Histol Histopathol (2013) 28: 1629-1638
DOI: 10.14670/HH-28.1629

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Upregulation of GPR34 expression affects the progression and prognosis of human gastric adenocarcinoma by PI3K/PDK1/AKT pathway

Weidong Yu[1]*, Shuyun Ma[1,2]*, Lihong Wang[2]*, Bo Zuo[1,2], Mei Li[1], Zhengguo Qiao[1], Xiuying Pan[1], Yulan Liu[1,2] and Jingtong Wang[2]

[1]Institute of Clinical Molecular Biology and [2]Department of Gastroenterology, Peking University People's Hospital, Beijing, China

*Weidong Yu, Shuyun Ma and Lihong Wang contributed equally to this study

Summary. Purpose. G-protein coupled receptor 34 (GPR34), which belongs to the G-protein coupled receptors superfamily, is reportedly expressed highly in the spread of several solid tumors. However, its expression in gastric primary tumor and potential role in gastric cancer development and progression have not been determined. Methods. Immunohistochemistry, real-time RT-PCR and western blot methods were used to determine GPR34 expression in human gastric cancer tissues/cell lines and matched adjacent tissues/ normal mucosal cell line. A statistical analysis was performed to establish the potential correlation between GPR34 expression and the patients' clinicopathological characteristics, tumor progression, and prognosis. Stably transfected NCI-N87 cell lines with either GPR34 over-expression or knock-down were constructed to determine the effect of GPR34 on gastric cancer cell invasion and migration, and to explain the preliminary molecular mechanism of GPR34 in gastric cancer metastasis. Results. GPR34 is up-regulated in primary gastric cancer tissues/cell lines compared with matched adjacent tissues/normal mucosal cell line, and when the relationship between GPR34 expression and the the clinicopathological characteristics was analyzed, it was shown that GPR34 expression is significantly correlated with tumor differentiation, infiltration depth, and lymph node status and had a significant influence on prognosis. Furthermore, GPR34-overexpression increased while GPR34-knockdown inhibited NCI-N87 cell invasion in vitro by PI3K/PDK1/AKT pathway. Conclusions. Taken together, up-regulation of GPR34 expression in human gastric carcinoma may play a critical role in tumor progression and in determining patient prognosis. GPR34 may be a useful diagnostic or prognostic molecular biomarker, and a potential target for therapeutic intervention.

Key words: G-protein coupled receptor 34 (GPR34), Gastric cancer, Metastasis, Prognosis

Offprints requests. Dr. Jington Wang, Department of Gastroenterology, Peking University People´s Hospital, Beijig 100044, China. e-mail: wangjingtong@pkuph.edu.cn

Introduction

Gastric cancer is one of the most common epithelial malignancies and the secondary leading cause of cancer-related death in the world (Anderson et al., 2010). In China, more than 390,000 new patients are diagnosed with gastric cancer and more than 300,000 patients are killed by the terrible disease annually (You et al., 2006). Despite the advances in treatment and research efforts over the past few decades, the poor outcome of gastric cancer is largely because many gastric cancers fail to be diagnosed at an early stage, tumor recurrence and metastasis including major lymph node metastasis (D'Ugo et al., 2010). As a significant prognostic factor in gastric cancer, lymph node metastasis is related to a variety of intracellular events, including activation of various oncogenes, inactivation of tumor suppressor genes, and abnormal expression of G-protein coupled receptors (GPRs) (Gao et al., 2006; Qin et al., 2009; D'Ugo et al., 2010). These perturbations result in an increased lymph node metastasis for gastric cancer cells. G-protein coupled receptors (GPRs) represent a large family of proteins with the characteristic feature that

they have seven α-helical transmembrane domains. GPRs regulate key biological functions including cellular motility, growth and differentiation, gene transcription, but also appear to be involved in cancer progression (Qin et al., 2011)

As a member of the GPR super family, G-protein coupled receptor 34 (GPR34) is a 7-transmembrane receptor reportedly expressed highly in mast cells but poorly expressed in B cells (Sugo et al., 2006; Engemaier et al., 2006; Iwashita et al., 2009; Bédard et al., 2007). Its elevated expression was found in the spread of several solid tumors, such as melanoma metastasis (Qin et al., 2011) and MALT lymphoma (Frasch et al., 2007; Hamoudi et al., 2010; Baens et al., 2012). Qin et al. found that GPR34 was statistically significantly differentially expressed between melanoma metastases and benign nevi (Qin et al., 2011). In MALT lymphoma, dysregulation of GPR34 is commonly found and overexpression of GPR34 results in activation of the NF-κB and MAP kinase pathways and may be a novel mechanism by which MALT lymphoma occurs (Frasch et al., 2007; Hamoudi et al., 2010; Baens et al., 2012). In BCR-Abl wild type and mutant transformed BaF3 cell, we found Gpr34 to be involved in BCR-Abl induced leukemogenesis (unpublished data). These characteristics indicate that GPR34 is not only essential for degranulation of mast cells, but is also implicated in cancer development and progression, such as lymph node metastasis (Engemaier et al., 2006; Sugo et al., 2006; Bédard et al., 2007; Frasch et al., 2007; Iwashita et al., 2009; Hamoudi et al., 2010; Qin et al., 2011; Baens et al., 2012). Whether GPR34 plays a potential role in gastric cancer development and progression is unknown. In the present study, it has been demonstrated that expression of GPR34 in gastric cancer cells contributes to the biology and metastastic destination of tumor cells.

