ranged from 42 to 78 years of age. Informed consent was obtained from each patient and the study was approved by the Institute Research Ethics Committee at the Cancer Center of Xi'an Jiaotong University.

Stomach adenocarcinoma cell lines (including AGS, BGC-823, SGC-7901 and MKN-45) and a GES-1 cell line were obtained from the Shanghai Genechem Co., Ltd. (Shanghai, China). The cells were grown at 37˚C in a 5% CO_2 incubator, in RPMI-1640 culture media supplemented with fetal bovine serum (FBS).

RNA extraction and quantitative real-time PCR. Total RNA was isolated from prepared gastric issues and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), measured spectrophotometrically using NanoDrop (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). cDNA was synthesized by using PrimeScript® RT reagent kit (DRR037A; Takara, Dalian, China), according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using the SYBR-Green PCR kit (Takara), and was conducted in the IQ5 Optical System real-time PCR machine. The relative expression of APPL1 genes was calculated with the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Table I.

Western blot analysis. Protein expression levels were assessed using western blot analysis. In brief, total cell lysates from different experiments were obtained by lysing the cells in RIPA buffer. Following protein concentration assay, equal amounts of protein were run on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature, and incubated with the various primary antibodies overnight at 4˚C, including mouse monoclonal antibody (mAb) anti-APPL1 (dilution 1:1,000; sc-271901; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit mAb anti-E-cadherin (dilution 1:1,000; ab-133597; Abcam), rabbit mAb anti-N-cadherin (dilution 1:5,000; ab76011; Abcam), rabbit mAb anti-vimentin (dilution 1:1,000; ab-92547; Abcam), rabbit mAb anti-Akt2 (dilution 1:1,000; #2964; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit mAb anti-phospho-Akt2 (p-Akt2) (dilution 1:1,000; #4060; Cell Signaling Technology), rabbit mAb anti-MMP2 (dilution 1:1,000; ab92536; Abcam), rabbit mAb anti-MMP9 (dilution 1:1,000; ab76003; Abcam) and mouse mAb anti-β-actin (dilution 1:5,000; 66009-1-Ig; Proteintech Group, Inc., Rosemont, IL, USA). The PVDF membranes

Table I. DNA primer and miRNA sequences.

DNA/siRNA	Genes	Sequences
PCR primer	APPL1	F: 5'-ACGGGCCCTCTAGACTCGAGCGCCACCATGCCGGGGATCGACAAGCTGCCC-3'
		R: 5'-AGTCACTTAAGCTTGGTACCGATGCTTCTGATTCTCTCTTCTTTCCTC-3'
		F: 5'-GCCCGCAGACAAGGTCTTTA-3'
		R: 5'-TGAGGTCAGGTGTGTTGCTG-3'
	β-actin	F: 5'-CCAACCGCGAGAAGATGA-3'
		R: 5'-CCAGAGGCGTACAGGGATAG-3'
APPL1 3'-UTR	wt	5'-CGACATAAAGATTTGA**_AACTG_**GAAC-3'
		5'-TCGAGTTCCAGTTTCAAATCTTTATGTCGAGCT-3'
	mut	5'-CGACATAAAGATTTGA**_CGAC_**GAAC-3'
		5'-TCGAGTTCGTCGTCAAATCTTTATGTCGAGCT-3'
siRNA	si-APPL1	Top: 5'-GCUCAGAUAAGUUUCUUUA-3'
		Bottom: 5'-UAAAGAAACUUAUCUGAGC-3'
	NC	Top: 5'-AAUUCUCCGAACGUGUCACGU-3'
		Bottom: 5'-ACGUGACACGUUCGGAGAAUU-3'

were then incubated with the corresponding secondary antibodies [horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, 1:2,000, SA00001-1; Proteintech Group; HRP-conjugated goat anti-rabbit IgG, 1:2,000, SA00001-2; Proteintech Group] for 1 h at room temperature and washed three times with TBST. Finally, the membranes were incubated with ECL (Pierce, Rockford, IL, USA) for chemiluminescence detection. Luminescent signals were detected and recorded by Syngene's G:BOX (Syngene, Cambridge, UK). Relative protein expression of APPL1 was then normalized to β-actin levels in each sample.

siRNA synthesis and transfection. Small interfering RNAs (siRNAs) were designed for APPL1 gene silencing, and were synthesized by Shanghai GenePharma. Scramble siRNA was used as negative control (NC siRNA). The sequences are listed in Table I. MNK-45 and AGS cells were cultured for 24 h in plates and the siRNAs were transiently transfected into the cells using jetPRIME® reagent (Polyplus-transfection) according to the manufacturer's protocol.

