GASTRIC CANCER

Reduced expression of epidermal growth factor receptor related protein in gastric cancer

W S Moon, A S Tarnawski, J Chai, J T Yang, A P N Majumdar

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See end of article for authors' affiliations

Correspondence to: Professor A S Tarnawski, VA Medical Center/University of California-Irvine, 5901 East 7th St, Long Beach, CA 90822-5201, USA; andrzej.tarnawski@med.va.gov

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Objectives: The recently cloned epidermal growth factor receptor related protein (ERRP) has been proposed to be a negative regulator of the epidermal growth factor receptor (EGFR). Because of the causal involvement of EGFR and its ligands in gastric cancer growth, we investigated expression of ERRP and cell proliferation in human gastric cancer.

Methods: We examined ERRP expression and localisation in surgical specimens of gastric cancers from 47 patients versus non-malignant gastric mucosa and determined their relationship to cell proliferation and differentiation. We also examined expression of ERRP by western blotting in three different gastric cancer cell lines. To further determine the functional properties of ERRP, we examined the effect of ERRP on epidermal growth factor (EGF) induced EGFR phosphorylation essential for its activation in MKN-28 gastric cancer cells.

Results: ERRP expression was dramatically reduced in gastric cancers (34% of all specimens positive) compared with non-malignant gastric mucosa (66% of specimens positive). Expression of ERRP in cancer cells inversely correlated with cell proliferation and grade of malignancy. Cell lines derived from metastatic gastric cancers had reduced ERRP expression compared with cell lines derived from a non-metastatic cancer. Exogenous ERRP protein markedly inhibited EGF induced EGFR phosphorylation in gastric cancer cells providing a novel molecular mechanism of its action.

Conclusions: Our data indicate that downregulation of ERRP could play an important role in gastric cancer differentiation and progression. ERRP is a negative regulator of tumour cell proliferation and may exert its inhibitory effect, in part, by attenuating EGFR activation.

The epidermal growth factor receptor (EGFR) is a 170 kDa protein that consists of an extracellular domain, a transmembrane domain, and a cytoplasmic domain. Ligand binding to the receptor activates its intrinsic kinase activity that ultimately results in increased cell proliferation and differentiation.¹ Gastric cancer is the second most common cancer in the world, accounting for ~9% of all malignancies worldwide. Increased expression and activation of EGFR and its ligands are causally associated with progression of gastric cancer and poor prognosis.^{2–3} The recently isolated EGFR related protein (ERRP) is proposed as a negative regulator of EGFR activation.^{4–6} Although the precise mechanism(s) of increased EGFR activation in gastric cancer cells is not known, we hypothesised that this increase is due, at least in part, to downregulated ERRP expression. We examined ERRP expression and localisation in surgical specimens of gastric cancers compared with non-malignant gastric mucosa and determined its relationship to cell proliferation and differentiation. In addition, we examined expression of ERRP by western blotting in three different gastric cancer cell lines representing various degrees of differentiation and metastasis. To further determine the functional properties of ERRP, we examined the effect of ERRP on epidermal growth factor (EGF) induced EGFR phosphorylation, essential for its activation in MKN-28 gastric cancer cells.

MATERIALS AND METHODS

Materials

The Human Ethics Committee of Chonbuk National University Medical School and the Human Study Subcommittee of the VA Medical Center, Long Beach, approved this study. Surgical specimens of 47 formalin fixed paraffin embedded gastric cancers with adjacent non-involved normal gastric mucosa and 10 specimens of gastric dysplasia were

obtained from Surgical Pathology Archives of Chonbuk National University Hospital, Korea. Of the 47 patients, 30 were males and 17 were females, aged 34–76 years (mean 60.2 years). Gastric cancers were classified according to the WHO classification⁷ as well differentiated (n = 10), moderately differentiated (n = 10), poorly differentiated (n = 12), and signet ring cell carcinomas (n = 15). According to the Lauren classification,⁸ 32 specimens were intestinal type and 15 were diffuse type.

Specimens were examined by two independent experienced pathologists who also evaluated haematoxylin-eosin (H&E) and Giemsa stained slides for the presence of *Helicobacter pylori*.

The human gastric cancer cell lines AGS (non-metastatic gastric cancer derived from poorly differentiated gastric adenocarcinoma), NCI-N87 (derived from a liver metastasis) (both from ATCC, Manassas, Virginia, USA), and MKN-28 (derived from a lymph node metastasis but from a well differentiated gastric adenocarcinoma with intestinal type differentiation; RIKEN Cell Bank, Tsukuba, Japan) were cultured according to cell bank instructions.

