expression during colorectal carcinogenesis. *Int J Cancer*. 1997;74: 35–44.
21. Merchant SH, Amin MB, Tamboli P, et al. Primary signet-ring cell carcinoma of lung: immunohistochemical study and comparison with non-pulmonary signet-ring cell carcinomas. *Am J Surg Pathol*. 2001;25:1515–1519.
22. Mitani Y, Oue N, Matsumura S, et al. Reg IV is a serum biomarker for gastric cancer patients and predicts response to 5-fluorouracil-based chemotherapy. *Oncogene*. 2007;26:4383–4393.
23. Nguyen MD, Plasil B, Wen P, et al. Mucin profiles in signet-ring cell carcinoma. *Arch Pathol Lab Med*. 2006;130:799–804.
24. Oue N, Hamai Y, Mitani Y, et al. Gene expression profile of gastric carcinoma: identification of genes and tags potentially involved in invasion, metastasis, and carcinogenesis by serial analysis of gene expression. *Cancer Res*. 2004;64:2397–2405.
25. Oue N, Mitani Y, Aung PP, et al. Expression and localization of Reg IV in human neoplastic and non-neoplastic tissues: Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma. *J Pathol*. 2005;207: 185–198.
26. Oue N, Kuniyasu H, Noguchi T, et al. Serum concentration of Reg IV in patients with colorectal cancer: overexpression and high Reg IV serum level is associated with liver metastasis. *Oncology*. In press.
27. Raju U, Ma CK, Shaw A. Signet ring variant of lobular carcinoma of the breast: a clinicopathologic and immunohistochemical study. *Mod Pathol*. 1993;6:516–520.
28. Randolph TL, Amin MB, Ro JY, et al. Histologic variants of adenocarcinoma and other carcinomas of prostate: pathologic criteria and clinical significance. *Mod Pathol*. 1997;10: 612–629.
29. Sanada Y, Oue N, Mitani Y, et al. Down-regulation of the claudin-18 gene, identified through serial analysis of gene expression data analysis, in gastric cancer with an intestinal phenotype. *J Pathol*. 2006;208:633–642.
30. Sjodin A, Guo D, Hofer PA, et al. Mammaglobin in normal human sweat glands and human sweat gland tumors. *J Invest Dermatol*. 2003;121:428–429.
31. Tornos C, Soslow R, Chen S, et al. Expression of WT1, CA 125, and GCDFP-15 as useful markers in the differential diagnosis of primary ovarian carcinomas versus metastatic breast cancer to the ovary. *Am J Surg Pathol*. 2005;29:1482–1489.
32. Tot T. The role of cytokeratins 20 and 7 and estrogen receptor analysis in separation of metastatic lobular carcinoma of the breast and metastatic signet ring cell carcinoma of the gastrointestinal tract. *Apmis*. 2000;108:467–472.
33. Tsuta K, Ishii G, Yoh K, et al. Primary lung carcinoma with signet-ring cell carcinoma components: clinicopathological analysis of 39 cases. *Am J Surg Pathol*. 2004;28:868–874.
34. Tsuta K, Ishii G, Nitadori J, et al. Comparison of the immunophenotypes of signet-ring cell carcinoma, solid adenocarcinoma with mucin production, and mucinous bronchioloalveolar carcinoma of the lung characterized by the presence of cytoplasmic mucin. *J Pathol*. 2006;209:78–87.
35. Tung SY, Wu CS, Chen PC. Primary signet ring cell carcinoma of colorectum: an age- and sex-matched controlled study. *Am J Gastroenterol*. 1996;91:2195–2199.
36. Watson MA, Fleming TP. Mammaglobin, a mammary-specific member of the uteroglobin gene family, is overexpressed in human breast cancer. *Cancer Res*. 1996;56:860–865.
37. Watson MA, Dintzis S, Darrow CM, et al. Mammaglobin expression in primary, metastatic, and occult breast cancer. *Cancer Res*. 1999;59:3028–3031.
38. Yamashina M. A variant of early gastric carcinoma. Histologic and histochemical studies of early signet ring cell carcinomas discovered beneath preserved surface epithelium. *Cancer*. 1986;58:1333–1339.
39. Yokozaki H, Takekura N, Takanashi A, et al. Estrogen receptors in gastric adenocarcinoma: a retrospective immunohistochemical analysis. *Virchows Arch A Pathol Anat Histopathol*. 1988;413:297–302.