Plasmid construction and transfection. The APPL1 expression vector was constructed by Shanghai Genechem. The full-length of APPL1 cDNA was generated by PCR amplification (The primer sequences are listed in Table I). The PCR product was subcloned into GV141 plasmid using the *Xho*I/*Kpn*I restriction sites. Proper construction of plasmids was verified by automated sequencing. Transfections into BGC-823 and SGC-7901 cells were carried out using jetPRIME® reagent, according to the manufacturer's protocol.

Wound healing assay. A wound healing assay was performed to examine the capacity for cell metastasis. Briefly, cells were cultured to 90% confluence in 12-well plates. A 200-μl disposable pipette tip was used to create a linear scratch wound. Photographs were taken immediately after wound induction

and following 48-h incubation. The extent of wound closure was measured using ImageJ software.

Transwell assay. Cells ($2.0x10^4$) in serum-free medium were plated into the upper chamber (8 μm pore size; Millipore, Billerica, MA, USA), and the bottom wells were filled with complete medium. The cells were allowed to migrate across the membrane for 48 h. Following incubation, the cells were removed from the upper surface of the filter by using a cotton swab. The migrant cells that adhered to the bottom of the membrane were stained with 1% crystal violet. Quantitative analysis of migration rates was performed by solubilization of crystal violet and obtaining optical density (OD) at 570 nm.

Bioinformatic analysis. Information about human miR-145 was registered and obtained in miRBase (http://www.miRBase.org/). The prediction of miRNA targets was acquired from TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/) public databases.

Dual-Luciferase reporter assay. The primary transcript of miR-145 was synthesized and cloned into the pcDNA6.2GW/EmGFP vector using *Eco*RI/*Hin*dIII restriction sites. The 3'-UTR of APPL1 containing miR-145 binding sites was cloned downstream of the luciferase reporter in the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). This pmirGLO-APPL1-3'-UTR vector was cotransfected with pcDNA6.2GW/EmGFP-miR-145 vector into BGC-823 cell lines. The pmirGLO vector and a vector containing mutual binding site were used as the control (The sequences are listed in Table I). The reporter gene assays were performed 48 h post-transfection using the Dual-Luciferase reporter assay system (Promega), according to the manufacturer's instructions. The normalized firefly luciferase activity (firefly luciferase activity/*Renilla* luciferase activity) for each construct was compared with that of the pmirGLO
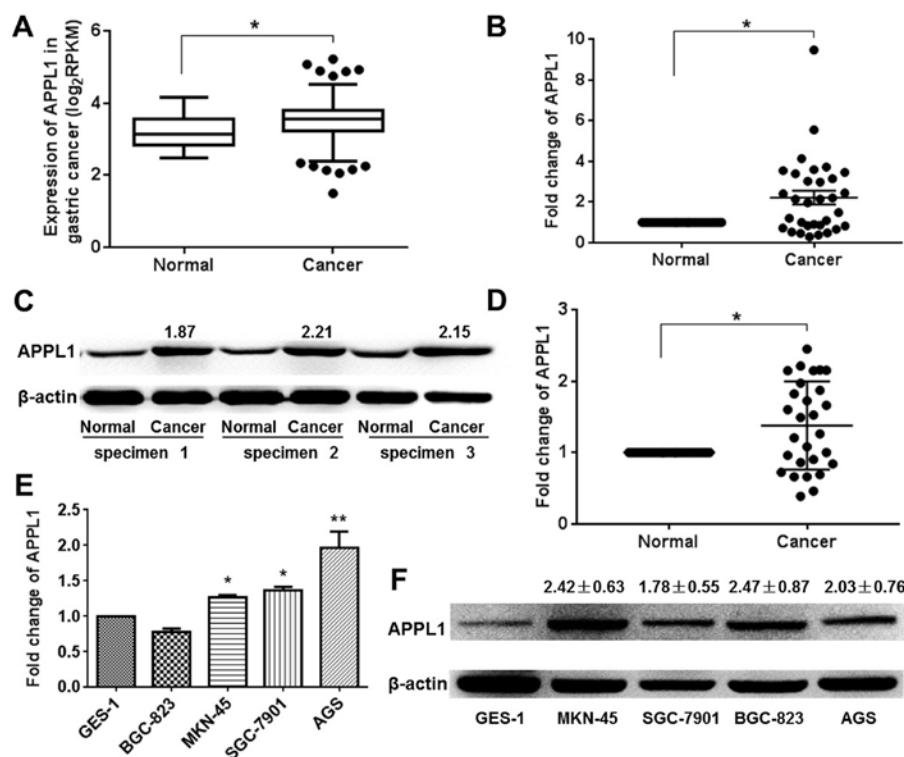
Figure 1. APPL1 is frequently highly expressed in gastric cancer tissues and cells. (A) TCGA datasets showing APPL1 expression (log₂RPKM) was signifi-cantly upregulated in 380 gastric carcinoma compared with 37 adjacent normal stomach tissues. Data are presented as a box plot with median and 25th-75th percentiles (boxes) and 5th-95th percentiles (whiskers). (B) qRT-PCR was performed to examine APPL1 mRNA expression in 32 paired human gastric carci-noma and adjacent normal tissues. (C) Representative western blot results for APPL1 protein expression for three patient samples. (D) Western blot analysis was performed to examine APPL1 protein expression in 28 paired human gastric carcinoma and adjacent normal tissues. (E and F) qRT-PCR and western blot analyses of APPL1 mRNA and protein expression in normal gastric mucosa epithelial cell and gastric carcinoma cells. (Student's t test; *P<0.05; **P<0.01).