Immunohistochemistry

For immunohistochemical staining, an immunoperoxidase method was used with a streptavidin biotinylated horse-radish peroxidase complex (Dako, Carpinteria, California, USA). Sections of formalin fixed paraffin embedded tissue blocks were deparaffinised and subsequently incubated in methanol containing 0.3% hydrogen peroxide at room temperature for 20 minutes to quench endogenous peroxidase

Abbreviations: EGF, epidermal growth factors; EGFR, epidermal growth factor receptor; ERRP, epidermal growth factor receptor related protein; PCNA, proliferation cell nuclear antigen; LI, labelling index; H&E, haematoxylin-eosin

activity and then treated with pepsin for 10 minutes at room temperature. Sections were incubated with protein block serum free (Dako) at room temperature for 10 minutes to block non-specific staining and then incubated for two hours at room temperature with rabbit polyclonal anti-ERRP antibody. After washing, sections were incubated with a biotin conjugated secondary antibody at room temperature for 30 minutes and finally with peroxidase conjugated streptavidin at room temperature for 30 minutes. Peroxidase activity was detected with the enzyme substrate 3-amino-9-ethyl carbazole. For negative controls, sections were treated in the same way except that they were incubated with Tris buffered saline instead of the primary antibody. In addition, to assure ERP antibody specificity, in selected experiments, for immunostaining we used as a new control ERP antibody preabsorbed (4°C for 16 hours) with a 50-fold excess of ERP peptide instead of normal ERP antibody.

Polyclonal antibody against ERP was generated in rabbits as described previously^{5,6} utilising an epitope from the "U" region comprising 15 amino acids (AVTRPLHPLAQNRVS) that showed no homology with any known sequence in the database. Previous study by one of the authors (APNM) demonstrated the specificity of this ERP antibody.⁶

Double immunostaining for ERP and PCNA

To determine the relationship between ERP expression and proliferative activity, we performed double immunostaining for proliferating cell nuclear antigen (PCNA, the nuclear antigen present only in proliferating cells) and ERP on the same specimens. Paraffin embedded tissue sections were double immunostained using the Envision Doublestain System (Dako). Tissue sections were deparaffinised and slides were treated with a microwave antigen retrieval procedure in 0.01 M sodium citrate buffer for 10 minutes. After quenching the endogenous peroxidase activity with peroxidase blocking reagent (Dako), sections were incubated with mouse monoclonal anti-PCNA antibody (Dako) for one hour at room temperature. Slides were rinsed with washing buffer and incubated with labelled polymer-horseradish peroxidase-antimouse and antirabbit antibodies (Envision Doublestain System step 3) for 30 minutes at room temperature. Peroxidase activity was detected with the enzyme substrate 3,3'-diaminobenzidine tetrachloride (Envision Doublestain System step 4). After quenching the enzyme reaction, slides were incubated in Doublestain Block (Envision Doublestain System step 5) at room temperature for five minutes to block endogenous phosphatase. Then, slides were incubated anti-ERP antibody for one hour at room temperature. After washing, slides were incubated with labelled polymer-alkaline phosphatase antimouse and anti-rabbit antibody (Envision Doublestain System step 7) for 30 minutes at room temperature. Fast red solution was used for visualisation of the bound antibody. Sections were counterstained with Mayer's haematoxylin. To examine the role of ERP tumour cell proliferation, we counted PCNA positive cancer cells in both ERP negative and positive cancer cells. The PCNA labelling index (PCNA-LI) was defined as the percentage of cells with nuclei stained positively for PCNA.

ERP expression

Samples immunostained for ERP were rated according to a score calculated by adding the intensity of the stain to the area of the stain. The intensity of cell staining was graded according to the following scale; 0, no staining; 1+, mild staining; 2+, moderate staining; and 3+ strong staining. The area of staining was evaluated using the following scale: 0, <10% of cells stained positive; 1+, 10–30% stained positive;

2+, 30–70% stained positive; and 3+, >70% stained positive. The maximum combined score was 6 and the minimum was 0.

