Kinase D-interacting substrate of 220kDa is overexpressed in gastric cancer and associated with local invasion

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Running title: Kidins220 in gastric cancer

Abstract:

Background: Kinase D-interacting substrate of 220kDa (Kidins220), also known as ankyrin repeat-rich membrane spanning protein (ARMS) is a transmembrane scaffold protein. It has been indicated in various malignancies including melanoma, glioma, neuroblastoma, prostate cancer, pancreatic cancer, and ovarian cancer. **Materials and Methods:** In the current study, Kidins220 expression was determined at transcript and protein levels. Kidins220 knockdown cell model was established to identify its role in cellular functions including cell cycle, proliferation, and invasion. The relevant cell signalling was analysed by protein array and TCGA gastric cancer cohort. **Results:** Kidins220 transcript was significantly increased in gastric tumors in comparison with adjacent normal tissues. More advanced tumors (TNM3 and TNM4) exhibited higher protein levels of Kidins220 compared with early-stage tumors (TNM1 and TNM2). Increased expression of Kidins220 in gastric cancer was associated with poorer overall survival. Loss of Kidins220 promoted cell invasion and adhesion of gastric cancer and correlated to EMT and MMP signalling. Knockdown of Kidins220 allowed more cells to enter into G2/M phase in gastric cancer and attribute to cell proliferation with corresponding alteration in cell cycle regulators. **Conclusion:** Our study identified an increased expression of Kidins220 in gastric cancer, which is associated with disease progression and poor prognosis. The disease progression in gastric cancer can be promoted by the loss of Kidins220 via EMT, MMP and cell cycle signalling.

Introduction

Approximately 990,000 people are diagnosed with gastric cancer (GC) globally, and about 738,000 patients die from this disease every year (1). It is hard to detect GC at an early stage, most patients are diagnosed when diseases are fairly progressed (2). Although great advances have been made in surgery, radiotherapy, and chemotherapy for the treatment of GC, the 5-year survival rate for patients with advanced GC is still less than 30% (3).

Kidins220/ARMS is a transmembrane scaffold protein with multiple binding domains (4). It was first identified as a substrate for protein kinase D (PKD) in neural cells and was mainly related to neurotrophin (5). It acts as a downstream regulator of several neuronal growth factors and regulates neuronal differentiation, survival, and cytoskeleton remodelling (6-8). The substantial involvement of Kidins220 has been revealed in malignancies (9). In melanoma, Kidins220 inhibited the stress-induced apoptosis of melanoma cells through MAPK signalling pathway (10). Moreover, Kidins220 played a positive role in regulating the cell proliferation of neuroblastoma through a regulation of cyclin D1 and cyclin-dependent kinase 4 (CDK4) (11). As a direct target gene of miR-4638-5p, Kidins220 was involved in regulating angiogenesis via VEGF and PI3K/AKT pathway in prostate cancer (12). In pancreatic cancer, Kidins220 mediated the metastasis of pancreatic cancer through EGFR/Erk signalling(13). A recent study revealed that XPR1-Kidins220 complex was vital for the cellular

distribution and function of XPR1 and its regulated phosphate efflux. Impaired XPR1 function led to the accumulation of intracellular phosphate and reduce the viability of ovarian cancer cells (14).

To date, the role played by Kidins220 in gastric cancer remains unknown. We aimed to examine the involvement of Kidins220 in the disease progression of gastric cancer and explore how it affects cellular functions including cell proliferation and invasion of gastric cancer cells.

Methods and Materials:

Cell lines and cell culture. HGC27 and AGS gastric cancer cell lines were purchased from the American Type Culture Collection (ATCC, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum. Materials and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless stated.

Collection of clinical cohorts. Gastric tumors (n=324) together with paired adjacent (n=183) background tissues were collected immediately after the surgery and stored at -80°C until use, with written consent from the patients at the Peking University Cancer Hospital. All protocols and procedures of the tissue collection were approved by Peking University Cancer Hospital Research Ethics Committee.