vector control. All experiments were performed at least three times.

Statistical analysis. Each experiment was repeated at least three times. Data are presented as means ± SD. Unless otherwise indicated, the statistical significance of differences between each pair of groups was analyzed using a Student's t-test (two-tailed). All statistical analyses were performed using GraphPad Prism version 7.0 for Windows (Graphpad Software, Inc., San Diego, CA, USA).

Results

APPL1 is frequently highly expressed in human gastric cancer tissue samples and cell lines. In order to evaluate and analyze APPL1 expression in gastric carcinoma, an indepen-dent cohort of 417 patient specimens (including 380 gastric carcinoma tissues and 37 adjacent normal tissues) was down-loaded from TCGA datasets. The results showed that the mean expression value of APPL1 was increased in gastric carcinoma tissues compared to adjacent normal tissues (3.52±0.025 vs. 3.19±0.007; P<0.05) (Fig. 1A). To validate APPL1 expression observed in the TCGA datasets, we examined the expression of APPL1 in human gastric cancer tissue samples collected from the Pathology Department of our affiliated hospital using qRT-PCR and western blot analysis. The results of qRT-PCR showed that most gastric cancer patients (21 of 32, 65.6%) showed higher APPL1 expression levels, compared

with corresponding adjacent normal tissues (Fig. 1B). The upregulation of APPL1 protein in gastric carcinoma was also detected by using western blot analysis. Three representative western blot results were selected and shown in Fig. 1C. The quantitative statistical results showed there were 13 gastric cancer patients with higher APPL1 expression levels (Fig. 1D). It was observed that the level of APPL1 mRNA and protein were significant upregulated in four gastric carcinoma cell lines, including MKN-45, SGC-7901, BGC-823 and AGS cell lines, compared with normal gastric mucosa epithelial cell line GES-1 (Fig. 1E and F). The results suggested that increased APPL1 expression was a frequent event in human gastric cancer tissues, which was interestingly consistent with the statistical results of APPL1 expression in TCGA datasets.

Association of APPL1 mRNA expression with clinicopatho-logical outcomes in GC. In order to investigate the role of increased expression of APPL1 in gastric carcinoma, we analyzed the association of APPL1 mRNA expression with the clinicopathological outcomes. Based on qRT-PCR results, the association of APPL1 expression with the clinicopatho-logical outcome of 32 patient specimens was analyzed and listed in Table II. Since the sample size is limited, there was no significant association between the expression of APPL1 with clinicopathological features of patients from our insti-tute (P>0.05). In order to effectively enlarge the sample size, we downloaded 190 patient specimens clinicopathological outcomes from TCGA datasets. The results shown here are

Table II. Association between the expression of APPL1 with clinicopathological features in 32 patients collected by ourselves.

Variables	Patients High (n=21)	Patients Low (n=11)	Percentage High	Percentage Low	P-value
Sex					
Male	13	5	61.90%	45.45%	0.3730
Female	8	6	38.10%	54.55%	
Age (years)					
≥60	19	8	90.48%	72.73%	0.1891
<60	2	3	9.52%	27.27%	
Pathologic stage					
I+II	14	6	66.67%	54.55%	0.8639
III+IV	7	5	33.33%	45.45%	
Tumor stage					
T1/T2	7	4	33.33%	36.36%	0.6515
T3/T4	14	7	66.67%	63.64%	
Lymph node status					
N0	6	4	28.57%	36.36%	0.3606
N1/N2/N3	15	7	71.43%	63.64%	
Metastasis stage					
M0	15	10	71.43%	90.91%	0.2055
M1	6	1	28.57%	9.09%	

Table III. Association between the expression of APPL1 with clinicopathological features in 190 patients based on TCGA datasets.

