GASTRIC CANCER

Elevated plasma osteopontin associated with gastric cancer development, invasion and survival

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Objective: Osteopontin (OPN) has been found to be valuable in diagnosis and predicting the prognosis of a variety of malignancies. The aims of the present study are to evaluate the usefulness of plasma OPN level for predicting gastric cancer development, invasion and survival.

Patients and Methods: One hundred and thirty two gastric cancer patients and 93 healthy controls were enrolled. Real-time quantitative reverse-transcription polymerase chain reaction and immunohistochemical staining were used to detect OPN expression in gastric cancer tissues. Plasma levels of OPN were measured by enzyme-linked immunosorbent assay. Plasma OPN levels were compared with gastric cancer development, clinicopathological features and outcomes.

Results: Expression of OPN mRNA was significantly higher in gastric cancer tissues compared with non-tumour tissues. Most OPN immunoactivity was localised to cancer cells. The median plasma OPN level was significantly higher in patients than in controls ($p < 0.0001$), and significantly higher in patients with advanced stages, serosal invasion, lymph node metastasis, lymphatic invasion, venous invasion and liver metastasis. Logistic regression showed that high plasma OPN level (greater than 67.3 ng/ml) is significantly associated with advanced stages, serosal invasion, lymph node metastasis, lymphatic invasion, venous invasion and liver metastasis. Plasma OPN level demonstrated significant association with patient survival ($p < 0.0001$), especially in the subgroups with invasive phenotypes. On Cox multivariate analysis, elevated plasma OPN level was an independent risk factor for poor survival ($p < 0.0001$).

Conclusions: Elevated plasma OPN level is significantly associated with gastric cancer development, invasive phenotypes and survival. Plasma OPN level may have potential usefulness as a diagnostic and prognostic factor for gastric cancer.

Gastric cancer remains a leading cause of cancer mortality, despite a worldwide decline in incidence. In Asian countries, gastric cancer is one of the most prevalent tumours and the leading cause of cancer death.¹ In the Western world, more than 80% of gastric cancer patients have advanced cancer on diagnosis with poor prognosis.² Complete resection of the tumour and adjacent lymph nodes is the only proven, effective curative treatment.³ Unfortunately, the accuracy of current preoperative staging is limited, particularly for depth of invasion, lymph node involvement and distant metastasis.⁴ Developing new biomarkers to identify the subgroup of gastric cancer patients with invasive phenotypes will be helpful for avoiding inappropriate attempts at curative surgery.

RNA-based global gene expression strategies are powerful approaches to identifying new cancer markers. Two studies using these strategies have suggested that osteopontin (OPN) is a potential marker for gastric cancer invasiveness.^{5–6} Lee *et al*⁵ used cDNA microarrays to identify differentially expressed genes in gastric cancer tissues and their surrounding gastric mucosa tissues and found 55-fold OPN overexpression in gastric cancer tissues. Using a highly metastatic gastric cancer cell line model, Fukui *et al*. found that OPN expression in liver metastatic variant cells is 2.7–10.2 times higher than in the parent cells.⁶ In an immunohistochemical staining study, co-immunoreaction of OPN and its receptor, CD44v9, in gastric cancer tumour cells correlated with the degree of lymphatic vessel invasion and lymph node metastasis.⁷ Recent studies have consistently reported that OPN mRNA and protein expression in cancer tissues is closely related to invasion and metastasis of gastric cancer.^{8–9}

OPN tissue expression and plasma levels have been reported to be diagnostic tumour markers and correlate well with prognosis for a variety of malignancies.^{15–18} However, the application of plasma OPN level as a biomarker for gastric cancer has not been investigated. In the present study, we evaluate the usefulness of plasma OPN level for predicting gastric cancer development, invasion and survival.

PATIENTS AND METHODS

Study subjects

Since January 1998, blood samples have been prospectively collected from individuals participating in two national projects to investigate the risk factors of gastric cancer in Taiwan (the National Taiwan University Hospital cohort). All patient-derived specimens were collected and archived under protocols approved by the institutional review boards of the parent institutions. A full verbal explanation of the study was given to all participants. They consented to participate on a voluntary basis. Patients with newly diagnosed gastric cancer undergoing gastrectomy in the inpatient unit and outpatient cancer clinics of four major medical centres in Taiwan were enrolled. Inclusion and exclusion criteria were detailed previously.^{19–20} Gastric adenocarcinoma was histopathologically confirmed by surgical specimens. No patients in this study had been prescribed chemotherapy before surgery, and there was no evidence of any other malignancy. In total, we studied 132 consecutive patients with gastric cancer, for whom complete

Abbreviation: OPN, osteopontin

Figure 1 Relative quantitation of osteopontin (OPN) in gastric cancer tissues and neighbouring non-tumour tissues. Expression differences were obtained for samples of 11 gastric cancer tissues and neighbouring non-tumour tissues using paired Student's *t*-test on log-transformed values ($p < 0.0001$). Each value is expressed as the mean of duplicate assays.

clinical data and a plasma sample were available. All recruited patients had been followed up for at least 5 years.

Control blood samples were obtained from 93 individuals who visited health examination clinics with minimal gastritis or normal appearance of the gastric mucosa on gastroscopic examination. The controls were matched by age (± 3 years) and date of blood collection (± 3 months).