ANTICANCER RESEARCH *28*: 1169-1180 (2008)

Overexpression of *RegIV* in Peritoneal Dissemination of Gastric Cancer and Its Potential as A Novel Marker for the Detection of Peritoneal Micrometastasis

K. MIYAGAWA[1*], C. SAKAKURA[1*], S. NAKASHIMA[1], T. YOSHIKAWA[1], K. FUKUDA[1], S. KIN[1], Y. NAKASE[1], K. SHIMOMURA[2], N. OUE[3], W. YASUI[3], H. HAYASIZAKI[4], Y. OKAZAKI[5], H. YAMAGISHI[6], A. HAGIWARA[1] and E. OTSUJI[1]

Department of Surgery and Regenerative Medicine, Divisions of [1]Surgery and Physiology of the Digestive System and [6]Surgery and Oncology of the Digestive System, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kawaramachi-dori, Kyoto 602-8566; [2]Department of Surgery, Kyoto Second Red Cross Hospital, Kamigyo-ku, Kamanza-dori, Kyoto 602-8026; [3]Department of Molecular Pathology, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551 [4]Genomic Sciences Center, RIKEN, Yokohama Institute 1-7-22 Suehiro-cho, Tsurumi-district, Yokohama 230-0045; [5]Research Center for Genomic Medicine, Saitama Medical School, 1397-1 Yamane, Hidaka city, Saitama 350-1241, Japan

Abstract. *Background: Regenerating gene type IV (RegIV) is a candidate marker for cancer and inflammatory bowel disease. In this study, its potential as a novel marker for the detection of gastric cancer peritoneal micrometastases was examined. Patients and Methods: RegIV mRNA levels in the peritoneal washes of 95 gastric cancer patients and 22 with benign disease were quantified by real-time RT-PCR. To examine whether expression of RegIV enhance tumorigenicity or not, thirty two mice were injected intraperitoneally or subcutaneously with RegIV transfectants of TMK-1 cells, parental TMK-1 cells, or neomycin control transfectants. Results: RegIV expression was markedly higher in patients with peritoneal metastases compared to those without. The level of RegIV mRNA in gastric cancer patients was related to the extent of wall penetration. A cut-off value for RegIV-positive expression was based on an analysis of negative control patients with benign disease, and gastric cancer patients above the cut-off value constituted the micrometastasis (MM+) group. Based on this criteria, 3 out of 43 T1 or T2 cases were MM+ (93% specificity). Among 15 patients with peritoneal dissemination (7 out of 15 cases were positive by cytology), 14 cases were positive for RegIV expression (93% sensitivity), while analysis of carcinoembryonic antigen (CEA) mRNA failed to detect micrometastases in 4 cases (73% sensitivity). Combined analysis of CEA and RegIV improved the accuracy of diagnosis to 100%. The prognosis of RegIV-positive cases was significantly worse than that of RegIV-negative cases. Multivariate analysis using the Cox proportional hazards model suggested that RegIV may be an independent prognostic factor. Stable expression of RegIV significantly enhanced peritoneal metastasis in an animal model of gastric cancer. Conclusion: These findings suggest that RegIV mRNA expression has the potential to serve as a novel marker for detecting peritoneal dissemination in gastric cancer.*

*Both authors contributed equally to this study.

Abbreviations: RT-PCR: Reverse transcriptase-polymerase chain reaction; CY: cytology; MM: micrometastasis; *RegIV*: regenerating gene type IV.

Correspondence to: Kouji Miyagawa, MD, Ph.D., Department of Surgery and Regenerative Medicine, Division of Surgery and Physiology of Digestive System, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kawaramachi-dori, Kyoto 602, Japan. Tel: +81 75 2515527, Fax: +81 75 2515522, e-mail: k-miya@koto.kpu-m.ac.jp

Key Words: RegIV, gastric cancer, peritoneal metastasis, RT-PCR.