Variables	Patients High (n=95)	Patients Low (n=95)	Percentage High	Percentage Low	P-value
Sex					
Male	58	62	61.1%	65.3%	0.5475
Female	37	33	38.9%	34.7%	
Age (years)					
≥60	67	62	73.6%	66.0%	0.2564
<60	24	32	26.4%	34.0%	
Pathologic stage					
I+II	41	47	45.6%	50.0%	0.5463
III+IV	49	47	54.4%	50.0%	
Tumor stage					
T1/T2	21	31	22.6%	32.6%	0.1235
T3/T4	72	64	77.4%	67.4%	
Lymph node status					
N0	30	36	32.6%	37.9%	0.4495
N1/N2/N3	62	59	67.4%	62.1%	
Metastasis stage					
M0	89	80	93.7%	84.2%	**0.0373**
M1	6	15	6.3%	15.8%	

The results shown here are based upon data generated by the TCGA datasets. A total of 190 patient specimens of clinicopathological outcomes were downloaded from TCGA datasets, in which 95 specimens have high APPL1 expression and the other 95 specimens have low APPL1 expression. The number of patients is inconsistent in the statistical results, because the clinicopathological outcomes of individual specimens are not intact in the TCGA datasets.

based upon data generated by the TCGA datasets. The selection for specimens with altered expression of APPL1 was performed according to the methods previously reported (21). Tumors with APPL1 expression levels greater than the 75th percentile (3.807) were defined as having high APPL1 expression, whereas tumors that fell below the 25th percentile (3.221) were defined as low APPL1-expressing tumors. As shown in Table III, no significant correlation was observed between APPL1 expression with age, sex, pathologic stage, tumor stage, or lymph node status (P>0.05). However, APPL1 expression was correlated with metastasis stage (P=0.037), indicating that the high expression of APPL1 may play an important role in gastric carcinoma metastasis.

Silencing of APPL1 suppresses gastric cancer cell migration. As shown in Table III, the expression of the APPL1 gene was correlated with tumor metastasis. We used a Transwell assay and a wound healing assay to analyze the effect of APPL1 on tumor cell migration. Firstly, the expression of the APPL1 gene was silenced using specifically designed siRNAs in AGS and MKN-45 cells. In the wound healing assay, AGS cells in the si-APPL1 group migrated more slowly than the control group. Similar results were obtained in the MKN-45 cell line (Fig. 2A and B). In the Transwell assay, we stained the invaded cells to measure the directional metastasis ability of the AGS and MNK-45 cells after silencing APPL1 expression. The invasiveness of cells transfected with si-APPL1

was dramatically decreased compared with the control cells (Fig. 2C and D). Using western blot analysis, the expression of mesenchymal markers was detected in AGS and MKN-45 cells, including E-cadherin, N-cadherin and vimentin. The results showed that APPL1 silencing dramatically increased the expression of E-cadherin (1.52 ± 0.11 and 1.40 ± 0.08 in AGS and MKN-45 cells, respectively), but attenuated the expression of N-cadherin and vimentin (Fig. 2E and F). These results support the hypothesis that APPL1 plays a role in suppression of invasive cell migration and metastasis.