To verify the diagnostic and prognostic values of plasma OPN level, we used an independent cohort including 39 patients and 50 health controls from Taichung Veterans General Hospital (the VGH validation cohort). The controls were matched by age (± 3 years) and date of blood collection (± 3 months). We used the same inclusion and exclusion criteria as the first study cohort.

Real-time quantitative reverse transcription polymerase chain reaction

Detection of OPN mRNA in gastric cancer tissues and surrounding non-tumour tissues was performed using the

method and primer sequences described in the manufacturer's instructions. OPN primers and probes were purchased from ABI (Applied Biosystems, Foster City, California, USA). Total RNA was isolated using Trizol reagent, and 2 μ g of RNA from each sample were reverse transcribed using SuperScript II RT (Invitrogen, Carlsbad, California, USA) in total reaction volume of 20 μ l. One microlitre of reverse-transcription product (cDNA) was amplified by polymerase chain reaction (PCR) with Taq DNA polymerase. Expression of TBP for each template was detected in the same PCR run as an internal control for efficiency of reverse transcription and amount of RNA. There were a total of 35 PCR cycles, with each cycle consisting of a denaturation step (95 $^{\circ}$ C for 1 min), an annealing step (60 $^{\circ}$ C for 1 min) and an elongation step (72 $^{\circ}$ C for 1 min).

Immunohistochemical analysis

Tissue sections from gastric cancer samples were deparaffined in xylene and rehydrated in ethanol, then immersed in 3% hydrogen peroxide-methanol solution for 10 min at room temperature to inhibit endogenous peroxidase activity. Sections were washed twice in phosphate-buffered saline (PBS) buffer for 5 min, and placed in 10% non-immune serum (goat) for 10 min. Sections were then incubated with mouse anti-OPN monoclonal antibodies (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) at a dilution of 1:50 overnight at 4 $^{\circ}$ C, and washed twice in PBS, before being incubated in appropriate biotinylated secondary antibody for 10 min. The sections were washed again in PBS twice for 5 min. Slides were incubated in streptavidin/peroxidase conjugate (Zymed Laboratories, Inc.) for 10 min, before being washed twice in PBS for 5 min. Slides were subsequently incubated in DAB (3,3'-diamino-benzidine) solution for 5 min, then washed in water, counterstained with haematoxylin for 2 s, washed thoroughly in running tap water, dehydrated and mounted.

Measurement of plasma OPN concentration

OPN Quantikine ELISA kits were purchased from R&D Systems Europe (product code DOST00). This assay kit pre-coats a monoclonal antibody specific for OPN onto a microplate to bind OPN in the samples. The sensitivity limit of the assay was

Figure 2 Relative quantitation of osteopontin (OPN) expression in gastric cancer tissues. A–D, Immunolocalisation of OPN in human gastric tissues. OPN expression is indicated by brown staining and is immunolocalised using anti-OPN monoclonal antibody and avidin-biotin peroxidase complex and method with diaminobenzidine as the chromogen. The sections were counterstained with haematoxylin. A, Healthy gastric epithelial cells with no expression of OPN. B, Epithelial cells of atrophic gastritis with weak expression of OPN. C and D, Gastric adenocarcinoma with OPN expression in cancer cells. Magnification: A, B $\times 250$; C $\times 400$; D $\times 600$.

Table 1 Cutoff values of plasma osteopontin levels and their sensitivity, specificity and accuracy for predicting gastric cancer occurrence, serosal invasion, lymph node metastasis and liver metastasis

	Cutoff value (ng/ml)	Predictive probability (%)		
		Sensitivity	Specificity	Accuracy
All subjects				
Occurrence	67.3	58.3	69.9	63.6
Serosal invasion	78.6	61.3	65.4	62.9
Lymph node metastasis	73.0	65.1	65.3	65.2
Liver metastasis	111.2	87.5	83.1	83.3
<i>Helicobacter pylori</i> seropositive subjects				
Occurrence	67.3	58.6	77.8	65.2
Serosal invasion	80.0	60.4	66.7	63.0
Lymph node metastasis	80.7	60.9	71.4	65.4
Liver metastasis	129.8	80.0	88.2	87.7

0.011 ng/ml. Plasma OPN concentrations were measured in samples prepared in tubes coated with lithium heparin. All samples were preserved at -80°C . Plasma samples were diluted and the immunoassays were performed following the manufacturer's instructions. All assays were duplicated. A subset of samples was reassayed six times in every ELISA plate for quality control. The inter-assay variability was 4.8%.

Statistical analysis

The relative measures of OPN expression, $2^{-\Delta\Delta\text{CT}}$, on real-time RT-PCR in gastric cancer tissues and surrounding non-tumour tissues were compared using paired Student's *t*-test on log-transformed values. The demographic characteristics of patients and controls were compared using the χ^2 -square test and Student's *t*-test. Because of the non-Gaussian distribution of data for the plasma OPN levels in study subjects, the differences

in plasma OPN values were compared using a nonparametric Mann Whitney U test. Analyses of nonparametric receiver-operating characteristics (ROC) were performed to calculate the cutoff values according to the most accurate value obtained. Logistic regression analyses were conducted to evaluate the associations between plasma OPN levels and clinicopathological features of gastric cancer patients. To determine whether plasma OPN concentration is an independent prognostic factor for survival, hazards ratios were calculated using the Cox proportional hazards model. Data were analysed via the SPSS program for Windows, version 11.0 (SPSS Inc., Chicago, Illinois, USA). Analyses of nonparametric ROCs were performed via the STATA program for Windows, version 8.0 (Stata Corporation, College Station, Texas, USA).