Materials and methods

Tissue samples and patient information

This retrospective study included tissue samples that were obtained from 297 patients who underwent total or partial gastrectomy in our hospital from January 2000 to December 2009. For immunohistochemical analysis, 1 tissue sample was obtained from each of the 297 patients represented primary tumor tissue and 1 from each 182 patients represented adjacent tissue (Table 1). Only patients with primary tumors and without other known malignancies at the time of diagnosis and follow-up were included, and a total of 172 patients with complete follow-up records were selected to perform survival analysis. None of the patients received chemotherapy or radiotherapy before surgery. Histological diagnosis was established on the basis of standard hematoxylin-and-eosin-stained sections of each sample. The cancer pathological type was classified according to the 2000 WHO guidelines (Hamilton and Aaltonen, 2000), and the tumor-node-metastasis (TNM) stage was classified according to the UICC staging system of 2002 (Mullaney et al., 2002). As described in Table 1, the patients included 203 men (68.4%) and 94 women (31.6%) with an average age of 62.3 years (range 23-93 years). One hundred and ten of these cases were differentiated adenocarcinoma (pap, tub) and 187 cases were undifferentiated adenocarcinoma (por. sig, muc). Among the 297 cases, 38 were diagnosed at stage I, 85 at stage II, 137 at stage III and 43 at stage IV. Two hundred and twenty-two cases had lymph node metastasis, whereas 75 cases had no lymph node metastasis. The details of the patients' characteristics are shown in Table 1. The median follow-up duration was 32.9 months (range 0-120). At the end of follow-up, 75 patients were still alive, whereas 97 had died. Overall survival was measured from the time of surgery to the date of tumor-related death. Patients were censored at the time of their last follow-up appointment or upon their death from causes unrelated to the tumor.

Immunohistochemistry

Immunohischemistry was performed as described in reference (Cui et al., 2010). In brief, tissue sections were deparaffinized in xylene, then rehydrated in a decreasing ethanol series. Endogenous peroxidase was blocked by incubating with 3% hydrogen peroxide for 15 min and non-specific binding was blocked by incubating with 5% normal goat serum (Zymed, USA) for 30 min. Sections were then incubated with Mouse-anti GPR34 monocolonal antibody (1:400 dilution; MAB4617, R&D Systems, MN, USA) at 4°C overnight, followed by a second-step incubation with ChemMateTMEn-Vision /HRP anti-mouse reagent according to the manufacturer's instruction (Gene Tech, Shanghai, China). The sections were stained with DAB, rinsed gently and counterstained with hematoxylin. Human liver and colon cancer sections were used as a positive control, whereas the primary antibody was omitted as a negative control. Two pathologists, who were blind to patient outcome, independently examined and scored the sections. Digital images were acquired using a Leica DM4000B digital camera microscope (Leica, Germany). A modified semi-quantitative scoring system (Hamm et al., 2008) was used to evaluate cytoplasmic GPR34. The staining was evaluated by scoring the percentage of positive cells according to the following scale: 0, negative; 1, 0-10%; 2, 10-50%; 3, 51-80%; and 4, >80% positive cells. The staining intensity was assessed using

Table 1. GPR34 expression in gastric cancer tissues and adjacent tissues.

GPR34	N	GPR34 low-expression n%	GPR34 high-expression n%	P χ^2 test
Adjacent tissue	182	65 (35.71)	117 (64.29)	
Tumor tissue	297	80 (26.94)	217 (73.06)	0.042

the following scale: 0, negative; 1, low; 2, moderate; and 3, strong. The final total score was obtained by multiplying the score of the percentage of positive cells by the staining intensity score, yielding the total scores between 0 and 12. Based on this score, tissues were classified as either GPR34 low-expression (score 0-5) or GPR34 high-expression (score 6-12).

Human gastric normal mucosal cell line and gastric cancer cell lines

Human gastric normal mucosal cell line GES-1 was kindly gifted by Dr Ke (Cui et al., 2010). The human gastric adenocarcinoma cell lines AGS, BGC823, HGC-27, MGC-803, MKN45 and NCI-N87 were purchased from the American Type Culture Collection. Among them, the GES-1and HGC-27 cell line were cultured in MEM, the others in DMEM (Gibco, USA) containing 10% fetal bovine serum (Hyclone, German) and penicillin/streptomycin (10 μl/ml) at 37°C in a humidified atmosphere with 5% CO_2.

Construction of GPR34 overexpression and Knock-down NCI-N87 cell models

Two MSCV-based retroviral vectors containing cDNAs encoding GPR34 and short hairpin RNA (shRNA) that specifically target GPR34 transcript were purchased from Open Biosystems (Huntsville, AL, USA). Sense sequence of GPR34 shRNA is as follows: CAGTTTGGATCGCTATATA. The expression of shRNAs was driven by the U6 promoter. Amplification and purification of plasmid DNAs were performed as specified by the manufacturer's instructions. Lipofectin 2000 was used to transfect MSCV-empty vector (Clontech, USA), MSCV-GPR34 and MSCV-ShRNA plasmids into NCI-N87 gastric cancer cell according to manufacturer's instruction (Invitrogen USA). The stable transformed clones were selected by puromycin (2 mg/mL) and used for subsequent experiments.

Quantitative real-time RT-PCR and Western blotting analyses

Quantitative real-time RT-PCR was performed using an OPTICON 2 real-time PCR detection system (Bio-Rad, USA) using human GPR34 primers (forward: 5'-CTC CCA CAG AAT GCG CTT TAT A-3', reverse: 5'-CAA CCA GTC CCA CGA TGA AAA-3') and ß-actin primers (forward: 5'-TCC TCC TGA GCG CAA GTA CTC-3', reverse: 5'-CAT ACT CCT GCT TGC TGA TCC A-3') combined with SYBR Green Master Mix (Applied Biosystems, USA). In brief, total RNA was extracted from cells with the RNeasy mini kit (Qiagen, USA) and cDNA was subsequently generated with Super-Script III (Invitrogen, USA). The PCR reactions began at 94°C for 2 min, followed by 40 cycles of 94°C for 20 s,59°C for 20 s, 72°C for 30 s, 74ºC for 1 s.

Western blotting analyses were performed by conventional protocols as described by ref (Cui et al., 2010) ß-actin (CB100997) primary antibody was obtained from Proteintech Company (IL, USA), PI3 Kinase Ab Sampler Kit (#9655) and P-Akt pathway Sampler Kit (#9916) were purchased from CST Company (MA, USA). In brief, Nylon membranes (Pharmacia, UK) were then blocked with 5% skimmed milk in TBS containing 0.1% Tween 20 (TBST) followed by an overnight incubation with primary antibodies (1:2000 or 1:1000) at 4°C. Membranes were then washed with TBST and incubated with secondary antibodies conjugated to HRP (Jackson, USA). Signals were detected using the SuperSignal_West Pico Trial Kit (Thermo Scientific, USA) and images were acquired using a MXP102 X-ray film processor (Kodak, USA).