IHC of gastric adenocarcinoma tissue microarray (TMA). Immunohistochemical staining was conducted on a gastric adenocarcinoma tissue microarray (TMA) (OD-CT-DgStm01-007, Biomax, Rockville, MN, USA). Proteins was probed with

Kidins220 rabbit monoclonal antibody at 1:50 concentration (SC-48738, Santa Cruz Biotechnology, UK). The secondary antibody solution consisted of 100µl biotinylated antibody stock at 5ml dilution (Vectastain Universal Elite ABC Kit, PK-6200, Vector Laboratories, Peterborough, UK). The intensity of Kidins220 staining was calculated using Image J software by determining 10-20 cancerous cells by a subtraction of background of empty area for each sample from the duplicate cores.

RNA extraction cDNA synthesis and RT-PCR. Total RNA was isolated using TRI Reagent (Sigma-Aldrich, Poole, Dorset, UK) from a $25cm^2$ flask. The cDNA was then synthesised from 500 ng of RNA using the GoScriptTM Reverse Transcription System kit (Promega, Corporation, Madison, WI, USA). PCR was performed with initial denature at 94°C for 5min, followed by 30-35 cycles of amplification at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes, whilst GAPDH was determined as a house-keeping control.

Quantitative polymerase chain reaction (QPCR). Kidins220 and GAPDH in the gastric tissue samples were determined using the AmpliflourTM system (Intergen company, New York, NY, USA) with following conditions: 94 °C for 10 min, 90 cycles of 94 °C for 10s, 55 °C for 35s, and 72°C for 20s. Gene expression in cell lines was determined using Sybr Green master mix (Sigma Aldrich, Poole, UK).

GAPDH (forward:5'-TGCACCACCAACTGCTTAGC-3' and reverse:5'-GGCATGGACTGTGGTCATGAG-3').

Western Blot. Proteins from gastric cancer cell lines were extracted using RIPA lysis buffer and then quantified using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hemel-Hempstead, UK). After separation of protein samples in the SDS-PAGE gel, proteins were transferred onto PVDF membranes and subsequently blocked with 10% milk for 1 hour at room temperature. The membrane was subsequently incubated with primary antibody and a corresponding peroxidise-conjugated secondary antibody. Antibodies against Kidins220 (sc-48738, 1:1000) and GAPDH (sc-47724, 1:2000) were purchased from Santa Cruz, the protein bands were eventually visualised using a chemiluminescence detection kit (Supersignal™ West Dura kit, Pierce Biotechnology, Rockford, IL, USA).

Establishment of Kidins220 knockdown gastric cancer cell line. Knockdown of Kidins220 was carried out in HGC27 and AGS gastric cancer cell lines. Anti-Kidins220 ribozyme was designed based on the secondary structure of Kidins220 mRNA. The ribozymes were synthesised using touch-down PCR and subsequently cloned into a pEF/V5 HIS TOPO TA plasmid vector. The transfected cells were selected with 5μg/ml blasticidin and maintained with 0.5μg/ ml blasticidin in DMEM culture medium. RT-PCR and western blot were used to verify the knockdown of Kidins220.

Cell cycle assay. HGC27 gastric cancer cells were cultured in serum-free medium to synchronise the cell cycle for 36 hours. The cells were subsequently cultured in 10% FCS medium for 16 hours. Propidium iodide (PI) was used to fix and stain the cells. DNA content was determined with FACS Canto TM II (BD UK Ltd, West Sussex, UK), and the cell cycle analysis was determined using FCS Express (v4.0, De Novo software, CA, USA).

In vitro cell adhesion assay. The gastric cancer cells (20,000 cells/well) were seeded into a 96-well plate which was pre-coated with 5µg Matrigel (Corning Incorporated, Flintshire, UK). After an incubation of 40 minutes, adhered cells were then fixed with 4% formaldehyde and stained with 0.5% crystal violet. Absorbance of crystal violet was measured to quantify the adhered cells.

In vitro cell invasion assay. The 24-well transwell inserts with 8µm pores (Greiner Bio-One Ltd., Stonehouse, UK) were coated with 50 µg/well Matrigel. After air drying and rehydration, 20,000 cells were seeded. Cells that had invaded were fixed and stained after 72 hours of culture. Absorbance of the crystal violet was determined.