RESULTS

OPN expression in gastric cancer tissues

To validate overexpression of OPN in gastric cancer, gastric cancer tissues and surrounding non-tumour tissues were examined using real-time RT-PCR with non-tumour tissue of sample 10 as the reference. Significantly higher OPN expression was observed in the 11 samples of gastric cancer tissues compared with their surrounding non-tumour tissues ($p < 0.0001$) (fig 1).

Immunohistochemical staining was performed to localise OPN expression in gastric cancer tissues. OPN immunoreactivity was not observed in the epithelium or stroma of the healthy gastric tissues. In atrophic gastritis, mild OPN immunoreactivity was found in epithelial cells, but not in stromal cells. In gastric cancer tissues, most positive staining was localised to the cancer cell cytoplasm with diffuse staining pattern (fig 2).

Associations between plasma OPN levels and occurrence of gastric cancer

Plasma OPN levels were analysed in 132 gastric cancer patients and 93 controls. There were no age or gender differences between gastric cancer patients and controls. The median plasma concentration of OPN was significantly higher in gastric cancer patients than in control subjects (78.6 vs 55.6 ng/ml, $p < 0.0001$). To further examine the predictive values of plasma OPN levels, cutoff values were calculated on analyses of ROCs, according to the most accurate value obtained, and predictive probability was determined. Gastric cancer occurrence, serosal invasion, lymph node metastasis and liver metastasis could be predicted with accuracies of 63.6%, 62.9%, 65.2% and 83.3%, respectively (table 1).

In subjects who were *Helicobacter pylori* seropositive, the accuracies of plasma OPN to predict gastric cancer occurrence,

Table 2 Plasma median osteopontin levels in patients with gastric cancer with different clinicopathological features

	Plasma OPN level (ng/ml) Median (mean $\pm$ SE)	p Value*
<i>Helicobacter pylori</i>		
Seronegative (n = 51)	78.6 (94.2 $\pm$ 10.9)	NS
Seropositive (n = 81)	78.0 (86.2 $\pm$ 7.2)	
Stage		
I (n = 42)	59.3 (66.1 $\pm$ 8.2)	NS†
II (n = 23)	63.7 (74.5 $\pm$ 8.2)	
III (n = 40)	89.9 (96.9 $\pm$ 10.6)	
IV (n = 27)	97.0 (126.9 $\pm$ 18.8)	<0.001‡
Serosal invasion		
Absent (n = 52)	66.3 (73.6 $\pm$ 7.4)	0.017
Present (n = 80)	84.2 (99.5 $\pm$ 8.7)	
Lymph node metastasis		
Absent (n = 49)	63.3 (68.9 $\pm$ 7.1)	0.003‡
Present (n = 83)	90.2 (101.6 $\pm$ 8.5)	
pN1 (n = 56)	82.9 (94.7 $\pm$ 9.4)	
pN2, N3 (n = 27)	92.4 (115.8 $\pm$ 17.3)	0.003‡
Lymphatic invasion		
Absent (n = 76)	64.5 (76.0 $\pm$ 6.9)	0.003
Present (n = 56)	91.8 (107.5 $\pm$ 10.5)	
Venous invasion		
Absent (n = 77)	63.7 (82.8 $\pm$ 8.3)	0.035
Present (n = 55)	84.1 (98.5 $\pm$ 8.9)	
Liver metastasis		
Absent (n = 124)	75.6 (82.2 $\pm$ 5.3)	0.002
Present (n = 8)	166.5 (200.2 $\pm$ 45.3)	

*p Value calculated by non-parametric Mann Whitney U test.

†Compared with stage I.

‡Compared with no lymph node metastasis.

NS, non-significant; SE, standard error.

Table 3 Association between plasma osteopontin levels and clinicopathological features of gastric cancer

	Plasma OPN levels		Odds ratio (95% CI)*	p Value
	Normal	Elevated		
<i>Helicobacter pylori</i>	(≤ 67.3 ng/ml)	(>67.3 ng/ml)		
Seronegative	20 (35.7%)	31 (40.8%)	1	
Seropositive	36 (64.3%)	45 (59.2%)	0.81 (0.40 to 1.65)	NS
Age	(≤ 67.3 ng/ml)	(>67.3 ng/ml)		
≤ 60 years	29 (51.8%)	20 (26.3%)	1	
>60 years	27 (48.2%)	56 (73.7%)	3.01 (1.45 to 6.25)	0.003
Sex	(≤ 67.3 ng/ml)	(>67.3 ng/ml)		
Female	23 (41.1%)	23 (30.3%)	1	
Male	33 (58.9%)	53 (69.7%)	1.61 (0.78 to 3.31)	NS
Stage	(≤ 67.3 ng/ml)	(>67.3 ng/ml)		
I	26 (46.4%)	16 (21.1%)	1	
II	12 (21.4%)	11 (14.5%)	1.49 (0.53 to 4.17)	NS†
III	12 (21.4%)	28 (36.8%)	3.79 (1.51 to 9.51)	0.004†
IV	6 (10.7%)	21 (27.6%)	5.69 (1.89 to 17.09)	0.002†
Serosal invasion	(≤ 78.6 ng/ml)	(>78.6 ng/ml)		
Absent	34 (52.3%)	18 (26.9%)	1	
Present	31 (47.7%)	49 (73.1%)	3.21 (1.49 to 6.92)*	0.003
Lymph node metastasis	(≤ 73 ng/ml)	(>73.0 ng/ml)		
Absent	32 (51.6%)	17 (24.3%)	1	
Present	30 (48.4%)	53 (75.7%)	3.88 (1.75 to 8.61)*	0.001
Lymphatic invasion	(≤ 67.3 ng/ml)	(>67.3 ng/ml)		
Absent	40 (71.4%)	36 (47.4%)	1	
Present	16 (28.6%)	40 (52.6%)	3.16 (1.45 to 6.91)*	0.004
Venous invasion	(≤ 67.3 ng/ml)	(>67.3 ng/ml)		
Absent	42 (75.0%)	35 (46.1%)	1	
Present	14 (25.0%)	41 (53.9%)	4.24 (1.88 to 9.56)*	0.001
Liver metastasis	(≤ 111.2 ng/ml)	(>111.2 ng/ml)		
Absent	103 (98.1%)	21 (77.8%)	1	
Present	2 (1.9%)	6 (22.2%)	11.09 (2.04 to 60.29)*	0.005