Gastric cancer is the most common malignancy of the gastrointestinal tract in Japan and in certain Southeast Asian populations, and the second most common cause of cancer-related deaths in the world (1). The prognosis of patients with gastric cancer that has invaded the gastric serosa is poor, with a 5-year survival rate of less than 35% (2). In such cases, peritoneal dissemination is reported to be the most frequent type of recurrence after curative resection (3, 4). Free cancer cells derived from serosal invasion may be an indicator of early peritoneal seeding with subsequent

formation of metastatic colonies. Thus, their detection represents a potentially valuable predictor of outcome for patients with advanced gastric cancers (5, 6). Cytological examination of peritoneal washes from laparotomies to detect free metastatic cancer cells has been used to evaluate the risk of recurrent disease (6, 7). Conventional cytology, however, lacks sufficient sensitivity, as some patients with negative cytology results have presented with recurrence in the form of peritoneal dissemination (5). Carcinoembryonic antigen (*CEA*)-specific RT-PCR has been used to detect cancer cells in peritoneal fluids (8); however, the results indicate that *CEA* expression is not 100% accurate as a marker, suggesting that more reliable markers are needed.

Previously, we examined global differential gene expression in gastric cancer cell lines established from a primary tumor and from metastases to the peritoneal cavity (9). Using a high-density cDNA microarray, we analyzed the expression of approximately 21,168 genes. The results of this study revealed that 24 genes were up-regulated and 17 genes were down-regulated in gastric cancer cell lines established from metastases to the peritoneal cavity. One of the up-regulated genes was *RegIV*. *RegIV* is a member of the regenerating gene (Reg) family, which is part of the larger calcium-dependent lectin (C-type) gene superfamily (10). Reg family members are a group of small secretory proteins which can function as acute phase reactants, lectins, antiapototic factors, or growth factors for pancreatic β cells, neural cells and epithelial cells in the digestive tract (11). The Reg family proteins also play an important role in the injury response in the gastrointestinal mucosa. *RegIV* expression is up-regulated in response to mucosal injury in active Crohn's disease and ulcerative colitis, and is increased in most colorectal cancers compared to normal tissue (10, 12-14). Recently, *RegIV* expression in gastric cancer was reported and was found to be closely related to the infiltrating potential of the carcinoma (15-17). In one study, RT-PCR analysis was used to show a high level of *RegIV* expression in gastric cancer (16). However, while overexpression of *RegIV* in gastric cancer has been reported, the role of RegIV in gastric cancer peritoneal dissemination has not been investigated. In this study, amplification of *RegIV* by quantitative RT-PCR from peritoneal lavage cells was used to develop a highly sensitive method for detecting micrometastases of cancer cells. This detection method has the potential to predict peritoneal recurrence in gastric cancer patients with a higher level of accuracy than previous methods.

Patients and Methods

Cell culture. The gastric cancer cell lines SNU-1, SNU-5, SNU-16, and SNU-719 were established previously by Park *et al.* (18). The mesothelial cell line Met5A was established by Duncan *et al.* (19, 20). The gastric cancer cell lines KATO-III and GT3TKB, and the acute myeloid leukemia cell line HL60 were purchased from Riken Cell Bank (Tsukuba, Japan). Another gastric cancer cell line, TMK-1 was kindly donated by Professor Tahara, of Hiroshima University. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in high-glucose RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, penicillin and streptomycin.

Clinical samples and peritoneal washes. Patients underwent surgery at Kyoto Prefectural University of Medicine between 1999 and 2004 and underwent regular postoperative surveillance for at least 2 years or until death. One patient who died within 30 days of surgery as a result of perioperative complications was excluded. The current study population consisted of 95 patients with gastric cancer and 22 with benign disease, such as cholelithiasis. Of the 95 patients with resectable cancer, 77 underwent potentially curative R0 resection; the remaining 18 were treated with palliative resection. The 95 cases included 21 patients with T1 tumors (tumor confined to the mucosa or invading as far as the submucosa), 22 with T2 tumors (invasion beyond the submucosa but not as far as the serosa), 37 with T3 tumors (serosal invasion), and 15 with T4 tumors (invasion to adjacent tissues). The population included 15 patients with synchronous peritoneal metastasis.