Statistical analysis. KM plotter analysis (http://kmplot.com/) was performed to evaluate the prognosis of gastric cancer patients by comparing the mRNA expression of Kidins220 in gastric cancer and control. In this study, t-test was employed for normally distributed data whilst non-normally distributed data was analysed using a Mann-Whitney test, $p<0.05$ was advised as statistically significant.

Kaplan-Meier survival analysis was carried out using SPSS software (SPSS Standard version 13.0; SPSS Inc., Chicago, IL, USA).

Result

Overexpression of Kidins220 in gastric cancer and the clinical relevance. The transcript level of Kidins220 in gastric cancer (n=324) and adjacent normal control （n=183） was determined using QPCR. The clinical and pathological information together with average Kidins220 transcript levels was supplied in Table1. Kidins220 transcript was significantly increased in gastric tumor tissues compared with normal tissues. (p=0.015) (Fig.1A). Gastric tumors at advanced T stage(T III &T IV) presented a higher mRNA expression level of Kidins220 in comparison with early T stage (T I&T II) (p=0.02). More advanced tumors with lymph node metastases (TNM III) exhibited higher transcript levels of Kidins220 compared with early-stage tumors (TNMI) (p=0.038). To examine the protein expression of Kidins220, immunohistochemical staining was performed on a gastric tumor tissue microarray, the representative figures were shown in Figure 1B. The intensity of Kidins220 in gastric tumor tissues at TNMIII(p=0.0022) and TNMIV (p=0.0154) exhibited stronger staining of Kidins220 protein in comparison with tumors at TNMI (Fig 1C). Furthermore, gastric tumor tissues at early stages (TNMI-II) presented weaker staining of Kidins220 compared with advanced gastric tumor tissues (TNMIII-IV) (p=0.001).

The relevance of Kidins220 and the prognosis of gastric cancer patients. Kaplan-Meier survival analysis showed that gastric cancer patients with high expression of Kidins220 have a markedly shorter survival compared with patients with lower expression of Kidins220 (p<0.001) (Fig 2A). Patients with low expression of Kidins220 had a better free progression survival (p<0.001) (Fig 2B). By analyzing the recurrence of gastric cancer using public gene expression data (GSE26253), we found patients with high expression of Kidins220 had an increased recurrence possibility with gastric cancer (p=0.0047) (Fig 2C). Kaplan-Meier analysis using (GSE26253) database exhibited that high expression of Kidins220 in gastric tumors was associated with poorer overall survival (Fig 2D).

Kidins220 is involved in regulating metastasis of gastric cancer cells. In order to investigate how Kidins220 affects cellular function of gastric cancer cells, Kidins220 knockdown model was established using ribozyme in HGC27 and AGS gastric cancer cells. The knockdown of Kidins220 in both cell lines was then verified using RT-PCR and Western blot (Fig 3A), as well as qPCR (Fig 3B). Knockdown of Kidins220 increased cell adhesin in both HGC27 and AGC cancer cells (Fig 3C). Furthermore, an increased cell invasion was observed in HGC27 gastric cancer cells following the knockdown of Kidins220 (p<0.001). Knockdown of Kidins220 also promoted cell invasion in AGS gastric cancer cells(p<0.001) (Fig 3D). Since EMT (epithelial-mesenchymal transition) and MMPs (matrix metalloproteinases) are two major impactors in regulating invasion of gastric cancer cells. Our current study analyzed the correlation between Kidins220 and

EMT-related molecules (snail, slug, twist and vimentin) and MMPs using TCGA database. The result showed that Kidins220 had a significantly positive correlation with slug (Fig 3E). Kidins220 expression had a significantly negative correlation with MMP1, MMP3, MMP11, MMP12, and MMP15, while a positive regulation with MMP16, MMP19, and MMP21 (Fig 3F).