Overexpression of APPL1 facilitates gastric cancer cell migration. Subsequently, we increased APPL1 expression in BGC-823 and SGC-7901 cells by using a GV141 plasmid vector to further verify the effects of APPL1 on GC cell metastasis. As the overexpression vector carries other tags such as 3FLAG, the generated exogenous APPL1 protein is a fusion protein with more molecular weight than the endogenous protein. In addition, the western blot results showed two bands appeared in overexpression vector treated cells, which
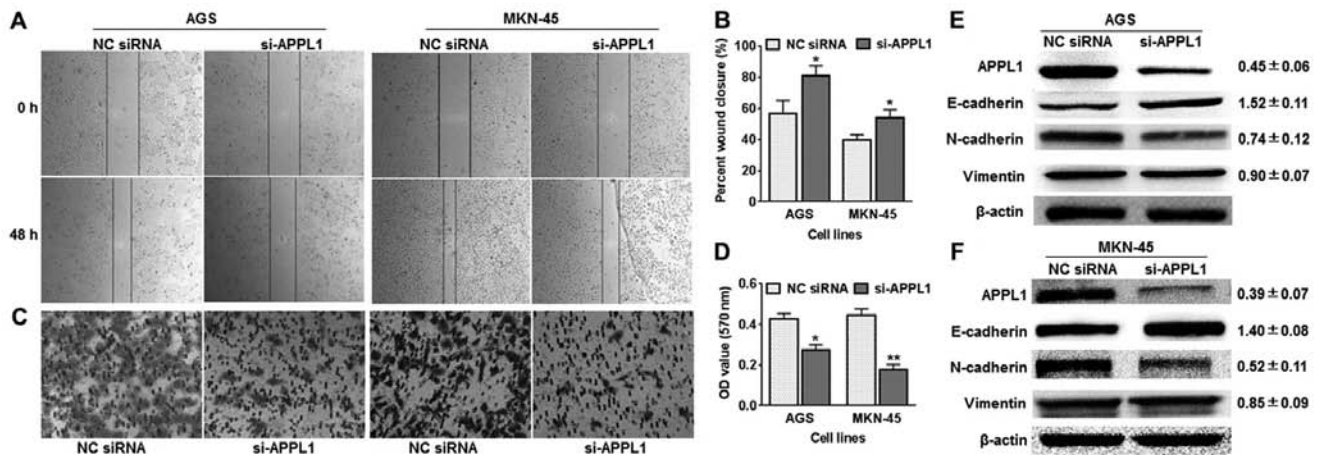
Figure 2. Silenced APPL1 suppresses gastric cancer cell migration. (A) A wound healing assay was used to analyze changes in cell migration ability of AGS and MKN-45 cells after treatment with si-APPL1. Representative images were captured at 0 and 48 h after transfection. (B) Cell migration quantified as the reduction of wound width, as denoted by the solid lines in (A). Data represent means ± SD from 3 experiments, using 7-10 images per group for quantification. (C) Cell migration examined using the Transwell assay in AGS and MKN-45 cells. (D) Quantitative analysis of migration rates by solubilization of crystal violet and spectrophotometry at OD 570 nm. Data are shown as means ± SD from three independent experiments ($^*P<0.05$, Student's t-test). (E and F) Western blot analysis was performed to assess protein levels of epithelial markers E-cadherin, mesenchymal markers N-cadherin and vimentin in AGS and MKN-45 cells, with β-actin as internal control.
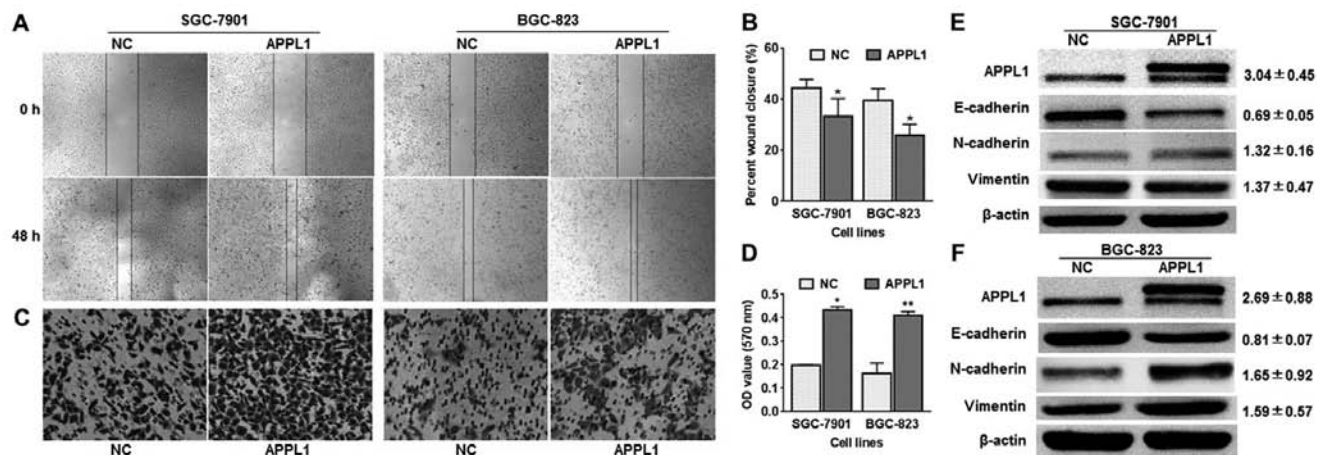

Figure 3. Overexpression of APPL1 enhances metastasis of gastric cancer cells. (A) A wound-healing assay was used to analyze the changes in cell migration ability of SGC-7901 and BGC-823 cells after treatment with APPL1 overexpression vector. Representative images were captured at 0 and 48 h after transfection. (B) Cell migration was quantified as the reduction of the wound width, as denoted by the solid lines in (A). Data represent means ± SD from 3 experiments, using 7-10 images per group for quantification. (C) Cell migration was examined using the transwell assay in SGC-7901 and BGC-823 cells. (D) Quantitative analysis of migration rates by solubilization of crystal violet and spectrophotometry at OD 570 nm. Data are shown as means ± SD from three independent experiments ($^*P<0.05$, Student's t-test). (E and F) Western blot analysis was performed to assess protein levels of epithelial markers E-cadherin, mesenchymal markers N-cadherin and vimentin in SGC-7901 and BGC-823 cells, with β-actin as internal control.

indicated that GV141 plasmid vector effectively increased APPL1 protein expression in BGC-823 and SGC-7901 cells. The results observed from Transwell and wound healing assays showed that both BGC-823 and SGC-7901 cell lines treated with APPL1 overexpression vector were distinctively more migratory than control cells at 48 h after transfection (Fig. 3A-D). In accordance with these observations, the results of western blot analysis showed that overexpression of APPL1 reversed the previously observed changes in mesenchymal markers expression (Fig. 3E and F). Among these, E-cadherin depressed 0.69±0.05-fold in SGC-7901 cells.