The peritoneal wash was collected from the Douglas cavity at laparotomy. Written informed consent was obtained from each patient prior to tissue acquisition. In the absence of ascites, 150 ml of saline was introduced into the Douglas cavity at the beginning of the operation and aspirated after general stirring. The washes were centrifuged at 2000 rpm for 10 minutes to collect intact cells, which were then rinsed with phosphate-buffered saline (PBS).

RNA preparation. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The mRNA from each cell line was extracted using the FAST Track Kit Ver.2 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Northern blot analysis. Northern blot analysis was performed as described elsewhere (21-23). In brief, mRNA was prepared from each cell line, then fractionated on 1% agarose/2.2 M formaldehyde gels. Probes were labeled with ^{32}P by random priming. Each blot was hybridized with the *RegIV* probe and a β-actin probe as a control. Hybridization signals were analyzed with a BAS 2000 image analyzer (Fuji, Tokyo, Japan) and calculated the degree of overexpression in comparison to the β-actin control.

Real-time quantitative RT-PCR. cDNA was produced from 2 μg of total RNA using the Superscript Preamplification System (BRL, Bethesda, MD, USA), according to the manufacturer's instructions. Briefly, RNA was heated to 70°C for 10 min in 14 μl of diethylpyrocarbonate-treated water containing 0.5 μg of oligo (dT) primer. Synthesis buffer (10x, 500 mM Tris-HCl, pH 8.3, 750 mM KCl, 30 mM $MgCl_2$), 2 μl of 10 mM dNTP mixture, 2 μl of 0.1 M DTT and reverse transcriptase (Superscript RT; 200U/μL, Gibco BRL, Gaithersburg, MD, USA) were added to the sample. The reaction mixture was incubated at 42°C for 50 min, and the reaction was terminated by incubation at 90°C for 5 min.

Quantitative PCR was performed using real time Taqman TM technology, as described by Nakanishi *et al.* (24). Results were analyzed on a Model 5700 Sequence Detector (Applied Biosystems Corp., Foster City, CA, USA).

The *RegIV* RT-PCR primers used were 5'-TCCTTGCAC TAGCTACATCC-3' and 5'-GGAATGTATGGCCCACATCA-3'. The *CEA* RT-PCR primers used were 5'-TCTGGAACT TCTCCTGGTCTCTCAGCTGG-3' and 5'-TGAAGCTGTTGCA