ERP expression in gastric cancer cell lines by western blotting

To further examine the role of ERP in cancer and invasiveness, we performed western blot analysis of ERP expression in three different gastric cancer cell lines: AGS, MKN-28, and NCI-N87. Briefly, cells were lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Nonidet p-40, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 5 mM sodium fluoride, and 1 mM sodium orthovanadate. The protein concentration of the lysates was determined by the bicinchoninic acid protein assay (Pierce Chemical, Rockford, Illinois, USA). Equal amounts of protein (150 µg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. ERP expression was determined by western blot analysis as previously described using polyclonal anti-ERP antibody. The membrane was incubated with ERP antibody at room temperature for one hour. The membrane was washed and incubated with antirabbit IgG peroxidase conjugate at room temperature for one hour. The signal of the bound antibody was visualised by chemiluminescence (Amersham Life Science, Arlington Heights, Illinois, USA). The membrane was stripped and reprobed with monoclonal anti-β-actin antibody (Sigma, St Louis, Missouri, USA). Quantification of the data was performed using ImageQuant software (Molecular Dynamics, Piscataway, New Jersey, USA). Each signal was normalised against a corresponding β-actin signal.

Effect of exogenous ERP protein on EGF induced EGFR phosphorylation in MKN-28 gastric cancer cells

ERP was affinity purified from CuSO₄ induced *Drosophila* S2 cell extracts using either a Ni chelated or a His antibody column.⁶ Serum starved MKN-28 cells were pretreated with 5 µg/ml human ERP protein for 60 seconds and then incubated with 50 ng/ml of recombinant human EGF protein (BD Transduction Laboratory, San Diego, California, USA) for an additional 15 minutes. Cells were lysed and EGFR was immunoprecipitated using mouse monoclonal EGFR antibody (BD Transduction Laboratory) conjugated to protein A Sepharose. Following extensive washing, phosphorylation levels of the immunoprecipitated EGFR was determined by western blot analysis using antibodies against EGFR and phosphorylated EGFR (Santa Cruz Biotechnology, Santa Cruz, California, USA). Protein from cells treated only with EGF served as a positive control while protein from cells without treatment was used as a negative control. Blots were stripped and reprobed for human β-actin as a loading control.

Statistical analysis

Values are expressed as mean (SEM). Data were analysed statistically using the Kruskal-Wallis test to determine the difference in ERP expression between each group. The Mann-Whitney rank sum test was used to determine differences in PCNA-LI between the two groups and ERP expression between intestinal and diffuse type gastric cancers. A p value <0.05 was considered statistically significant.

RESULTS

ERP expression and its relation to tumour cell proliferation and differentiation

Positive immunostaining for ERP was detected in 31 of 47 (66%) non-malignant gastric tissues where it showed a distinct perinuclear dot-like expression in surface epithelial

Figure 1 (A–F) Expression and localisation of epidermal growth factor receptor related protein (ERRP) in gastric tissue. (A) Normal pyloric gland mucosa. ERRP expression is localised to the perinuclear region (red signals) with no membrane staining. Inset: foveolar epithelium exhibit perinuclear dot-like staining. (B) Dysplastic cells showed perinuclear cytoplasmic staining for ERRP. (C, D) Representative gastric carcinoma specimens. (C) Moderately differentiated carcinoma. Cancer cells have minimal or absent (arrows) ERRP staining. (D) In infiltrating poorly differentiated carcinoma cells (arrows), ERRP staining is absent. (E, F) Gastric cancer specimens positive for ERRP. (E) Well differentiated carcinoma. Some cells exhibit intense luminal and/or circumferential membrane staining (arrows) (F) Moderately differentiated carcinoma. Cancer cells showed either intense luminal and circumferential membrane staining and/or cytoplasmic and membrane staining. (G–I) Double immunostaining for ERRP and proliferation cell nuclear antigen (PCNA). (G) In normal gastric tissue, proliferating cells (arrows) in the neck area of the gastric glands do not express ERRP. ERRP positive glandular cells (red signals) do not exhibit PCNA expression. (H) Only ERRP negative cancer cells display nuclear PCNA staining (arrows). Conversely, ERRP positive normal glandular cells (red signals) do not exhibit PCNA immunoreactivity. (I) ERRP positive cancer cells, with selective ERRP membrane staining (red signals), do not exhibit PCNA staining. In adjacent PCNA positive carcinoma cells (arrows), ERRP staining is negative, indicating an inverse relationship between ERRP and cell proliferation.