Migration assays

The activity of invasion and migration of cells was performed using 24-well transwell chamber with 8.0-Ìm pore polycarbonate filter inserts and coated with matrigel (Costar, Cambridge, MA, USA). Cells ($5x10^4$ cells/well) suspended in serum-free DMEM containing 0.2% BSA were overlaid in the upper chamber of each transwell. In each lower chamber, 600 μl of DMEM supplemented with 10% FBS was added. Then the inserts were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for overnight. The cells that had not penetrated the filters were removed using cotton swabs. The migrated cells attached to the bottom side were fixed in 4% PFA (Paraformaldehyde) for 10 min and stained in 0.1% crystal violet for 30 min, rinsed in PBS and examined under a bright-field microscope with 100x magnification. The value of migratory activity was expressed as the average number of migrated cells per microscopic field over the 5 fields in each assay from three independent experiments.

Statistical analysis

SPSS version 16.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. The chi-square test (χ^2) was used to determine the potential correlation between the clinicopathological characteristics and GPR34 expression in primary tumor. The Kaplan-Meier method was used to analyze the potential correlation between GPR34 expression and both 3-year survival and median survival time, with significance evaluated by the two-sided log-rank test. Also, the Cox regression model was used to compute univariate and multivariate hazards ratios for the study variables. P values less than 0.05 were considered significant.

Results

Immunohistochemical assay

Positive and negative controls are shown as Fig. 1A,B. Immunohistochemical staining of 182 matched

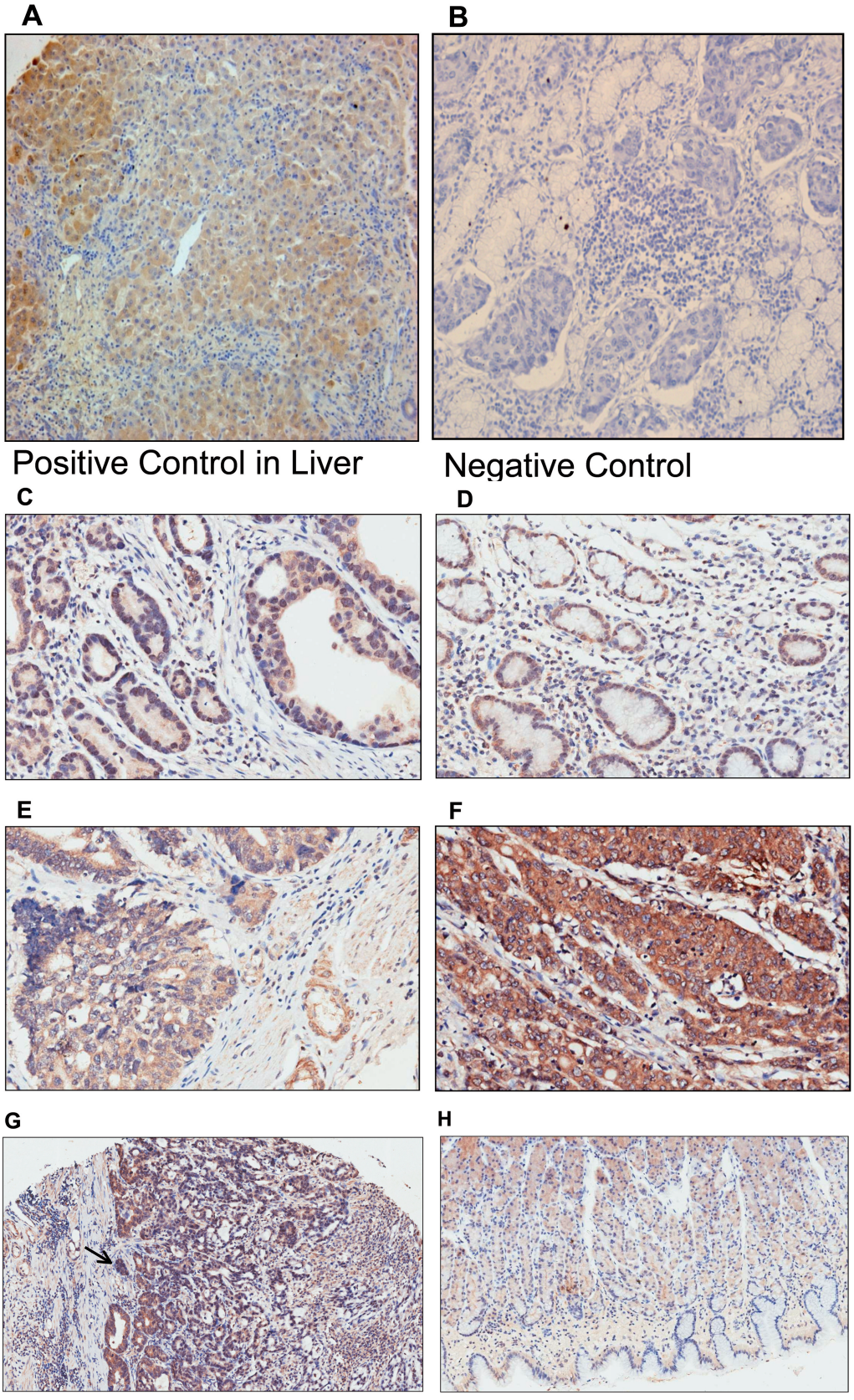

Fig. 1. Immunohistochemical detection of GPR34 expression in gastric cancer tissues. **A.** Positive controls in liver. **B.** Negative control, with primary antibody omitted, shows no staining. **C to F** show increased expression of GPR34 in T1, T2, T3 and T4. G shows surface and invasion front (arrow) of cancer tissue. H shows expression of GPR34 in normal gastric epithelium. A, B, G, H, x 1000; C-F, x 200

adjacent tissues and 297 primary cancerous tissues showed that GPR34 protein was abundant in the membranes and but scarce in the nucleolus of adjacent tissue, particularly at the base of the lamina propria, while in the cytoplasm and nucleolus of gastric cancer cells, GPR34 showed expression in 64.29% (117/182) of adjacent tissues and 73.06% (217/297) of gastric primary cancer tissues (Fig. 1A-H) (P=0.042) (Tables 1, 2).