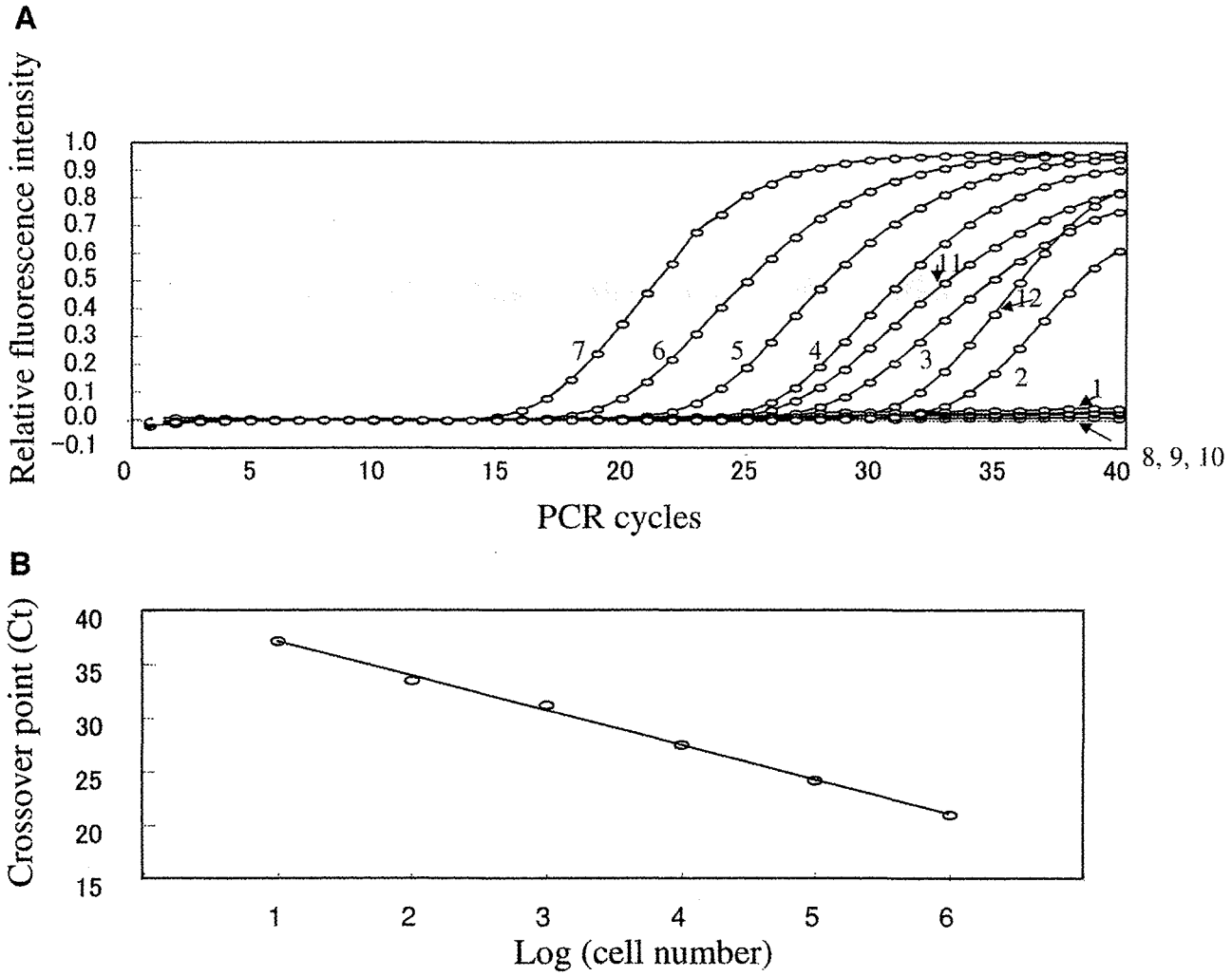

Figure 1. *Representative real-time RT-PCR result and calibration curve for estimating RegIV mRNA expression. A, Relative fluorescence vs. number of PCR cycles. Six external standards (lines 1-6) and two patient samples (lines 10 and 11) of unknown concentration were amplified with real-time 'Taqman TM' technology and analyzed with a Model 5700 Sequence Detector. Line 1=1, line 2=10, line 3=10^2, line 4=10^3, line 5=10^4, line 6=10^5, and line 7=10^6 SNU-16 gastric cancer cell equivalents of cDNA; line 8=Met 5A cells; line 9=HL60 cells; line 10=negative peritoneal wash; lines 11 and 12=positive peritoneal washes. B, Calibration curve for estimating RegIV mRNA expression. Curve was generated using data from the external controls in A by plotting the crossover points (Ct) against log SNU-16 cell number. Relative RegIV mRNA values in patient samples were calculated using this curve.*

AATGCTTTAAGGAAGAAGC-3'. Hybridization probes for detecting PCR products were labeled with a reporter dye (FAM), at the 5' end and a quenching dye (TAMRA), on the 3'end. The sequence of the *CEA* hybridization probe was 5'-(FAM) CATCTGGAACTTCTCCTGGTCTCTCAGC(TAMRA)-3'; the identification number for the hybridization probe for *RegIV* is Hs00230746 (Applied Biosystems Corp.).

For RT-PCR, the reaction mixture contained 1.25 units of Amp-Taq DNA polymerase, 1xPCR reaction buffer, 180 ng of each primer, 200 mM dNTP, 400 mM dUTP, 100 nM Taqman probe and 0.5 U Amplirase (Applied Biosystems Corp.). Serial dilutions of control cDNA were analyzed for each target gene. Crossover point (Ct) values were determined corresponding to the cycle number at which fluorescence emission reached a threshold standard deviation of ten above the mean baseline emission derived from 40 cycles. *CEA*- and *RegIV*-specific primers were used to generate standard curves from which the rate of change in the Ct value was determined for each patient sample (as shown in Figure 1). The cycling parameters were as follows: 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15s at 95°C and 1 min at 60°C.