Kdins220 may regulate cell proliferation and cell cycle through cell cycle regulators. The cell cycle of the HGC27 gastric cancer cells was detected by flow cytometric assay. There was no statistical significance when HGC27 knockdown and PEF control cells in G0/G1 and S phase. However, there was a higher percentage of Kidins220-knockdown cells entering the G2/M phase compared with the PEF control ($p<0.01$) (Fig 4A). Knockdown of Kidins220 also promoted the cell proliferation in HGC27 gastric cancer cells in comparison with PEF control on Day 3 ($p<0.001$) and Day 5 ($p<0.001$). The effects of Kidins220 in regulating cell cycle and cell growth inspired us to further explore its correlation with cell cycle regulators. The Pearson's correlation test showed Kidins220 had a significantly negative correlation with CyclinB1 by using TCGA gene expression array data ($p<0.01$) (Fig 4C), and the correlation of Kidins220 with cell cycle regulators was presented using a heatmap in Fig 4D. Fig 4E presented the correlation between Kidins220 and CDKs cell cycle-promoting molecules.

Discussion

Kidins220 has been found in several malignancies by acting as tumor suppressor or tumor promoter. Kidin220 expression significantly promoted primary malignant melanomas and metastatic melanoma in comparison with benign nevocellular lesions (10). Likewise, the expression of Kidins220 was drastically stimulated in primary and metastatic melanoma tissues（depths >1.0 mm）compared with benign tumors tissues (15). Increased expression of Kidins220 was found in melanoma and associated with shorter overall survival [16]. Overexpression of Kidins220 was also detected in neuroblastoma tissue samples(16). A reverse expression of Kidins220 was detected in pancreatic cancer, whereas Kidins220 transcript was significantly reduced in pancreatic tumors in comparison with adjacent normal tissues, and malignant tumors exhibited weaker staining of Kidins220 in comparison with adjacent normal pancreatic tissues and normal pancreas(13). Here we identified that Kidins220 transcripts was highly expressed in gastric tumor tissues in comparison with normal control, and overexpression of Kidins220 may predict the poor prognosis of gastric cancer.

In pancreatic cancer, more advanced pancreatic tumors (TNMIII and TNMIV) had lower Kidins220 transcripts compared with those of early stages (TNMI and TNMII), and knockdown of Kidins220 promoted the invasion and migration of pancreatic cancer cells. In melanoma, Kidins220 promoted tumor migration/invasion through MEK/ERK signalling(15), however, loss of Kidins220 did not affect migration of neuroblastoma cells(16). Our current study found that the expression Kidins220 was promoted in advanced tumor stage both in mRNA and protein level, which

provides the evidence for personal management when evaluating its value for those patients with different cancer types. Knockdown of Kidins220 promoted cell invasion and focal adhesion in both HGC27 and AGS gastric cancer cells. As a scaffold protein, Kidins220 acts as a binding domain for protein-protein interactions. It recruits receptor substrates to activate the downstream signaling pathways and attributes them to cellular activities of neural cells(17). Considering its multiple binding domains in regulating cell signaling, we speculated that Kidins220 did not reflect the dependent manner when regulating the cell invasion of gastric cancer. Future studies may focus on exploring the multiple signaling pathway regulated by Kidins220 in cell invasion of gastric cancer. A previous study has indicated the role of Kidins220 in regulating the disease progression of pancreatic cancer with the involvement of EMT and MMPs, two important factors in the cell migration and invasion of cancer metastasis (13). During tumor progression, the majority of tumors undergo EMT to acquire infiltrating and metastasizing properties(18). In gastric cancer aggressiveness, the tumor epithelial cells lose cell polarity and cell-cell adhesion to have mesenchymal phenotype and acquire properties of cell invasion and migration(19). By analysing TCGA gastric cancer cohort, we found that Kidins220 has a positive correlation with slug and vimentin and a negative correlation with snail and twist. MMPs are known for their role in mediating the tumor microenvironment during tumor progression(20). MMPs enable the degradation of the barriers including extracellular cellular matrix and basement membrane, facilitating the metastasis

of tumor cells(21). An analysis was performed for the expression profile of MMPs in gastric cancer using TCGA online data, the result showed that Kidins220 has a significantly positive correlation with MMP19, MMP16, and MMP21 and a significantly negative correlation with MMP1, MMP3, MMP11, MMP12, and MMP15. It is speculated that Kidins220 regulated gastric cancer cell invasion with the involvement of EMT and MMPs. However, the specific mechanism targeting cell invasion needs to be verified in the future study.