Correlation of GPR34 expression with clinicopathological characteristics

We analyzed the association in 297 patients between GPR34 expression and clinicopathological variables that are used to describe the progression and aggressiveness of a tumor. By means of the chi-square test and the Spearman correlation test, we found that GPR34 expression in gastric primary tumors had no relationship with sex (P=0.351), age (P=0.173), clinical stages and distance metastasis (P=0.243) (Table 1), GPR34 expression showed a increasing trend as stage ranged from I to IV cancer tissue, although this was not statistically significant(P=0.093); GPR34 expression showed a highly correlation with tumor differentiation (P=0.000), infiltration depth (P=0.018), and lymph node metastasis (P=0.019). There were significant differences of GPR34 expression in gastric primary tumors between lymph nodes metastasis group and lymph nodes non-metastasis group [76.58% (170/222) vs. 62.67% (47/75), (P=0.019)] (Table 2).

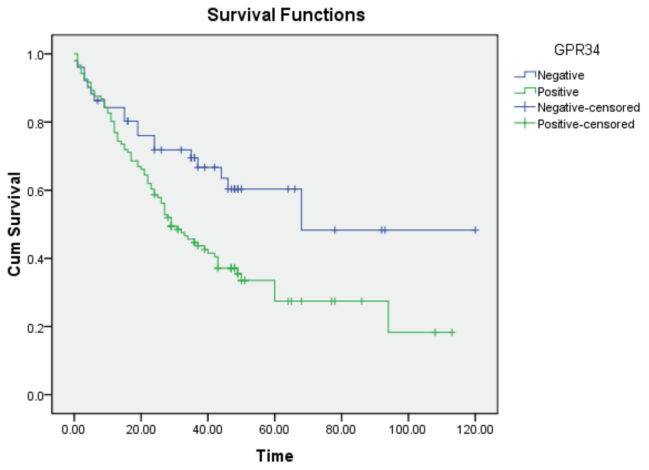

Fig. 2. Kaplan-Meier survival curves of gastric cancer patients who were positive and negative for GPR34 expression. The survival of GPR34-positive patients (n=121) was significantly greater than that of GPR34-negative patients (n=51) (P=0.025).

Table 2. Correlation between GPR34 expression and clinicopathological features of patients with gastric cancer.

Characteristics	N	GPR34 low-expression %	GPR34 high-expression %	p- values (χ^2 test)
Sex				
Male	203	28.6	71.4	
Female	94	23.4	76.6	0.351
Age at diagnosis (y)				
≥65	136	22.8	77.2	
<65	161	29.8	70.2	0.173
Differentiation				
Differentiated type (DT)	110	16.4	83.6	
Undifferentiated type (UDT)	187	33.2	66.8	0.000
TNM stage				
I	32	34.4	65.6	
II	85	29.4	70.6	
III	137	20.4	79.6	
IV	43	37.2	62.8	0.093
Infiltration depth				
T1	22	45.4	54.6	
T2	33	39.4	60.6	
T3	188	21.3	78.7	
T4	54	31.5	68.5	0.018
Lymph node metastasis				
N0		75	37.3	62.7
N1	222	23.4	76.6	0.019
Distance metastasis				
M0	263	25.9	74.1	
M1	34	35.3	64.7	0.243

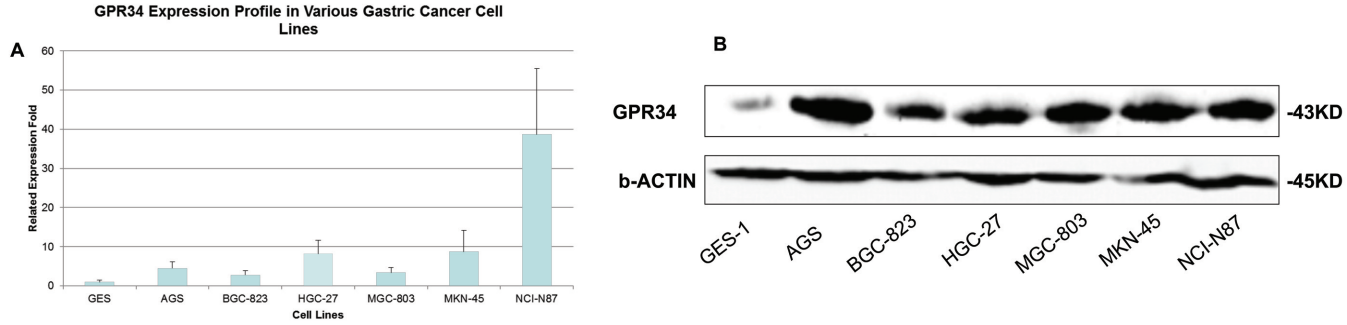

Fig. 3. The GPR34 expression in GES-1 and gastric cancer cell lines (AGS, BGC-823, HGC-27, MGC-803, MKN-45 and NCI- N87). A Real-time RT-PCR assay GPR34 mRNA expression in gastric cancer cell lines b. Western blotting showing specific expression of GPR34 in these cell lines.

Table 3. Multivariate analyses of overall survival of gastric cancer patients.