To minimize errors arising from variations in the amount of starting RNA among the samples, amplification of β-actin mRNA was analyzed as an internal reference. The values from target RNAs were then normalized to β-actin mRNA. The primers and the probe for β-actin were purchased from Applied Biosystems. Normalized results are expressed as the ratio of number of copies of target gene to β-actin.

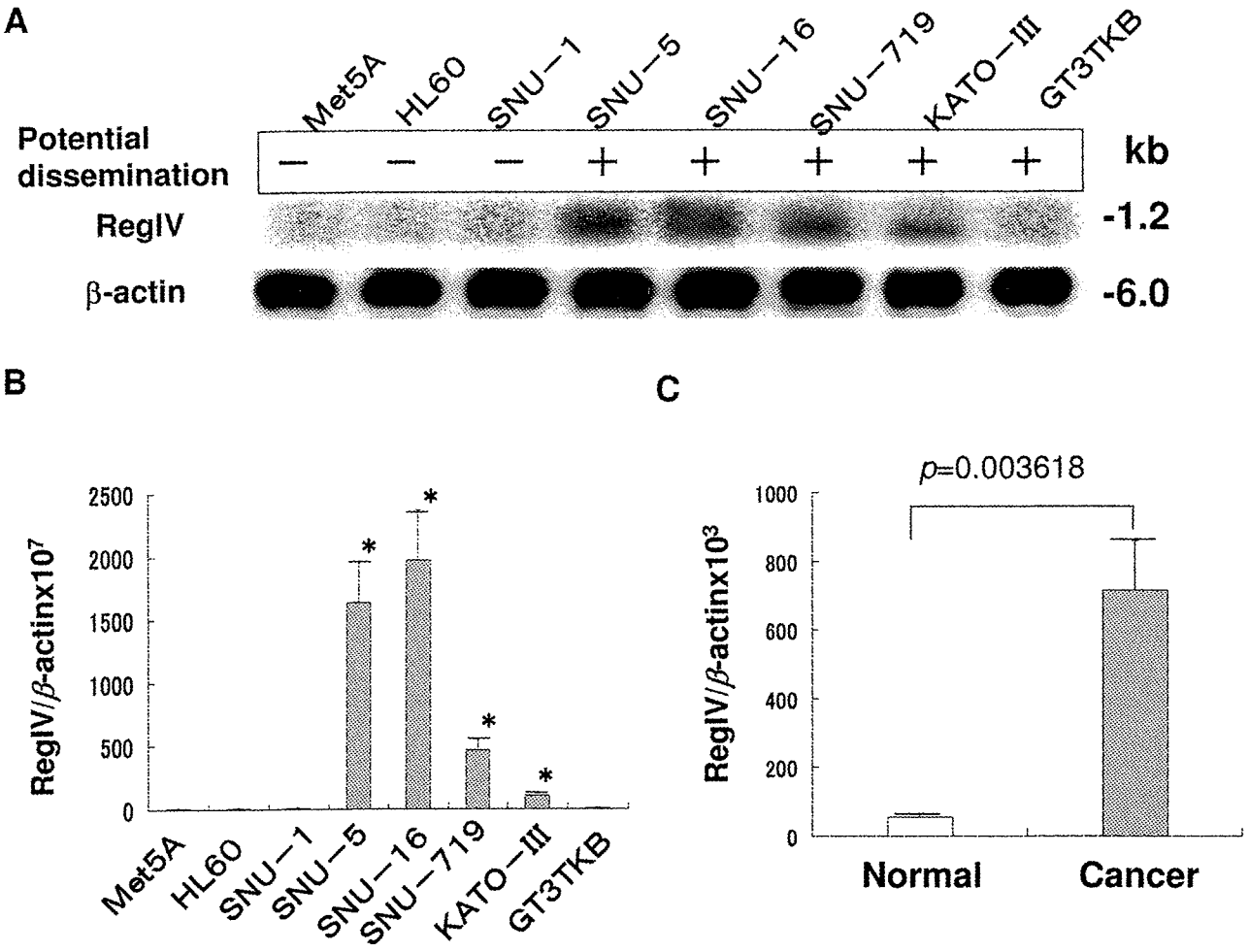

Figure 2. *RegIV mRNA expression in gastric cancer cell lines and primary gastric cancer specimens. A) RegIV mRNA expression in eight gastric cancer cell lines analyzed by Northern blot. RegIV was up-regulated in gastric cancer cells derived from malignant ascites compared to cells derived from a primary lesion. β-Actin was probed as a control for loading variations in each lane. (+) and (−): peritoneal dissemination potential. B) RegIV mRNA expression in primary gastric cancer cells, mesothelial cells, leukemia cell, and gastric cancer cells from malignant ascites was analyzed by quantitative RT-PCR, as described in Material and Methods. RegIV expression in gastric cancer cells from malignant ascites was significantly higher than in primary gastric cancer cells, mesothelial cells, leukemia cell (*p<0.01). C) RegIV mRNA expression in primary gastric cancers and normal gastric mucosa was analyzed by quantitative RT-PCR. Expression levels of RegIV were normalized to β-actin. Note that RegIV expression in gastric cancer tissue was significantly higher than in gastric mucosa (p=0.003168).*