*Odds ratio adjusted for age and sex.

†Compared with stage I.

95% CI, 95% confidence interval; NS, non-significant.

serosal invasion, lymph node metastasis and liver metastasis were 65.2%, 63.0%, 65.4% and 87.7% respectively. Plasma OPN did not have significantly better diagnostic value in subjects who were *H. pylori* seropositivity (table 1). We also evaluated the potential interaction between *H. pylori* serology and plasma OPN level. We found the medians of plasma OPN levels between patients with gastric cancer who were *H. pylori* seropositive and *H. pylori* seronegative were not significantly different (table 2).

Figure 3 Kaplan–Meier estimates of survival for patients with different plasma OPN levels. The survival rates for patients with plasma OPN >67.3 ng/ml were significantly lower than for patients with plasma OPN ≤ 67.3 ng/ml ($p < 0.0001$ by the log-rank test).

Associations between plasma OPN levels and clinicopathological characteristics of gastric cancer

We then compared plasma OPN levels among gastric cancer patients with different clinicopathological features. Median plasma OPN concentrations were significantly higher in patients with advanced stages (stage III, $p = 0.003$; stage IV, $p < 0.001$), serosal invasion ($p = 0.017$), lymph node metastasis ($p = 0.003$), lymphatic invasion ($p = 0.003$), venous invasion ($p = 0.035$), and liver metastasis ($p = 0.002$) (table 2). Among the patients with lymph node metastasis, those with pN2 or pN3 stages had a higher median plasma OPN levels than those with pN1 stage (table 2).

To examine the predictive value of plasma OPN for different clinicopathological characteristics, we conducted logistic regression analyses. We defined elevated plasma OPN levels according to the best predictive values calculated on ROC analyses for serosal invasion (>78.6 ng/ml), lymph node metastasis (>73.0 ng/ml), liver metastasis (>111.2 ng/ml) and used the criteria >67.3 ng/ml for analyses of other parameters. Elevated plasma OPN level was associated with age ($p = 0.003$), advanced stages (stage III: $p = 0.004$; stage IV: $p = 0.002$), serosal invasion ($p = 0.003$), lymph node metastasis ($p = 0.001$), lymphatic invasion ($p = 0.004$), venous invasion ($p < 0.001$), and liver metastasis ($p = 0.005$) (table 3).

Associations between plasma OPN levels and survival

Kaplan–Meier estimates of survival for patients with different plasma OPN levels are shown in Figure 3. The survival rates for patients with elevated plasma OPN level (>67.3 ng/ml) were significantly lower than the survival rates of those with normal plasma OPN level ($P < 0.0001$). Subgroup analyses according to stages, lymph node metastasis and serosal invasion status are shown in fig 4. Elevated plasma OPN level was associated with poor survival in gastric cancer patients with stage II disease

Figure 4 Kaplan-Meier curves for overall survival according to different plasma OPN levels. Stage I gastric cancer (panel A), stage II gastric cancer (panel B), stage III gastric cancer (panel C), stage IV gastric cancer (panel D), without lymph node metastasis (panel E), with lymph node metastasis (panel F), without serosal invasion (panel G) and with serosal invasion (panel H). Patients with elevated plasma OPN (>67.3 ng/ml) had significantly worse prognosis, especially in the stage II, stage III, lymph node metastasis and serosal invasion subgroups.

($p = 0.0008$) and stage III disease ($p = 0.03$), patients with lymph node metastasis ($p = 0.001$), patients without serosal invasion ($p = 0.05$) and patients with serosal invasion ($p = 0.001$). Patients with elevated plasma OPN level had significantly poorer prognosis, especially in the subgroups with invasive phenotypes, such as those with advanced stage, lymph node metastasis and serosal invasion.