APPL1 is a target gene of miR-145. In order to uncover the mechanisms by which APPL1 mediates metastasis, we studied the upstream and downstream regulation networks of the APPL1 gene. In our previous study, it was reported that miR-145 expression was lower in gastric carcinoma (22), which was consistent with the statistical results of miR-145 expression based upon TCGA datasets (Fig. 4A). The data obtained from TCGA datasets also demonstrated that the expression of miR-145 was negatively low correlation with that of APPL1 in gastric cancer (R=-0.189) (Fig. 4B). In the TargetScan and miRanda databases, the region complementary to the miR-145 seed region was found in the 3'-UTR of APPL1 mRNA (Fig. 4C). Dual-Luciferase reporter system containing wild-type (AACTG) or mutant (CGAC) 3'-UTR of APPL1 was used to verify an interaction between APPL1 and miR-145. As shown in Fig. 4D, miR-145/APPL1
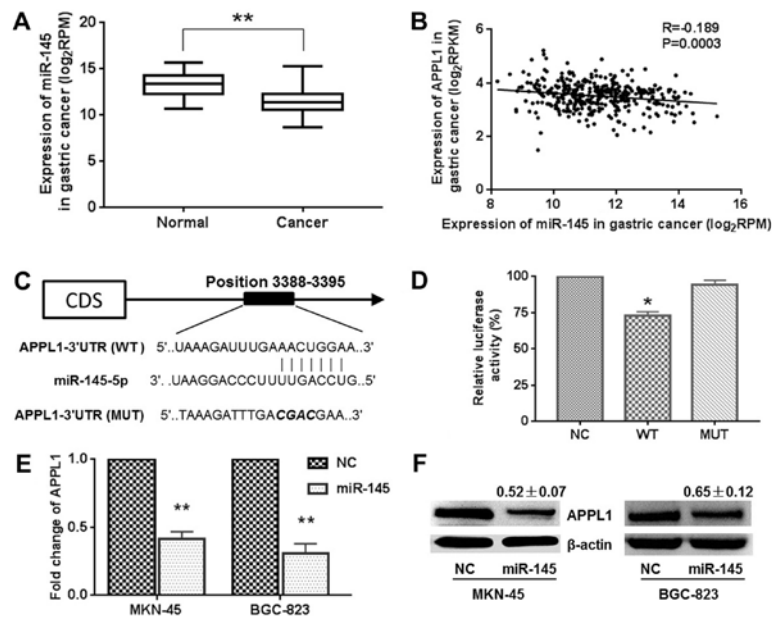
Figure 4. APPL1 is a direct target of miR-145. (A) TCGA datasets showing miR-145 expression (log$_2$RPM) was significantly downregulated in 380 gastric car-cinoma compared with 37 adjacent normal stomach tissues. (B) Inverse correlation between miR-145 and APPL1 expression in GC tissues. Statistical analysis was performed using Pearson's correlation coefficient (R=-0.189, P=0.0003). (C) Scheme of the potential binding sites of miR145 on the 3'-UTR of APPL1 mRNA. (D) Luciferase assay in BGC-823 cells. Pre-miR-145 vector was cotransfected with target gene reporter construct (pmirGLO-APPL1-WT vector or pmirGLO-APPL1-MUT vector) in BGC-823 cells. Relative repression of luciferase expression was standardized to the β-gal signal. Luciferase activity in the pmirGLO-APPL1-WT group denoted a statistically significant decrease following expression of miR-145. (E and F) APPL1 mRNA and protein expression were measured by using qRT-PCR and western blot analysis in MKN-45 and BGC-823 cells after transfection with pre-miR-145 vector.
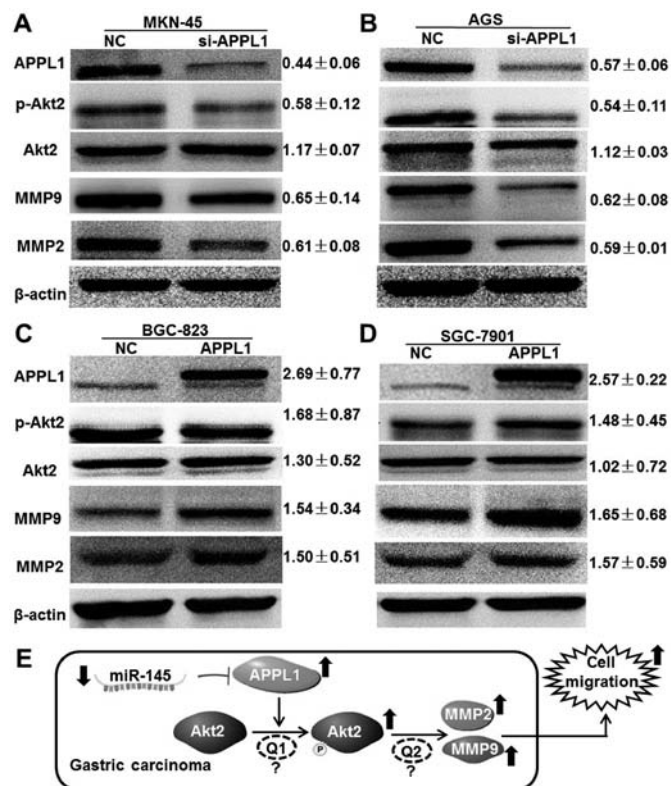