The ratio of *RegIV/β-actin* mRNA derived from negative control patients (patients with benign disease undergoing surgery) was determined and the highest value was adopted as the cut-off value (Figure 3A, broken line). Samples with ratios that were greater than this limit were regarded as positive (*RegIV* +).

Plasmids and transfection. To obtain stable RegIV-expressing TMK-1 transfectants, pEF-BOS-RegIV was transfected into TMK-1 cells using LipofectAMINE, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). G418 (600 Bg/mL)-resistant colonies were selected and subcultured as described elsewhere (23). Independent clonal cell lines that strongly expressed RegIV were identified by Northern blot analysis. TMK-1 cells transfected with pEF-BOS-neo and treated as described above were obtained as controls.

Experimental model of gastric cancer in nude mice. Four-week-old male C3H nude mice (Clea Japan, Inc., Osaka, Japan) were inoculated with $2x10^7$ TMK-1 gastric cancer cells intraperitoneally in 0.5 mL PBS, or subcutaneously in 0.3 mL PBS. Mice were injected with *RegIV* transfectants of TMK-1 cells, parental TMK-1 cells, or neomycin control transfectants in PBS. Six mice were injected intraperitoneally, and seven were injected subcutaneously. Five weeks after injection, the presence of disseminated foci or ascites was determined. Six weeks after inoculation intraperitoneally and five weeks after inoculation subcutaneously, the mice were sacrificed and examined. All animal experiments were conducted in accordance with our institutional guidelines for animal welfare. Representative whole mount specimens of tumors from the abdominal cavity and in the subcutaneous tissue in animals that received stable *RegIV* transfectants or control neomycin transfectants of TMK-1 cells were used to calculate tumor weight.