cells and in some glandular cells but not in cells of the proliferative zone (neck area) of gastric glands (fig 1A). Cytoplasmic perinuclear ERRP expression was detected in seven of 10 (70%) dysplasia samples (fig 1B). In gastric cancers, there was a marked decrease in ERRP expression (fig 1C, D), with only 16 of 47 (34%) specimens exhibiting positive staining, often only as a focal reaction (table 1). Interestingly, in ERRP positively stained gastric cancer specimens (34% of all cancer specimens), two patterns of ERRP expression were observed: (a) selective membrane expression and (b) dense membrane and cytoplasmic expression with loss of polarised distribution in

normal gastric mucosal cells. In the case of membrane staining, some cell within the same malignant gland showed luminal membrane staining as well as staining of luminal contents while other cells showed circumferential membrane staining (fig 1E, F). While ERRP expression was present in 60% of well differentiated cancers, it was markedly decreased (to 23%) or absent in dedifferentiated cancers. The ERRP staining score was 2.9 (0.3) in normal gastric mucosa, and 2.6 (0.6), 0.9 (0.6), and 1.0 (0.5) in well, moderately, and poorly differentiated cancers, respectively. Overall, the decrease in ERRP expression inversely correlated with histological grade of malignancy ($p = 0.01$). We found

Table 1 Epidermal growth factor receptor related protein (ERRP) expression in non-malignant gastric mucosa, dysplasia, and gastric cancers

	No of ERRP positive specimens	ERRP expression score (mean (SEM))
Non-malignant mucosa (n = 47)	31 (66%)	2.9 (0.3)
Dysplasia (n = 10)	7 (70%)	3.2 (0.9)
Total gastric cancer (n = 47)	16 (34%)	1.4 (0.3)
Well differentiated carcinoma (n = 10)	6 (60%)	2.6 (0.6)
Moderately differentiated carcinoma (n = 10)	2 (20%)	0.9 (0.6)
Poorly differentiated carcinoma (n = 12)	3 (25%)	1.0 (0.5)
Signet ring cell carcinoma (n = 15)	5 (33%)	1.3 (0.5)

Kruskal-Wallis test showed an inverse relationship between ERRP expression and gastric carcinoma differentiation. In gastric cancers there was a marked decrease in ERRP expression with 31 of 47 (66%) specimens exhibiting negative staining for ERRP. In ERRP positively stained gastric cancer specimens (34% of all cancer specimens), ERRP was localised to the membrane or membrane and cytoplasm.

Table 2 Association between epidermal growth factor receptor related protein (ERRP) expression and *Helicobacter pylori* infection in non-malignant gastric mucosa and gastric cancers

	<i>H pylori</i> positive	<i>H pylori</i> negative	Total
Non-malignant gastric mucosa*			
ERRP positive	5	26	31
ERRP negative	4	12	15
Total	9	38	47
Gastric cancer tissue: non-malignant cells in proximity to cancer cells†			
ERRP positive	5	11	16
ERRP negative	4	27	31
Total	9	38	47

* $p = 0.46$ χ^2 test.

† $p = 0.12$ χ^2 test.

no staining when the antigen preabsorbed ERRP antibody was used while sections from the same block gave positive staining with the regular (non-preabsorbed) ERRP antibody. This confirmed the specificity of the ERRP antibody. Double immunostaining for ERRP and PCNA revealed a negative relationship between ERRP and PCNA expression. Normal gastric gland and cancer cells expressing ERRP exhibited only a very faint or no PCNA immunoreactivity. PCNA-LI reflecting cell proliferation was also significantly (~ 3.5 fold) lower in ERRP positive cancer cells (9.4 (0.6)%) than in ERRP negative cancer cells (35.3 (1.1)%; $p < 0.001$). This pattern clearly distinguished ERRP expressing cells from adjacent proliferating cells that expressed PCNA (fig 1G–I). There was no difference in ERRP expression between intestinal type (1.45 (0.46)) and diffuse type (1.36 (0.37)) gastric cancer.

Evaluation of H&E and Giemsa stained slides demonstrated that only nine specimens (19%) showed the presence of *H pylori* while in remaining specimens *H pylori* was not detectable (table 2). Of nine *H pylori* positive specimens, five were ERRP positive and four were ERRP negative (table 2). These data showed a lack of association between ERRP positivity and *H pylori* presence determined by histological staining.

ERRP levels in gastric cancer cell lines

To further determine whether and to what degree ERRP expression is associated with the invasive properties of gastric cancer cells, we examined, by western blotting, ERRP expression levels in one non-metastatic and two metastatic gastric cancer cell lines. ERRP expression levels were markedly decreased in the metastases derived cells MKN-28 (1.7-fold) and NCI-N87 (twofold) compared with the non-metastatic gastric cancer cell line (AGS) which is poorly differentiated (fig 2).