Figure 5. APPL1 induces gastric cancer cell migration via the Akt2 sig-naling pathway. The activation of Akt2 and the expression of downstream genes MMP9, or MMP2 were detected by using western blot analysis in (A) MKN-45, (B) AGS, (C) BGC-823 and (D) SGC-7901 cells, with β-actin as internal control. (E) Proposed model for the effects of APPL1 on GC migration. APPL1 activates the Akt2 phosphorylation and the elevation of matrix metalloproteinases (MMP2 and MMP9), leading to the enhanced ability of GC migration. The currently unknown mechanism is indicated by 'Q1' and 'Q2'.

wild-type 3'-UTR-transfected cells showed a significant reduction (~70.6%) of luciferase activity. However, miR-145 failed to inhibit the luciferase activity of the reporter vector containing mutant binding sites, indicating that miR-145 may suppress gene expression through its binding sequences at the 3'-UTR of APPL1. Furthermore, a reduction of the APPL1 mRNA and protein expression levels was observed in MKN-45 and BGC-823 cells transfected with pcDNA6.2GW/EmGFP-miR-145 compared with control vector-transfected cells (Fig. 4E and F). For example, the APPL1 protein decreased 0.52±0.07- and 0.65±0.12-fold in MKN-45 and BGC-823 cells, respectively. These results indicate that miR-145 directly recognizes the 3'-UTR of APPL1 mRNA and inhibits APPL1 translation.

APPL1 facilitate gastric cancer cell migration via regulation of Akt2 phosphorylation. Because APPL1 was initially identified as an Akt2-interacting protein in a yeast two-hybrid screen (15), we hypothesized that APPL1 facilitates gastric cancer cell migration via regulation of Akt2 phosphorylation. Using western blot analysis, the expression of downstream molecules was demonstrated following changes in APPL1 expression. When APPL1 expression was silenced by siRNA in MKN-45 and AGS cells, we found that although the expression of Akt2 was not affected, its phosphorylation level was significantly suppressed (0.58±0.12 and 0.54±0.11 in MKN-45 and AGS cells, respectively). The expression of downstream effectors of Akt2, MMP2 and MMP9 was also reduced (Fig. 5A and B). Accordingly, APPL1 overexpres-sion in BGC-823 and SGC-7901 cells not only promoted phosphorylation levels of Akt2 but also increased the expres-sion of MMP2 and MMP9 (Fig. 5C and D). Taken together,

these results suggest that APPL1 facilitates gastric cancer cell migration by regulating Akt2 phosphorylation and downstream effector expression.

Discussion

Metastasis is the predominant cause of cancer-related mortality in gastric carcinogenesis and the mechanism of tumor metastasis is a multifactorial process associated with multiple genetic and epigenetic events. Previous studies have shown that adaptor proteins promote invasion and migration progression of some cancer types by altering early endosome biogenesis, which could have important implications for cancer cell biomarker release and intracellular signaling (3,7,8).

In the present study, we focused on changes in APPL1 expression in gastric cancer and the effect of APPL1 on cancer cell metastasis. The results showed that APPL1 protein and mRNA were upregulated both in gastric carcinoma tissue and cultured cell lines, which were consistent with findings previously reported in prostate (8), breast (20) and gastric cancer (19).