Cyclin B1 is a critical regulator of G2/M transition during the cell cycle. CyclinB1 accumulated progressively through G1/S phase and reach the peak in G2 phase, subsequently it forms a complex with CDK1(22). In neuroblastoma, knockdown of Kidins220 inhibits the growth of mouse neuroblastoma cell by slowdown G1 phase in cell cycle, which is regulated by the upregulation of a CDK inhibitor p21, and lead to decrease protein levels of cyclin D1 and CDK4(11). Here we found knockdown of Kidins220 allowed more cells to enter into G2/M phase in gastric cancer. Yasuda *et al* identified CyclinB1 was overexpressed in gastric cancer patients and associated with less aggressive tumor behavior (23). Our current study found that Kidins220 had a significantly negative correlation with CyclinB1. We also explored that Kidins220 has a significant correlation with most cell cycle-promoting molecules by using TCGA online data. This analysis provided the evidence of the correlation between Kidins220 and cell cycle. Certain experiment should be conducted in the future study.

Conclusion

In summary, Kidins220 was overexpressed in gastric cancer tissues, and the increased expression of Kidins220 was related to diseased progression and overall survival. Furthermore, knockdown of Kidins220 promoted invasion, focal adhesion, and proliferation of gastric cancer cells, leading to a higher percentage of Kidins220-knockdown cells entering the G2/M phase. Our current study provides possible mechanisms of gastric cancer progression affected by Kidins220, a potential therapeutic target for the treatment of gastric cancer.

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Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

LY and WGJ designed the study. SC, ZS, XG, KJ, FR, DS, XL, WGJ and LY did the experiments. SC, ZS, DS, XL, WGJ and LY contributed to data analyses. SC, ZS, WGJ and LY prepared the manuscript. SC, ZS, CH, NF, KF, FR, WGJ and LY revised and proof read the article.

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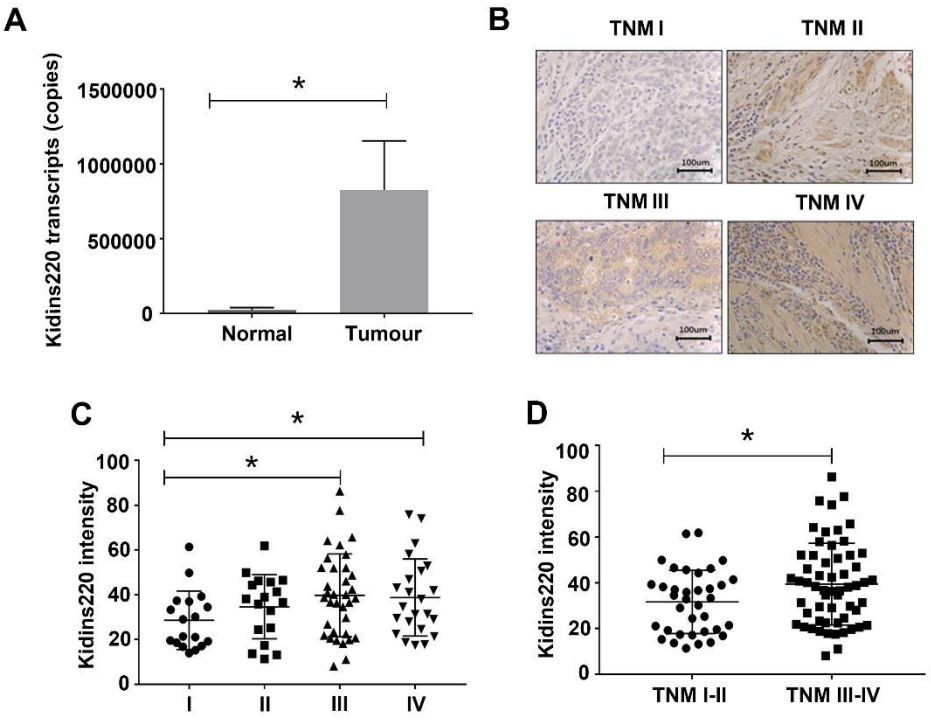

Figure1: Overexpression of Kidins220 is associated with disease progression in gastric cancer. A. Gastric clinical cancer cohort showed Kidins220 transcripts were increased in gastric tumor tissues compared with the adjacent normal tissues. Shown are average transcript levels of Kidins220 per 50ng RNA and error bars represent standard error of mean. B. Shown are representative images of IHC staining of the gastric tumor tissue microarray from different TNM stages. C. The expression of Kidins220 protein was assessed in a gastric tissue microarray (TMA XXX) with IHC staining (gastric tumor tissues from different TNM stages $*p<0.05$. Shown are semi-quantification of the staining intensity.