ERRP inhibits EGFR phosphorylation in EGF treated MKN-28 cells

EGF treatment induced a 3.5-fold increase in EGFR phosphorylation in MKN-28 cells. EGF induced phosphorylation of EGFR in MKN-28 cells was markedly inhibited (~ 45

Figure 2 Western blot analysis of epidermal growth factor receptor related protein (ERRP). ERRP expression levels were markedly decreased in the metastases derived gastric cancer cell lines (MKN-28 and NCI-N87) compared with the non-metastasis derived gastric cancer cell line (AGS).

Figure 3 Epidermal growth factor receptor (EGFR) activation reflected by the extent of EGFR phosphorylation (pEGFR). Exposure of MKN-28 gastric cancer cells to affinity purified ERRP protein caused marked inhibition of epidermal growth factor (EGF) induced EGFR phosphorylation.

fold) by ERRP. Western blotting revealed no significant differences in EGFR expression between ERRP treated cells and untreated cells, indicating that inhibition of EGFR activation by ERRP is direct and not the result of changes in EGFR expression levels (fig 3).

DISCUSSION

This study demonstrated that: (1) gastric cancer cells have significantly reduced, or complete lack of, ERRP expression, particularly cells of dedifferentiated cancers; (2) expression of ERRP in cancer cells is overall inversely correlated with proliferative activity and cell differentiation; (3) a major difference in ERRP cellular localisation exists between normal gastric cells and cancer cells; and (4) exogenous ERRP inhibits EGF induced phosphorylation of EGFR in MKN-28 gastric cancer cells. In agreement with our findings, recent study demonstrated that increased activation of EGFR and loss of ERRP in gastric mucosa occurs during aging and gastric cancer.⁹

In general, our results in resected human gastric cancer samples demonstrated that a decrease in ERRP expression is inversely correlated with grade of malignancy. However, three of 12 poorly differentiated carcinoma and five of 15 signet ring cell carcinomas in surgical resected specimens still had considerable ERRP expression. This indicates that the degree of differentiation does not always match ERRP expression in gastric cancer cells.

Metastasis derived gastric cancer cell lines have markedly lower ERRP expression levels than non-metastatic cell lines, although it does not correlate with cell differentiation. MKN-28 cells are metastases derived cells but they are from a well differentiated gastric adenocarcinoma with intestinal type differentiation.¹⁰ Conversely, AGS cells are from a non-metastatic gastric cancer but they are from a poorly differentiated gastric adenocarcinoma.¹¹ Therefore, there is a discrepancy between the results obtained in vivo (for example, the higher the cancer differentiation the higher the ERRP expression) and the results obtained in vitro where the highest level of expression of ERRP was in AGS cells, derived from a poorly differentiated adenocarcinoma, and lower in the well differentiated MKN-28 cell line. The reason(s) for this discrepancy between in vivo and in vitro results is unclear but gastric cancer specimens reflect better a real in vivo condition.

In normal gastric tissue, the proliferating cells (as determined by PCNA staining) at the neck area of the gastric gland did not express ERRP. Moreover, we found that ERRP expression was confined to normal foveolar epithelium and gland mucosa, which have less proliferating cells (as reflected by negative PCNA staining). These findings suggest that ERRP may be constitutively expressed at low levels in normal

resting cells where it inhibits cell proliferation. In the present study, we found selective membrane expression of ERRP in some cancer cells, and ERRP expressing cancer cells showed significantly less proliferation (decreased PCNA-LI) than cancer cells that did not express ERRP. The significance of ERRP membrane localisation in ERRP expressing cancer cells is uncertain. Although ERRP is not a product of the primary EGFR transcript, it has 85–90% homology to the extracellular ligand binding domain of EGFR.⁴ Three splice variants of the EGFR gene encoding the extracellular domain of the receptor have been isolated^{12–14} and accumulating evidence indicates that a truncated EGFR, that contains the EGF binding site but not the kinase domain, is associated with inhibition of cancer cell growth.¹⁵ This negative regulation of EGFR kinase may be due to dimeric interactions between full length EGFR and truncated EGFR.¹⁶ Enhanced expression of ERRP on the membrane of cancer cells demonstrated here suggest that ERRP may competitively inhibit EGFR activation. This contention was confirmed by demonstration that exogenous ERRP inhibited EGF induced EGFR phosphorylation essential for its activation in the MKN-28 gastric cancer cell line.