Variables	Univariate analyses			Multivariate analyses		
	HR	95% CI	P value	HR	95% (CI)	P value
Sex (male vs. female)	0.886	0.573-1.371	0.886	1.010	0.648-1.573	0.965
Age (years),(<65 vs.?65)	1.216	0.815-1.815	0.338	1.304	0.869-1.957	0.199
Differentiation (DT vs UDT)	0.637	0.390-1.025	0.063	0.584	0.354-0.962	0.035
Infiltration(T1, T2, T3, and T4)	2.137	1.162-3.933	0.015	1.778	0.827-3.824	0.141
TNM stage (I/II/III/IV)	1.261	1.009-1.575	0.042	1.042	0.735-1.477	0.817
Nodal metastasis (N0/N1)	1.607	0.969-2.665	0.066	1.275	0.690-2.357	0.437
GPR34 expression (Low/High)	1.955	1.183-3.230	0.009	2.276	1.359-3.811	0.002

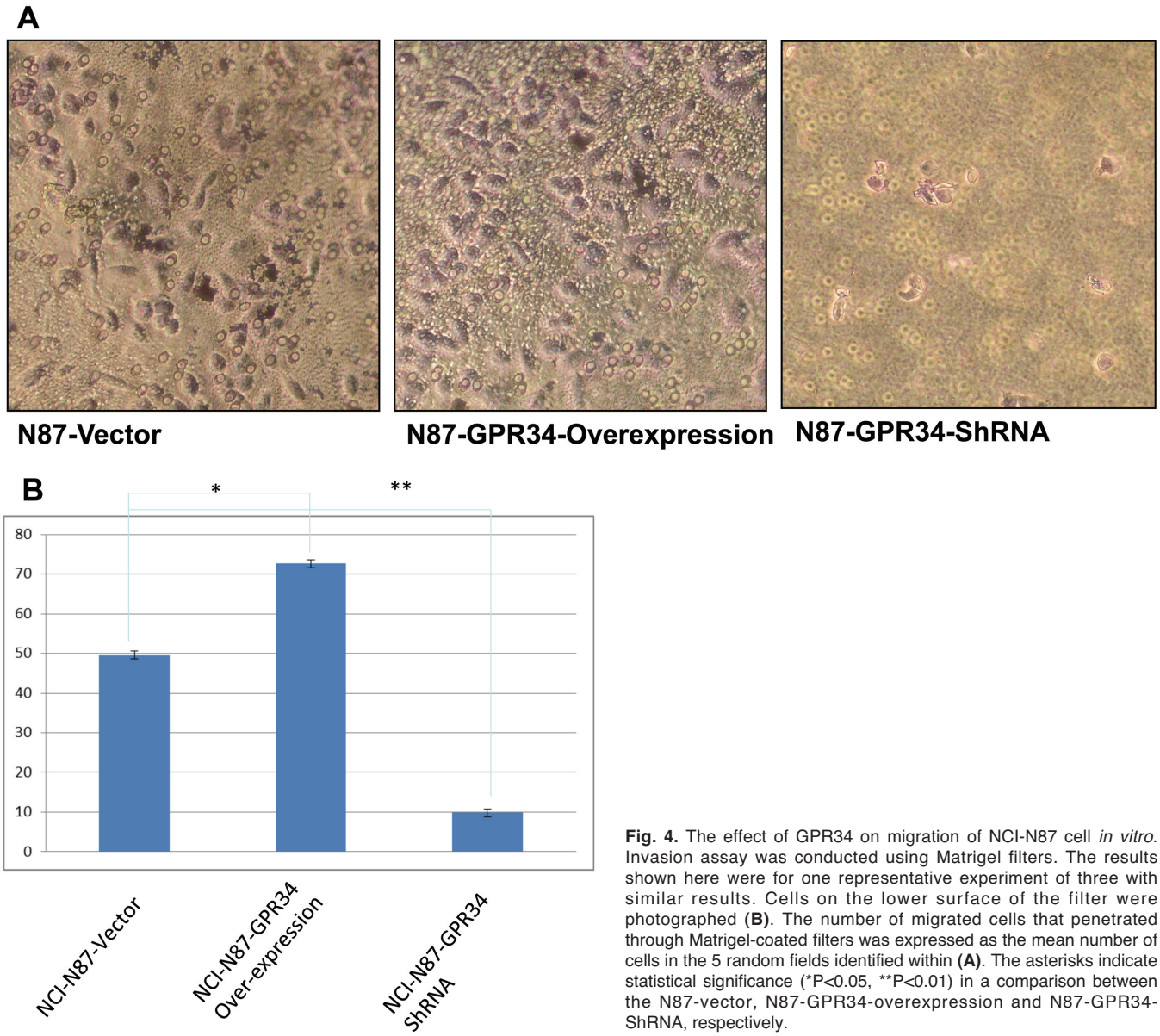

Fig. 4. The effect of GPR34 on migration of NCI-N87 cell *in vitro*. Invasion assay was conducted using Matrigel filters. The results shown here were for one representative experiment of three with similar results. Cells on the lower surface of the filter were photographed **(B)**. The number of migrated cells that penetrated through Matrigel-coated filters was expressed as the mean number of cells in the 5 random fields identified within **(A)**. The asterisks indicate statistical significance (*P<0.05, **P<0.01) in a comparison between the N87-vector, N87-GPR34-overexpression and N87-GPR34-ShRNA, respectively.

Correlation between GPR34 expression and patient survival

To test the hypothesis that changes in GPR34 expression are relevant to the outcome of patients with gastric cancer, we did a survival analysis using the Kaplan-Meier method. The increased GPR34 expression in primary cancer tissues was significantly associated with inferior survival duration. The 3-year survival rate was 85.2% in the GPR34-low-expression group and 72.9% in the GPR34-high-expression group. Median survival time in the GPR34-low-expression and GPR34-high-expression groups was 68.4 months (95% CI: 37.9.7-98.1) and 48.0 months (95% CI: 23.3-38.7), respectively. There was a significant difference between the two groups (χ^2=5.024, P=0.025) (Fig. 2). Multivariate analyses clearly showed that high GPR34 expression is an independent poor prognostic factor for overall survival (P=0.002, odds ratio, 2.276; 95% CI: 1.359-3.811) (Table 3).

Expression profile of GPR34 in human gastric normal mucosal cell line and gastric cancer cell lines

Both at mRNA and protein level, GPR34 expression was poor in human normal mucosal cell line GES-1, but high in gastric cancer cell lines AGS, BGC-823, HGC-27, MGC-803, MKN-45 and NCI-N87 (Fig. 3A,B).