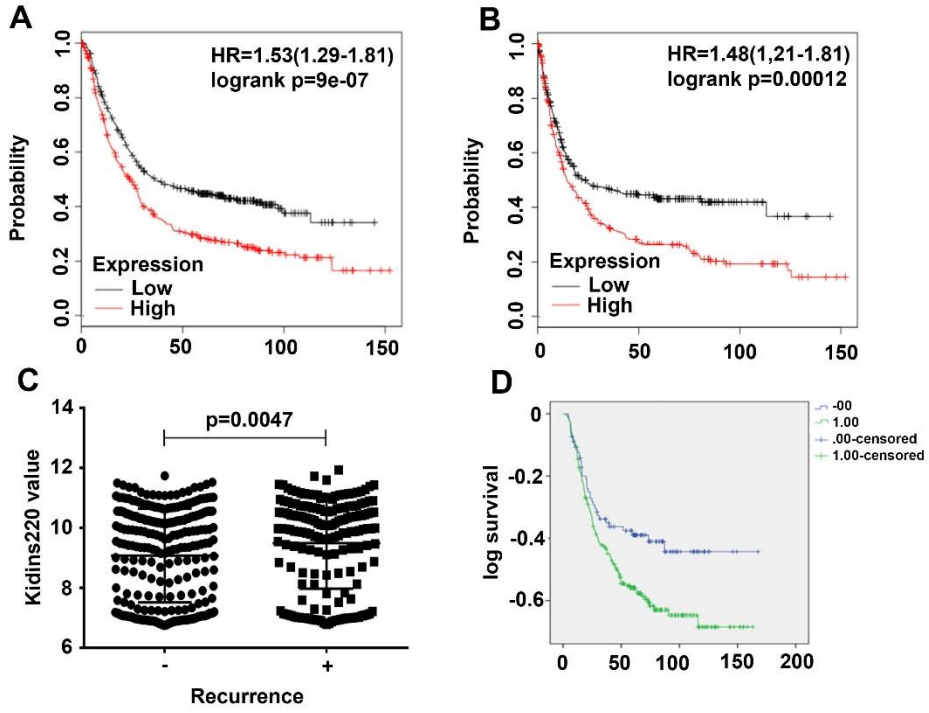

Figure2:Kidins220 and prognosis of gastric cancer. A. Correlation between Kidins220 expression (mRNA) and overall survival of patients with gastric cancer using Kaplan-Meier survival analysis. B. Correlation between Kidins220 expression (mRNA) and free progression of patients with gastric cancer using Kaplan-Meier survival analysis. C. Kidins220 expression and recurrence of gastric cancer. (GSE26253). D. Recurrence free survival of gastric cancer, kidins220 high=green, low=blue, cutoff value=8.1 (GSE26253).