Considering that the functional form of EGFR is normally localised to the plasma membrane, it is tempting to speculate that ERRP may compete with EGFR for ligand binding and/or may form inactive heterodimers with EGFR. Previous studies demonstrated that ERRP is a secretory protein⁶ and as such can exert local autocrine and paracrine actions. Furthermore, a recent study demonstrated that reduced EGFR phosphorylation was partly due to sequestration of EGFR ligands by ERRP, resulting in the formation of inactive heterodimers with EGFR, further supporting our contention.⁶ Previous studies have demonstrated that expression of EGFR and its various ligands is increased in gastric cancer, and that these increases are closely related and play an important role in the development and progression of gastric cancer.^{1–2, 17–19} Taken together with our present study, increased EGFR activation in gastric cancer might be due not only to a decrease in ERRP expression but also to increased expression of EGF related peptides.

We examined gastric cancer histological specimens for the presence of *H. pylori* using H&E and Giemsa staining. Of 47 patients, *H. pylori* was detected in only nine patients (19%); five of them were ERRP positive and four were ERRP negative. Compared with previous reports demonstrating the presence of *H. pylori* in gastric endoscopic biopsies in approximately 60–70% of the Korean population,^{20–21} the presence of *H. pylori* in this study using resection specimens was much lower (only 19%). However, our data are in agreement with previous observations in that while gastric intestinal metaplasia and gastric cancer are epidemiologically associated with *H. pylori* infection, the bacterium is often undetectable in these lesions by standard histological test stains.^{22–24} A recent study by Semino-Mora and colleagues²⁵ explained this apparent paradox by demonstrating intracellular localisation of *H. pylori* in patients with precancerous lesions and/or gastric adenocarcinoma. Intracellular *H. pylori* was detected only when using the combined techniques of light microscopy, laser confocal microscopy, transmission electron microscopy, in situ hybridisation, and immunohistochemistry. The staining method for detecting *H. pylori* used in our study was unlikely to detect intracellular *H. pylori*. A more detailed analysis using the combination of the above cited methods should provide further information regarding the possible relationship between *H. pylori* infection and ERRP expression.

In conclusion, our data indicate that downregulation of ERRP is associated with progression and dedifferentiation of gastric carcinoma. The inverse relationship between ERRP expression and cell proliferation suggests that loss of ERRP

likely plays a role in promoting tumour growth. Moreover, changes in ERRP localisation from perinuclear in normal gastric mucosa to membrane in gastric cancer cells, and its inhibitory effect on EGFR phosphorylation, suggest that ERRP is a negative regulator of EGFR and may exert its inhibitory effect partly by attenuating EGFR activation. As ERRP protein and/or cDNA can be easily synthesised, this study may provide a rationale for novel therapeutic approaches to gastric cancer.

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Authors' affiliations

W S Moon, Department of Medicine, VA Medical Center, Long Beach and the University of California, Irvine, California, USA, and Department of Pathology, Chonbuk National University Hospital, Chonju, Korea

A S Tarnawski, J Chai, J T Yang, Department of Medicine, VA Medical Center, Long Beach and the University of California, Irvine, California, USA

A P N Majumdar, John D Dingle VA Medical Center, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan, USA

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EDITOR'S QUIZ: GI SNAPSHOT

Robin Spiller, Editor

An unusual CT

Clinical presentation

A 50 year old man was admitted to the emergency room suffering from right upper quadrant pain and vomiting for two days. He had a history of diarrhoea and 10 kg weight loss in the past two months. On arrival, physical examination revealed a distended abdomen with muscular resistance and hypotension. Laboratory data were arterial pH 7.22, white cell count 17 000/ μ l, and total bilirubin 56.1 μ mol/l. Abdominal ultrasound was performed and air images were seen peripherally in the hepatic parenchyma. A computed tomography scan of the abdomen was obtained (fig 1).

Question

What is the diagnosis?

See page 249 for answer

This case is submitted by:

S Baydar, A Hatipoglu, A Sezer, C Ibis, I Coskun

Department of Surgery, Trakya University, School of Medicine, Edirne, Turkey

H Umit, A Tezel

Department of Gastroenterology, Trakya University, School of Medicine, Edirne, Turkey

Correspondence to: Dr H Umit, Gastroenteroloji Bölümü, Trakya Üniversitesi, Tıp Fakültesi, 6 kat, 22030, Edirne, Turkey; hasanumit@ttnet.net.tr

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Figure 1 Computed tomography scan taken on admission revealed gas shadows in the hepatic portal venous system.