Construction and validation of NCI-N87 GPR34 overexpression and knock-down cell models

NCI-N87 gastric cancer cell line, which derived from a liver metastasis of a well differentiated carcinoma of the stomach, was selected and used as a cell model in vitro to confirm the correlation between GPR34 expression in lymph nodes and gastric cancer metastasis. Both western blotting and real-time RT-PCR results indicated that the GPR34 over-expression and knockdown NCI-N87 cell models were successfully constructed (Fig. 5).

GPR34 over-expression promoted while its knock-down inhibited the migration of NCI-N87 in vitro by PI3K/PKD1/AKT pathway

To test the effect of GPR34 expression on migration of NCI-N87 gastric cancer cell, transwell chambers were used to determine the migration ability of NCI-N87-vector, NCI-N87-GPR34 over-expression, and NCI-N87-GPR34-ShRNA. The results show that GPR34 over-expression promoted while its knock-down inhibited the migration of NCI-N87 gastric cell (Fig. 4A,B).

Western blotting of p-PDK1, p-AKT and AKT show us that GPR34 over-expression increased while knock-down decreased the PDK1 and AKT Phosphorylation and indicated that GPR34 might play a important role in cell migration by PI3K/PDK1/AKT signal pathway (Fig. 5).

Discussion

The GPR34 gene lies on human chromosome Xp11.4 and encodes the GPR34 protein, a 7-transmembrane receptor (Engemaier et al., 2006). As a

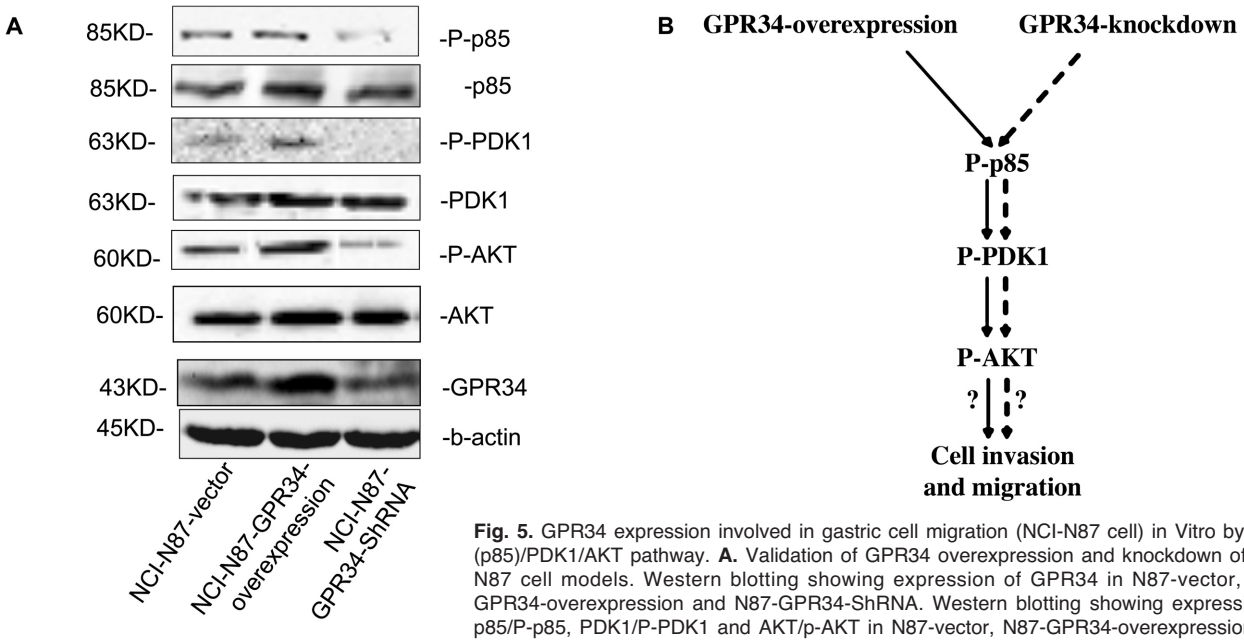

Fig. 5. GPR34 expression involved in gastric cell migration (NCI-N87 cell) in Vitro by PIK3 (p85)/PDK1/AKT pathway. **A.** Validation of GPR34 overexpression and knockdown of NCI-N87 cell models. Western blotting showing expression of GPR34 in N87-vector, N87-GPR34-overexpression and N87-GPR34-ShRNA. Western blotting showing expression of p85/P-p85, PDK1/P-PDK1 and AKT/p-AKT in N87-vector, N87-GPR34-overexpression and N87-GPR34-ShRNA. **B.** The hypothetic scheme of GPR34 role on the migration of gastric cancer cell NCI-N87. The arrow represents "promote" while the represents "inhibit".

member of the GPR superfamily, GPR34 plays essential roles in mast cell degranulation (Iwashita et al., 2009) and is preferentially expressed by microglia and upregulated during cuprizone-induced inflammation (Bédard et al., 2007).

G-protein coupled receptors (GPRs) have been implicated in the tumorigenesis and metastasis of human cancers (Qin et al., 2011). Aberrant GPR34 expression was recently described in several malignant solid tumors, including MALT lymphoma (Frasch et al., 2007; Hamoudi et al., 2010), melanoma (Qin et al., 2011), and glioma (Lee et al., 2008). We found that the GPR34 protein was mainly expressed in the membrane and nucleolus of gastric mucosal epithelia in adjacent tissues (Fig. 1H), while it was strongly expressed in the cytoplasm and nucleolus of gastric tumor specimens (Fig. 1F-P) (Table 1). We also confirmed that the expression of GPR34 was substantially increased in the gastric cancer cells AGS, BGC-823, HGC-27, MGC-803, MKN45 and NCI-N87 compared with GES-1 at both mRNA and protein levels (Fig. 3A,B). Interestingly, two transcript variants exist in human (http://www.ncbi.nlm.nih.gov/gene/2857), variant 1 differs in the 5' UTR compared to variant 4, but both of them encode the same protein. Our study shows that both variant 1 and 4 expressed in GES-1 (a normal gastric epithelial cell lines) with a rate of 1:1, while variant-1almost do not express in the gastric cancer cells AGS, BGC-823, HGC-27, MGC-803, MKN45 and NCI-N87 (unpublished Realtime-RT-PCR data, using variant-specific primers). Taken together with the different localized expression in adjacent tissues and cancer tissues, these results indicate the regulating mechanism of transcriptional level might determine the expression and roles of GPR34 in normal gastric epithelia and cancer cells and we need to pay more attention to this in future. The results of this study provide the first evidence that upregulated expression of GPR34 may play an important role in advanced gastric cancer. Migration assays (Fig. 4A,B) of GPR34 over-expression and knock-down NCI-N87 cells are consistent with the proposed role of GPR34 in other malignancies (Lee et al., 2008; Qin et al., 2011), and provide the first evidence that increased expression in metastastic lymph nodes of GPR34 may play a similarly important role in lymph node metastasis of gastric cancer. Thus, we provided both clinical and experimental evidence that GRP34 plays an important role in gastric cancer progression and metastasis.