On Cox univariate proportional hazards analysis, we found that patients with stage III or stage IV ($p < 0.001$), serosal invasion ($p < 0.001$), lymphatic invasion ($p = 0.002$), venous invasion ($p < 0.001$), lymph node metastasis ($p = 0.002$), liver metastasis ($p < 0.001$) and plasma OPN levels ($p < 0.001$) were of prognostic significance for poor overall survival (table 4). On multivariate analysis, only serosal invasion ($p = 0.001$), liver

Table 4 Univariate analysis for the predictors of mortality in gastric cancer patients

	Hazards ratio	(95% CI)	p Value
Age			
>60 vs ≤60	1.64	(0.88 to 3.03)	NS
Gender			
Male vs female	1.40	(0.72 to 2.71)	NS
Stage			
Stage III, IV vs stage I, II	3.98	(2.09 to 7.59)	<0.001
Depth of invasion			
Serosal vs non-serosal invasion	4.78	(2.21 to 10.35)	<0.001
Lymph node metastasis			
Yes vs no	2.89	(1.46 to 5.74)	0.002
Lymphatic invasion			
Yes vs no	2.55	(1.42 to 4.56)	0.002
Venous invasion			
Yes vs no	2.93	(1.63 to 2.25)	<0.001
Liver metastasis			
Yes vs no	6.02	(2.30 to 15.79)	<0.001
Osteopontin			
>67.3 vs ≤67.3	3.85	(1.95 to 7.61)	<0.001
Osteopontin			
As a continuous variable	1.009	(1.005 to 1.012)	<0.001

metastasis ($p = 0.009$) and high plasma OPN level ($p = 0.0001$) were independent risk factors for poor overall survival (table 5).

Because plasma OPN level is a continuous variable, the use of an arbitrary cut-off value of 67.3 ng/ml may be misleading. We also analysed plasma OPN level as a continuous variable on the Cox proportional hazard model. On univariable analysis, patients with high plasma OPN levels were of prognostic significance for poor overall survival ($p < 0.001$) (table 5). On multivariate analysis, only serosal invasion ($p < 0.001$) and high plasma OPN level ($p < 0.001$) were independent risk factors for poor overall survival (table 6).

Validation of the diagnostic and prognostic values

To assess the robustness of plasma OPN level as a diagnostic and prognostic factor in gastric cancer, we used an independent set of blood samples (the VGH validation cohort) to validate the results. We found that patients with gastric cancer had significantly higher median plasma OPN level compared with controls ($p < 0.001$). If we used the same cutoff value at 67.3 ng/ml, the sensitivity was 82.1% and specificity was 78.0%.

Median plasma OPN concentrations were significantly higher in patients with stage II ($p = 0.048$), stage III ($p < 0.001$), stage IV ($p = 0.003$), serosal invasion ($p = 0.002$), lymph node metastasis ($p = 0.002$), lymphatic invasion ($p < 0.001$), and venous invasion ($p = 0.001$) (table 7). Because the VGH validation cohort patients are followed shortly, we do not have sufficient survival data to conduct Cox proportional hazard analysis.

DISCUSSION

In the present study, we demonstrated that plasma OPN level is a potential new biomarker for gastric cancer development, invasion and survival. To the best of our knowledge, this is the first study to investigate the diagnostic and prognostic values of plasma OPN levels in gastric cancer. The usefulness of elevated plasma OPN level as a diagnostic marker has been reported in a variety of malignancies, including ovarian, colon, breast, lung, prostate, pancreatic and oesophageal cancers.^{15–18} Fedarko *et al.* used competitive ELISA to detect 20 serum samples from different types of cancer, including colon, breast, lung and prostate cancer.¹⁷ The sensitivities and specificities of elevated serum OPN level were, respectively: 65.0% and 55.7% for colon cancer; 74.1% and 55.7% for breast cancer; 94.7% and 55.7% for

Table 5 Multivariable Cox proportional hazards model analysis for prediction of mortality in gastric cancer patients

	Hazards Ratio	95% CI	p Value
Serosal invasion			
Yes vs no	3.97	(1.81 to 8.73)	0.001
Liver metastasis			
Yes vs no	3.67	(1.38 to 9.79)	0.009
Osteopontin			
>67.3 vs ≤67.3	3.38	(1.70 to 6.73)	0.001

prostate cancer; and 100% and 55.7% for lung cancer.¹⁷ In pancreatic cancer, the sensitivity and specificity of elevated serum OPN level were 80% and 97%, respectively.¹⁵ In oesophageal cancer, high plasma OPN level was found in 68.9% of patients.¹⁸ In ovarian cancer, elevated plasma OPN level showed specificity of 80.4% and sensitivity of 85.4% for late-stage cancer.¹⁶ In the present study, plasma OPN level at a cutoff value of 67.3 ng/ml predicted the occurrence of gastric cancer with sensitivity and specificity of 58.3% and 69.9%, respectively. Combination with *H. pylori* serology did not improve the accuracy of this assay. However, the sensitivity and specificity were 82.1% and 78.0% respectively in the VGH validation cohort. The different stage distribution may contribute to the discrepancy between these two study populations. In the National Taiwan University Hospital cohort, about half the patients were stage III (30.3%) and stage IV (20.5%). In contrast, more than three-quarters of the VGH validation cohort patients were stage III (51.3%) and stage IV (25.6%). Like other tumour markers, plasma OPN level has higher sensitivity to detect cancer development in a population with more advanced stage disease.