These results were also consistent with the statistical analysis results of TCGA datasets (Fig. 1A). TCGA is a collaboration between the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI). The tumor and normal tissues from more than 11,000 patients have been profiled, covering 37 types of genetic and clinical data for 33 types of cancer (23). The TCGA research center has published many studies identifying the mutations and dysregulations associated with tumors in comparison to matched normal tissue samples (24). When we focused on stomach cancer, the gene expression profile and phenotype data of 417 stomach cancer specimens were downloaded by using UCSC's Xena. We found that APPL1 in 380 gastric cancer tissues was significant overexpression, compared to the average of 37 normal tissues (3.52 ± 0.025 vs. 3.19 ± 0.007; $P<0.05$). More importantly, we demonstrated that APPL1 expression was significantly correlated with metastasis stage ($P=0.037$; Table III), indicating that APPL1 may act as an oncogene facilitating metastasis of gastric carcinomas. Since the size of patient specimens is limited, there was no significant association between the expression of APPL1 with clinicopathological features of gastric carcinoma patients collected from our institute ($P>0.05$; Table II). In this way, TCGA represents a rich resource for cancer prognostic studies. Besides identifying differentially expressed genes, we have also shown that study of clinicopathological features can be extended to the study of the function of differentially expressed genes.

Metastasis is the spread of cancer cells from their primary location to other parts of the body. Once cancer becomes metastatic, it cannot be effectively treated by surgical and radiation therapies (2). The process by which malignant cells become migratory and invasive is complex and requires overcoming barriers posed by constantly changing microenvironments. First, cells need to escape the surrounding matrix, adopting the phenotypes of mesenchymal cells, known as the epithelial-mesenchymal transition (EMT) (25,26). EMT is a process whereby tightly interacting and immotile epithelial cells acquire the phenotype of loosely adherent and motile mesenchymal cells, which facilitates invasion and metastasis of tumors (25,26). Pathological EMT is associated with E-cadherin repression, which has been shown to contribute to tumor progression (27,28). The present study showed that APPL1 silencing dramatically increased the expression of E-cadherin, but attenuated the expression of N-cadherin and vimentin (Fig. 2E and F). Conversely, overexpression of APPL1 reversed the expression changes of these mesenchymal markers (Fig. 3E and F). The results indicated that APPL1 might promote invasion and metastasis of gastric cancer cells by facilitating EMT.

Adaptor proteins are emerging as critical regulators of multiple aspects of the migration process. APPL1 was initially identified as an Akt2-interacting protein in a yeast two-hybrid screen (15). APPL1 interacts only with the inactive form of Akt2, anchoring it to the p110α subunit of PI3K in the cytoplasm (15,29). Akt2 is one of three closely related serine/threonine-protein kinases, which mediates serine and/or threonine phosphorylation of a range of downstream substrates. Over 100 substrates have been reported; therefore, Akt2 could regulate many processes, including metabolism, proliferation, cell survival, growth and metastasis (25,30-32). It has been established that Akt2 phosphorylation and activation is very important for elevation of matrix metalloproteinases (MMP2/MMP9), leading to the enhanced ability of migration and invasion in bladder cancer (33). Our results showed that APPL1 promotes Akt2 phosphorylation and activation in gastric cancer cells (Fig. 5A-D), which is in agreement with the findings of Mitsuuchi *et al* (15). In addition, activation of MMP2 and MMP9 was also observed when cell migration increased, suggesting that EMT was induced via cytoskeleton reorganization and activation of E-Cadherin repressors (30,31,34,35).

In summary, the present study demonstrated that expression of APPL1 protein and mRNA was upregulated in gastric carcinoma tissues and cell lines. The expression of APPL1 in GC was statistically associated with metastasis stage. Overexpression of APPL1 promotes invasion and metastasis of gastric cancer cells and the underlying molecular mechanism may facilitate EMT via Akt2 phosphorylation (Fig. 5E). Unfortunately, the molecular mechanism is still not determined, which was indicated by 'Q1' and 'Q2' in Fig. 5E. For example, the kinase activity of APPL1 for Akt2 phosphorylation has not yet been confirmed by experiment. The results of western blot analysis just indicated that the phosphorylation level of Akt was increased. More importantly, the Co-IP and GST pull-down assay are needed to confirm the interaction between APPL1 and inactive Akt2, and the recruitment of Akt2 from cytoplasm to cell membrane (15,29). Furthermore, the activation of Akt2 results in the elevation of MMP2/MMP9 via a unknown mechanism. It was reported that Akt2 phosphorylation and activation could play the regulatory role via several signal pathways (TGF-β, Wnt/β-catenin, JAK2/STAT3, PI3K/Akt and NF-κB) (2,32,36-38). In this event, whether Akt2 directly regulates the expression of MMP2/MMP9 through transcription factor NF-κB is worth further study. Importantly, a previous study reported that protein levels and phosphorylation levels of APPL1 were highly expressed in tissues from human hepatocellular carcinoma and breast cancer (3). In this study, we focused on the regulation of APPL1 expression by miR-145, which is a post-transcriptional regulation, no association with phosphorylation modification. Thus, we expect to analyze the phosphorylation levels of APPL1 in gastric cancer tissues and investigate the function of activated APPL1 in gastric cancer cells in future studies.

Acknowledgements

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