In this study, we found that the increased GPR34 expression in primary cancer tissue was correlated with differentiation (P=0.000), infiltration depth (Fig. 1C-F), (P=0.018) and lymph node metastasis (Table 1) (P=0.019), also, increased GPR34 expression was shown in the invasion front of cancer tissue. It is generally accepted that deeper infiltration depth and lymphatic metastasis predict a poor prognosis of gastric cancer. Our clinical evidence supports the hypothesis that the increased GPR34 level in primary gastric cancer may contribute to an increase in the migration of gastric cancer cells. Upregulation of GPR34 expression might thus become a marker indicating poor outcome in gastric cancer patients. However, it must be noted that we found no significant degree of correlation between GPR34 expression in primary cancer tissues and other clinicopathological factors, such as distance metastasis and clinical stages (Table 1), which are commonly used to assess the prognosis of gastric cancer.

It is generally accepted that surgical resection is the most effective means of improving prognosis after early diagnosis of gastric cancer (Cui et al., 2010). Unfortunately, most gastric tumors are unable to be diagnosed at an early stage. After surgery, patients often undergo radiochemical therapy, which leads to a high level of morbidity and does not appreciably diminish the high risk of recurrence. Thus, the 5-year survival rate in patients with advanced gastric cancer is extremely poor, ranging between 20 and 30% (Cui et al., 2010) with a median survival duration of 13 to 60 months (Hejna et al., 2006). It is thus essential to assess, as precisely as possible, the risk of recurrence of gastric cancer, so as to minimize adverse effects of treatment and maximize its effectiveness. Of the prognostic factors available for gastric cancer, the most important is the Union International Contra laCancrum (UICC)/American Joint Committee on Cancer (AJCC) TNM stage, which is determined by the depth of invasion, involvement of lymph nodes, and presence of distant metastasis. Unfortunately, using this system, the prognosis varies among patients at the same stage. It is thus necessary to find new prognostic and predictive factors other than the TNM stage (Cui et al., 2010). In this study, we found that the increased GPR34 expression in primary gastric cancer was correlated with survival, and specifically that an upregulation of GPR34 expression was significantly associated with inferior survival duration. This suggests that GPR34 expression may be a useful prognostic indicator in this disease. However, because the prognosis of gastric cancer patients is affected by a complex array of factors, the specific utility of GPR34 expression in evaluating prognosis needs to await further stratified studies that include a larger number of samples, although GPR34 had a significant independent prognostic effect (P=0.002, odds ratio, 2.276; 95% CI: 1.359-3.811) on gastric cancer by Cox-regression model analysis.

The mechanism by which GPR34 may play its roles in gastric cancer infiltration depth and lymph node metastasis remain unclear. Previous studies have shown that GPR34 mediated a number of signal pathways related to cancer cell proliferation, apoptosis, and migration (Lee et al., 2008; Hamoudi et al., 2010; Qin et al., 2011; Baens et al., 2012). In this study, NCI-N87, a cell line derived from a liver metastasis of a metastastic carcinoma of the stomach taken prior to cytotoxic therapy, was selected to confirm the roles of GPR34 in gastric cancer infiltration and lymph node metastasis. After successful construction of GPR34 overexpression and knock-down NCI-N87 cell models, our migration

assay results show that GPR34 over-expression increased while knock-down decreased the migration activity of NCI-N87 gastric cells (Fig. 4A,B). As Fig. 5A shows, p-P85, p-PDK1 and p-AKT are up-regulated in GPR34 over-expression NCI-N87 cells, but down-regulated in GPR34 knock-down NCI-N87 cells. This suggests a potential positive regulatory role for GPR34 in the signaling mediated by PI3K/PDK1/AKT (Fig. 5B).

Furthermore, in this study, we found that the increased GPR34 expression in primary cancer tissue was correlated with differentiation (P=0.000), and we need to perform more experiments to determine roles of GPR34 in gastric cancer cell differentiation in future.

In summary, our results suggest that GPR34 plays an important role in the infiltration depth and lymph node metastasis of human gastric cancer, and specifically that up-regulation of its expression in primary gastric cancer accompanies the progression of this disease. GPRs are considered amongst the most desirable targets for drug development (Qin et al., 2011). The observation that patients with increased expression of GPR34 have dramatically reduced survival durations suggests that GPR34 might not only be a useful prognostic marker, but may itself also represent an effective therapeutic target for gastric cancer, such as targeting GPR34 expression at transcriptional level, or developing a tumor-specific GPR34-related-drug transfer system. Further studies are necessary to more precisely define the molecular mechanisms of GPR34 signaling pathways in the development and progression of gastric cancer, further gastric cancer cell invasion and migration.

Acknowledgments. This study was supported by a grant from the National Natural Science Foundation of China (No.30872923) and the Peking University People's Hospital Research and Development Foundations (No.RDB2007-47, No.RDK2008-01 and RDB2011-26). We are grateful to Prof. Qingxuan Chen (Laboratory of Gene Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, People's Republic of China) for expert help in the preparation and revision comments of this manuscript.