The role of OPN in tumourigenesis can be explained by the multiple functions of OPN in cells.²¹ Several mechanisms have been proposed through studies using cultured cells. First, it is recognised that OPN has adhesive activity because its receptors all mediate cell adhesion. Second, the ability of cells to migrate may be directly tied to their tumourigenicity and OPN promotes the migration of diverse cells, including monocytes, macrophages and tumour cells, along OPN gradients.²² In addition, OPN-deficient cells are reported to be hypomotile.²³ Third, some experiments suggest that OPN inhibits apoptosis and stimulates survival and growth of cells with inducible OPN,²⁴ or with the addition of OPN to cell culture medium,²⁵ through an interaction with its receptor CD44.²⁶ Fourth, several studies have suggested that OPN increases tumour invasiveness by inducing proteinase, particularly uPA and MMPs, through complex signalling pathways, such as AP-1 activation, PI3-kinase/Akt-dependent or NIK-dependent NF- κ B activation.^{27–31} In the present study, we used real-time RT-PCR to demonstrate that OPN mRNA expression is significantly higher in gastric cancer tissues compared with surrounding non-tumour tissues. This observation is compatible with a previous report using a cDNA microarray method in which OPN was overexpressed in gastric cancer tissues.⁵

Table 6 Multivariable Cox proportional hazards model analysis for prediction of mortality in gastric cancer patients

	Hazards ratio	95% CI	p Value
Serosal invasion			
Yes vs no	4.32	(1.97–9.47)	<0.001
Osteopontin			
As a continuous variable	1.008	(1.003–1.012)	<0.001

Table 7 Plasma median OPN levels in the VGH validation cohort

	Plasma OPN level (ng/ml) Median (mean $\pm$ SE)	p Value*
Gastric cancer patients (n=39)	107.0 (110.8 $\pm$ 7.8)	
Control (n=50)	51.7 (49.4 $\pm$ 3.4)	<0.001
Stage		
I (n=6)	62.0 (55.7 $\pm$ 6.7)	
II (n=3)	75.0 (92.0 $\pm$ 20.5)	0.048†
III (n=20)	106.0 (109.7 $\pm$ 7.8)	<0.001†
IV (n=10)	143.6 (151.7 $\pm$ 17.0)	0.003†
Serosal invasion		
Absent (n=7)	63.9 (67.5 $\pm$ 13.7)	
Present (n=32)	116.3 (120.3 $\pm$ 8.1)	0.002
Lymph node metastasis		
Absent (n=7)	63.9 (66.9 $\pm$ 12.4)	
Present (n=32)	116.3 (120.4 $\pm$ 8.2)	0.002
Lymphatic invasion		
Absent (n=14)	70.5 (75.2 $\pm$ 8.5)	
Present (n=25)	119.9 (130.7 $\pm$ 8.9)	<0.001
Venous invasion		
Absent (n=18)	79.8 (86.5 $\pm$ 9.3)	
Present (n=21)	127.0 (131.6 $\pm$ 10.1)	0.001

*p Value calculated by non-parametric Mann Whitney U test.

†Compared with stage I.

We found that elevated plasma OPN not only correlates with gastric cancer occurrence, but also with invasive phenotypes and patient survival. In oesophageal cancer, high plasma OPN level has been reported to correlate with lymph node metastasis and overall survival, but not with depth of invasion.¹⁸ In metastatic breast cancer, elevated plasma OPN value has been associated with increased number of metastatic sites and poor survival.³² Similar associations between elevated OPN level and poor survival have been reported in many other kinds of tumours, including prostate cancer,³³ adult soft tissue tumours,³⁴ head and neck cancer receiving radiotherapy³⁵ and stage I non-small-cell lung cancer.³⁶⁻³⁷ Consistent with these observations, we found that median plasma OPN concentration is significantly higher in patients with advanced stages, serosal invasion, lymph node metastasis, lymphatic invasion, venous invasion, and liver metastasis. Logistic regression analyses confirmed that elevated plasma OPN level is associated with advanced stages, serosal invasion, lymph node metastasis, lymphatic invasion, venous invasion and liver metastasis. In the VGH validation cohort, we found similar associations. Thus, elevated plasma OPN level seems to represent a metastatic marker for gastric cancer.³⁸⁻³⁹

In contrast to the numerous reports on the usefulness of plasma OPN as a diagnostic biomarker, the role of plasma OPN as an independent prognostic indicator has not been well investigated. In prostate cancer, plasma OPN level is associated with the presence of metastases, and correlates negatively and independently with patient survival.⁴⁰ In breast cancer, increased plasma OPN level is the most prognostic value for poor survival.⁴¹ However, plasma OPN level is not an independent indicator of survival in oesophageal squamous cell carcinoma.¹⁸ In the present study, we found that patients with elevated plasma OPN level have significantly poorer survival rates, especially in the subgroups of patients with invasive phenotypes, such as those with advanced stage, lymph node metastasis or serosal invasion. On Cox multivariate analysis, high OPN level was an independent prognostic factor. However, further studies are needed to validate this observation.

Compared with previous studies and the mean OPN concentration reported by ELISA,¹⁵⁻¹⁸ our study cohorts had lower plasma OPN levels. There are several reasons that may

contribute to this discrepancy. First, most previous studies were conducted in Caucasians. Ethnic variation may lead to the different results. Second, many previous results, including ELISA, used mean instead of median to report the distribution of plasma OPN level without examining whether the distribution is Gaussian or not. In a non-Gaussian distribution, the value of mean tends to be overestimated, especially by extremely high-value outliers. To resolve this discrepancy, we suggest reporting median instead of mean of plasma OPN concentrations in the future studies. In addition, more studies based on different ethics will be also helpful.