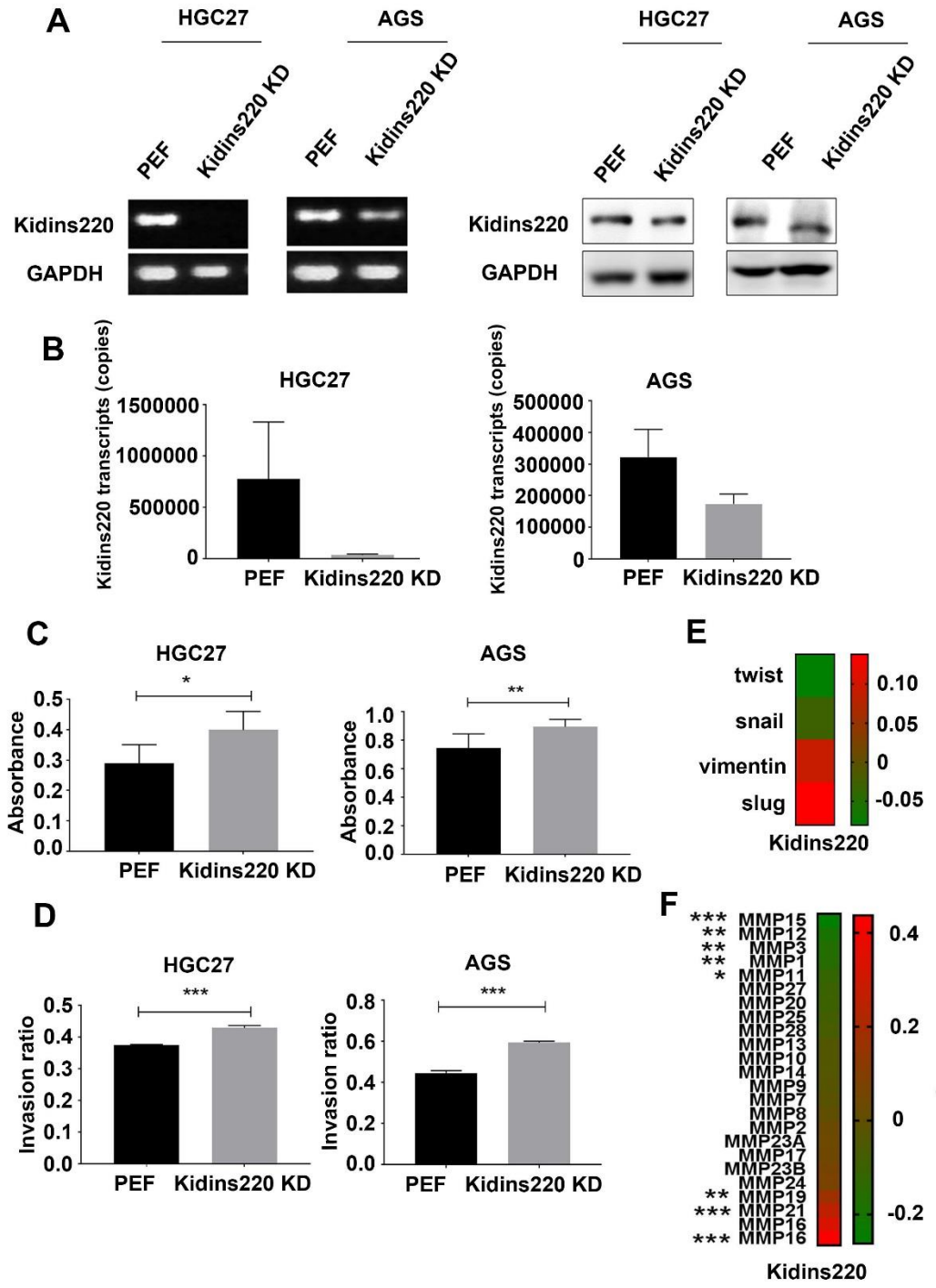

Figure3. Implication of Kidins220 in metastasis of gastric cancer. A.Knockdown of Kidins220 in HGC27 and AGS gastric cancer cells infected with Kidins220 ribozyme verified at both mRNA and protein levels. B. Verification of Kidins220 knockdown at

transcription level using QPCR. C. The impact of Kidins220 on cellular focal adhesion in vitro for HGC27 and AGS gastric cancer cell lines. D. The influence of Kidins220 on cell invasion of HGC27 and AGS cell lines. E. Kidins220 correlated with EMT markers in gastric cancer. F. The correlation of Kidins220 and MMPs in gastric cancer. * represents p<0.05; ** represent p<0.01; *** represent p<0.001.