References

Anderson W.F., Camargo M.C., Fraumeni J.F., Correa P., Rosenberg P.S. and Rabkin C.S. (2010). Age-specific trends in incidence of noncardia gastric cancer in US adults, JAMA 303, 1723-1728.

Baens M., Finalet Ferreiro J., Tousseyn T., Urbankova H., Michaux L., de Leval L., Dierickx D., Wolter P., Sagaert X., Vandenberghe P., De Wolf-Peeters C., and Wlodarska I. (2012). t (X;14)(p11.4;q32.33) is recurrent in marginal zone lymphoma and up-regulates GPR34. Haematologica 97, 184-188.

Bédard A., Tremblay P., Chernomoretz A. and Vallières L. (2007). Identification of genes preferentially expressed by microglia and upregulated during cuprizone-induced inflammation. Glia 55, 77-789.

Cui M., Yu W., Dong J., Chen J., Zhang X. and Liu Y. (2010). Downregulation of ABI1 expression affects the progression and prognosis of human gastric carcinoma. Med. Oncol. 27, 632-639.

D'Ugo D., Persiani R., Zoccali M., Cananzi F., Vigorita V., Mazzeo P., Tufo A. and Biondi A. (2010). Surgical issues after neoadjuvant treatment for gastric cancer, Eur. Rev. Med. Pharmacol. Sci. 14, 315-319.

Engemaier E., Römpler H., Schöneberg T. and Schulz A. (2006). Genomic and supragenomic structure of the nucleotide-like G-protein-coupled receptor GPR34. Genomics 87, 254-264.

Frasch S.C., Zemski-Berry K., Murphy R.C., Borregaard N., Henson P.M. and Bratton D.L. (2007). Lysophospholipids of different classes mobilize neutrophil secretory vesicles and induce redundant signaling through G2A. J. Immunol. 178, 6540-6548.

Gao Y., Kitagawa K., Hiramatsu Y., Kikuchi H., Isobe T., Shimada M., Uchida C., Hattori T., Oda T., Nakayama K., Nakayama K.I., Tanaka T., Konno H. and Kitagawa M. (2006). Up-regulation of GPR48 induced by down-regulation of p27Kip1 enhances carcinoma cell invasiveness and metastasis, Cancer Res. 66, 11623-11631.

Hamilton S.R. and Aaltonen L.A. (2000). Pathology and genetics of tumors of digestive system. In: WHO classification of tumors. Hamilton S.R. and Aaltonen L.A. (eds). IARC Press. Lyon. pp 38.

Hamm A., Veeck J., Bektas N., Wild P.J., Hartmann A., Heindrichs U., Kristiansen G., Werbowetski-Ogilvie T., Del Maestro R., Knuechel R. and Dahl E. (2008). Frequent expression loss of Inter-alpha-trypsin inhibitor heavy chain (ITIH) genes in multiple human solid tumors: a systematic expression analysis. BMC Cancer 28; 8, 25.

Hamoudi R.A., Appert A., Ye H., Ruskone-Fourmestraux A., Streubel B., Chott A., Raderer M., Gong L., Wlodarska I., De Wolf-Peeters C., MacLennan K.A., de Leval L., Isaacso n P.G. and Du M.Q. (2010). Differential expression of NF-kappaB target genes in MALT lymphoma with and without chromosome translocation: insights into molecular mechanism, Leukemia 24, 1487-1497.

Hejna M., Wohrer S., Schmidinger M., Raderer M. (2006). Postoperative chemotherapy for gastric cancer. Oncologist 11, 136-145.

Iwashita M., Makide K., Nonomura T., Misumi Y., Otani Y., Ishida M., Taguchi R., Tsujimoto M., Aoki J., Arai H. and Ohwada T. (2009). Synthesis and evaluation of lysophosphatidylserine analogues as inducers of mast cell degranulation. Potent activities of lysophosphatidylthreonine and its 2-deoxy derivative. J. Med. Chem. 52, 5837-5863.

Lee S.Y., Lee H.Y., Kim S.D., Jo S.H., Shim J.W., Lee H.J., Yun J. and Bae Y.S. (2008). Lysophosphatidylserine stimulates chemotactic migration in U87 human glioma cells. Biochem. Biophys. Res. Commun. 374, 147-151.

Mullaney P.J., Wadley M.S., Hyde C., Wyatt J., Lawrence G. and Hallissey M.T. (2002). Appraisal of compliance with the UICC/AJCC staging system in the staging of gastric cancer. Union Internacional Contra la Cancrum/American Joint Committee on Cancer. Br. J. Surg. 89, 1405-1408.

Qin J., Xie Y., Wang B., Hoshino M., Wolff D.W., Zhao J., Scofield M.A., Dowd F.J., Lin M.F. and Tu Y. (2009). Upregulation of PIP3-dependent Rac exchanger 1 (P-Rex1) promotes prostate cancer metastasis, Oncogene 28, 1853-1863.

Qin Y., Verdegaal E.M., Siderius M., Bebelman J.P., Smit M.J., Leurs R., Willemze R., Tensen C.P. and Osanto S. (2011). Quantitative expression profiling of G protein-coupled receptors (GPCRs) in metastatic melanoma: the constitutively active orphan GPCR GPR18 as novel drug target, Pigment Cell. Melanoma Res 24, 207-218.

Sugo T., Tachimoto H., Chikatsu T., Murakami Y., Kikukawa Y., Sato S.,

Kikuchi K., Nagi T., Harada M., Ogi K., Ebisawa M. and Mori M. (2006). Identification of a lysophosphatidylserine receptor on mast cells, Biochem. Biophys. Res. Commun. 341, 1078-1087.

You W.C., Brown L.M., Zhang L., Li J.Y., Jin M.L., Chang Y.S., Ma J.L., Pan K.F., Liu W.D., Hu Y., Crystal-Mansour S., Pee D., Blot W.J., Fraumeni J.F., Xu G.W. and Gail M.H. (2006) .Randomized double-blind factorial trial of three treatments to reduce the prevalence of precancerous gastric lesions, J. Natl. Cancer. Inst. 98, 974-983.

Accepted June 26, 2013