Although plasma OPN level seems to possess potential as a diagnostic and prognostic biomarker in gastric cancer, its potential as a specific marker is doubtful. Several conditions may influence plasma OPN levels, such as age, hyperlipidemia, cardiovascular disease, diabetes, kidney disease and sepsis.²¹⁻⁴² Even in patients known to have cancer, plasma OPN level may be elevated because of non-cancer causes. In the present study, we found that elderly patients had significantly higher plasma OPN level, which is consistent with a previous report showing significant positive correlation between plasma OPN level and age.⁴² Further studies using multivariate analyses for these confounders are required to determine whether OPN is an independent prognostic factor.

In addition to the systemic potential confounders, various sources of OPN may influence prognostic value.²¹ In breast cancer, it has been observed that OPN staining in cancer cells is associated with poor survival; whereas OPN immunopositivity in infiltrating host cells is not associated with patient survival.⁴³ Because OPN is not expressed specifically by tumour cells, but rather by several other tissues, it is suggested that any studies assessing the utility of OPN as a marker of tumour progression consider tissue expression specificity.²¹ In the present study, we found most OPN immunopositivity in gastric cancer cells, which may partly explain the significant association between plasma OPN expression and patient outcomes in gastric cancer.

There are several limitations to our study. First, selection bias could not be completely excluded in this hospital-based case-control study, although we tried to enrol cases from different hospitals in three different regions of Taiwan. Future studies based on community populations will be helpful in confirming the diagnostic and prognostic performances of plasma OPN level. Second, we did not study postoperative alteration of plasma OPN levels. To be a good tumour marker, sensitive postoperative change to represent the remission or recurrence of the disease is mandatory. Third, we did not investigate environmental factors, which also play important roles in the occurrence and progression of gastric cancer.

In conclusion, this investigation has validated OPN mRNA overexpression in gastric cancer tissues and localised OPN protein expression mainly in tumour cells. Moreover, elevated plasma OPN level was found to be significantly associated with gastric cancer development, invasive phenotypes and survival. Therefore, we suggest that OPN plays an important role in the tumour development and progression of gastric cancer and that plasma OPN level has the potential to be a useful diagnostic and prognostic factor for gastric cancer.