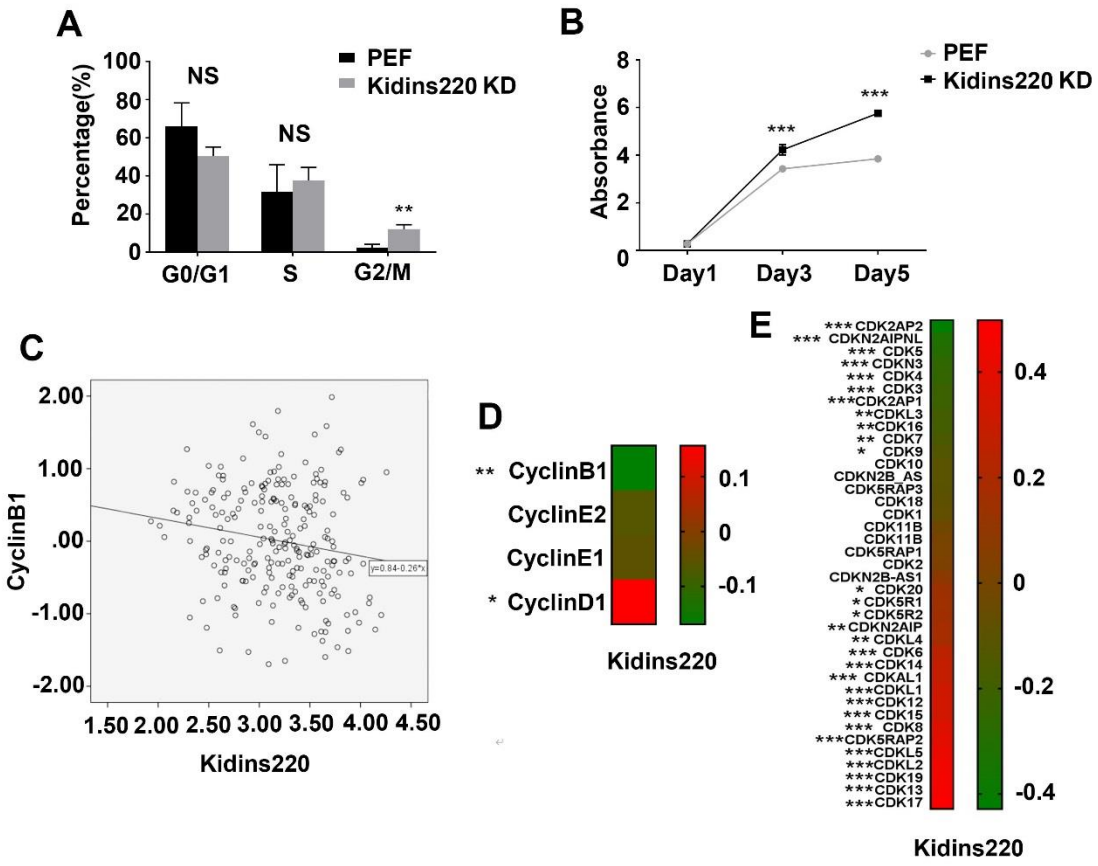

Figure4. Implication of Kidins220 in proliferation of gastric cancer. A. impact of Kidins220 in cell cycle of HGC27 gastric cancer cell lines. B. Knockdown of Kidins220 promoted cell growth in HGC27 gastric cancer cell lines. C. Kidins220 has a negative correlation with cyclinB1 in gastric cancer. D. The correlation of Kidins220 and cell cycle regulators in gastric cancer. E. The correlation of Kidins220 and CDKs are shown in heatmap. * represents p<0.05; ** represent p<0.01; *** represent p<0.001.

Table Ⅰ. Kidins220 transcripts in gastric cancer

Category		**No.**	**Mean (SEMean)**	***p***
Tumor	Tumor	322	826412 (328468)	
	Normal	183	21842 (17364)	0.015
Gender	Male	229	1131295(4604030)	
	Female	93	75679 (35369)	0.023
Invasion	Inv-WL	232	1081126(4542962)	
	InvSubSe	37	277222(1562622)	0.095
	InvMusc	30	32838(162766)	0.022
	InvMucs	11	115871(772817)	0.037
	Gastric	255	871501 (3985336)	
	Cardiac	52	765335 (565202)	0.88
	intersti	5	63627 (632645)	0.046
T stage	T1	16	79417 (54148)	
	T2	25	39406 (19322)	0.5
	T3	41	978768 (780698)	0.26
	T4	232	962057 (434486)	0.045
	T1+T2	41	55020 (239837)	
	T3+T4	273	964567 (386918)	0.02
TNM stage	I	25	62839(35367)	
	II	59	626778(539949)	0.3
	III	220	1023299 (458260)	0.038
	IV	9	204346 (132323)	0.33