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REFERENCES

- Gordon D. Luk. Tumors of the stomach. In: Feldman M, ed. *Gastrointestinal and liver disease: pathophysiology, diagnosis, management*. Philadelphia: WB Saunders, 2005:733–57.
- Roukos DH. Current status and future perspectives in gastric cancer management. *Cancer Treat Rev* 2000;**26**:243–55.
- Kim JP. Surgical results in gastric cancer. *Semin Surg Oncol* 1999;**17**:132–8.
- Miller FH, Kochman ML, Talamonti MS, et al. Gastric cancer. Radiologic staging. *Radiol Clin North Am* 1997;**35**:331–49.
- Lee S, Baek M, Yang H, et al. Identification of genes differentially expressed between gastric cancers and normal gastric mucosa with cDNA microarrays. *Cancer Lett* 2002;**184**:197–206.
- Fukui R, Nishimori H, Hata F, et al. Metastases-related genes in the classification of liver and peritoneal metastasis in human gastric cancer. *J Surg Res* 2005;**129**:94–100.
- Ue T, Yokozaki H, Kitadai Y, et al. Co-expression of osteopontin and CD44v9 in gastric cancer. *Int J Cancer* 1998;**79**:127–32.
- Sun XJ, Zuo WS, Ma H, et al. [Expression of osteopontin mRNA and its clinical significance in gastric cancer]. *Zhonghua Zhong Liu Za Zhi* 2005;**27**:292–5.
- Zhang DT, Yuan J, Yang L, et al. [Osteopontin expression and its relation to invasion and metastases in gastric cancer]. *Zhonghua Zhong Liu Za Zhi* 2005;**27**:167–9.
- Denhardt DT, Guo X. Osteopontin: a protein with diverse functions. *FASEB J* 1993;**7**:1475–82.
- Ashkar S, Weber GF, Panoutsakopoulou V, et al. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. *Science* 2000;**287**:860–4.
- Weber GF, Ashkar S, Glimcher MJ, et al. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* 1996;**271**:509–12.
- Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. *Science* 1987;**238**:491–7.
- Rangaswami H, Bulbule A, Kundu GC. Osteopontin: role in cell signaling and cancer progression. *Trends Cell Biol* 2006;**16**:79–87.
- Koopmann J, Fedarko NS, Jain A, et al. Evaluation of osteopontin as biomarker for pancreatic adenocarcinoma. *Cancer Epidemiol Biomarkers Prev* 2004;**13**:487–91.
- Kim JH, Skates SJ, Uede T, et al. Osteopontin as a potential diagnostic biomarker for ovarian cancer. *J Am Med Assoc* 2002;**287**:1671–9.
- Fedarko NS, Jain A, Karadag A, et al. Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. *Clin Cancer Res* 2001;**7**:4060–6.
- Shimada Y, Watanabe G, Kawamura J, et al. Clinical significance of osteopontin in esophageal squamous cell carcinoma: comparison with common tumor markers. *Oncology* 2005;**68**:285–92.
- Chen LT, Lin JT, Shyu RY, et al. Prospective study of *Helicobacter pylori* eradication therapy in stage (E) high-grade mucosa-associated lymphoid tissue lymphoma of the stomach. *J Clin Oncol* 2001;**19**:4245–51.
- Wu MS, Wu CY, Chen CJ, et al. Interleukin-10 genotypes associate with the risk of gastric carcinoma in Taiwanese Chinese. *Int J Cancer* 2003;**104**:617–23.
- Rittling SR, Chambers AF. Role of osteopontin in tumour progression. *Br J Cancer* 2004;**90**:1877–81.
- Denhardt DT, Giachelli CM, Rittling SR. Role of osteopontin in cellular signaling and toxicant injury. *Annu Rev Pharmacol Toxicol* 2001;**41**:723–49.
- Zhu B, Suzuki K, Goldberg HA, et al. Osteopontin modulates CD44-dependent chemotaxis of peritoneal macrophages through G-protein-coupled receptors: evidence of a role for an intracellular form of osteopontin. *J Cell Physiol* 2004;**198**:155–67.
- Wu Y, Denhardt DT, Rittling SR. Osteopontin is required for full expression of the transformed phenotype by the *ras* oncogene. *Br J Cancer* 2000;**83**:156–63.
- Chang PL, Cao M, Hicks P. Osteopontin induction is required for tumor promoter-induced transformation of preneoplastic mouse cells. *Carcinogenesis* 2000;**21**:1749–58.
- Lin YH, Huang CJ, Chao JR, et al. Coupling of osteopontin and its cell surface receptor CD44 to the cell survival response elicited by interleukin-3 or granulocyte-macrophage colony-stimulating factor. *Mol Cell Biol* 2000;**20**:2734–42.
- Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol* 2002;**4**:E131–6.
- Tuck AB, Arsenault DM, O'Malley FP, et al. Osteopontin induces increased invasiveness and plasminogen activator expression of human mammary epithelial cells. *Oncogene* 1999;**18**:4237–46.
- Das R, Mahabeshwar GH, Kundu GC. Osteopontin induces AP-1-mediated secretion of urokinase-type plasminogen activator through c-Src-dependent epidermal growth factor receptor transactivation in breast cancer cells. *J Biol Chem* 2004;**279**:11051–64.
- Rangaswami H, Bulbule A, Kundu GC. Nuclear factor-inducing kinase plays a crucial role in osteopontin-induced MAPK/IkappaBalpha kinase-dependent nuclear factor kappaB-mediated promatrix metalloproteinase-9 activation. *J Biol Chem* 2004;**279**:38921–35.
- Rangaswami H, Bulbule A, Kundu GC. JNK1 differentially regulates osteopontin-induced nuclear factor-inducing kinase/MEKK1-dependent activating protein-1-mediated promatrix metalloproteinase-9 activation. *J Biol Chem* 2005;**280**:19381–92.
- Singhal H, Bautista DS, Tonkin KS, et al. Elevated plasma osteopontin in metastatic breast cancer associated with increased tumor burden and decreased survival. *Clin Cancer Res* 1997;**3**:605–11.
- Forootan SS, Foster CS, Aachi VR, et al. Prognostic significance of osteopontin expression in human prostate cancer. *Int J Cancer* 2006;**118**:2255–61.
- Bramwell VH, Tuck AB, Wilson SM, et al. Expression of osteopontin and HGF/Met in adult soft tissue tumors. *Cancer Biol Ther* 2005;**4**:1336–41.
- Overgaard J, Eriksen JG, Nordmark M, et al. Plasma osteopontin, hypoxia, and response to the hypoxia sensitizer nimorazole in radiotherapy of head and neck cancer: results from the DAHANCA 5 randomised double-blind placebo-controlled trial. *Lancet Oncol* 2005;**6**:757–64.
- Donati V, Boldrini L, Dell'Omodarme M, et al. Osteopontin expression and prognostic significance in non-small cell lung cancer. *Clin Cancer Res* 2005;**11**:6459–65.
- Boldrini L, Donati V, Dell'Omodarme M, et al. Prognostic significance of osteopontin expression in early-stage non-small-cell lung cancer. *Br J Cancer* 2005;**93**:453–7.
- Weber GF. The metastasis gene osteopontin: a candidate target for cancer therapy. *Biochim Biophys Acta* 2001;**1552**:61–85.
- Chambers AF, Tuck AB. Ras-responsive genes and tumor metastasis. *Crit Rev Oncog* 1993;**4**:95–114.
- Hotte SJ, Winquist EW, Stitt L, et al. Plasma osteopontin: associations with survival and metastasis to bone in men with hormone-refractory prostate carcinoma. *Cancer* 2002;**95**:506–12.
- Bramwell VH, Doig GS, Tuck AB, et al. Serial plasma osteopontin levels have prognostic value in metastatic breast cancer. *Clin Cancer Res* 2006;**12**:3337–43.
- Takemoto M, Tada K, Nakatsuka K, et al. [Effects of aging and hyperlipidemia on plasma osteopontin level]. *Nippon Ronen Igakkai Zasshi* 1999;**36**:799–802.
- Tuck AB, O'Malley FP, Singhal H, et al. Osteopontin expression in a group of lymph node negative breast cancer patients. *Int J Cancer* 1998;**79**:502–8.