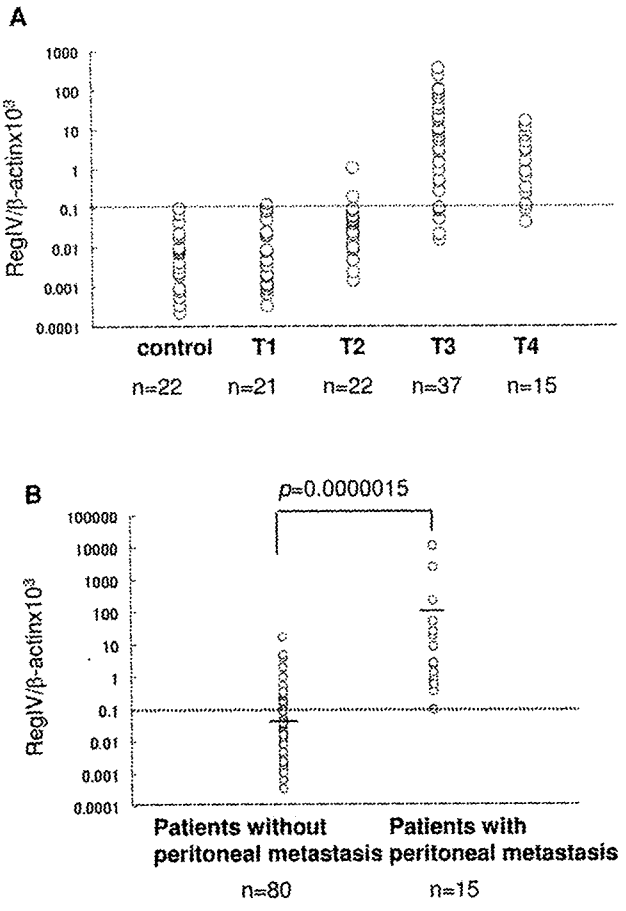

Figure 3. *RegIV/β-actin mRNA ratio in peritoneal washes from gastric cancer patients. A) RegIV/β-actin mRNA ratios in peritoneal washes from gastric cancer patients, grouped according to the depth of invasion (pT category). The broken line on the graph indicates the cut-off value for identifying RegIV+ samples, and was determined using data from negative control patients with benign disease. RegIV mRNA values correlated statistically with the depth of cancer invasion ($p<0.01$). B) RegIV mRNA values for peritoneal washes from gastric cancer patients with or without synchronous peritoneal metastasis. RegIV mRNA values for metastasis-positive patients were significantly higher than for metastasis-negative patients ($p=0.0000015$).*

Statistical analysis. Survival was analyzed with Kaplan-Meier curves, using death and clinical diagnosis of peritoneal carcinomatosis as the endpoints. For the analysis of survival with peritoneal metastasis as the endpoint, deaths resulting from other types of metastasis in the absence of clinical signs of peritoneal carcinomatosis were treated as censored cases.

The statistical significance of intergroup differences in *RegIV/β-actin* mRNA ratios were calculated using the Mann-Whitney *U*-test. *RegIV/β-actin* mRNA ratios of different groups, classified on the basis of their pT categories, were compared using the Kruskal-Wallis test. *P*-values of less than 0.05 were considered statistically significant. To identify independent prognostic factors, all 95 patients were analyzed by multivariate analysis using the Cox proportional hazards model.

Results

Quantitative RT-PCR using the Gene Amp 5700 sequence detection system. Real-time fluorescence RT-PCR using the Gene Amp 5700 sequence detection system allowed rapid, sensitive detection of *RegIV* mRNA from patient samples. With this method, 10 to 10^6 *RegIV*-expressing gastric cancer SNU-16 cells per 10^7 mesothelial cells were quantified (Figure 1A). No significant level of *RegIV* mRNA was detected in peripheral blood lymphocytes or mesothelial cells from healthy volunteers.

mRNA levels were quantified using Ct, which was the PCR reaction cycle when the fluorescence of a given sample rose above the background level to yield the maximal slope with respect to log-linear amplification. Figure 1B illustrates a standard curve generated by plotting on a log scale the number of SNU-16 cells (serial 10-fold dilutions) against their respective Cts. *RegIV* mRNA values for patient samples of unknown concentration were calculated using this calibration curve as a reference.

Expression of RegIV mRNA in gastric cancer cell lines, a mesothelial cell line, normal gastric mucosa, and cancerous tissues. Northern blot analysis showed a high level of expression of *RegIV* in cells with a high potential for peritoneal dissemination, and a low level of expression in cells with a low potential. Intense bands were observed in SNU-5, SNU-16, SNU-719 and KATO-III cells (5.8-, 8.1-, 2.3- and 1.5-fold, respectively compared to control Met5A and HL60 cells) (Figure 2A). The level of β-actin was probed as a control for loading variations. Quantitative RT-PCR yielded a similar pattern of expression of *RegIV* mRNA (Figure 2B).

RegIV expression was also detected in both normal gastric mucosa and clinical specimens of gastric cancer. In cancerous tissues, *RegIV* expression was significantly higher than in the normal mucosa (Figure 2C).

RegIV/β-actin mRNA ratio in peritoneal washes of gastric cancer patients varies with the degree of wall invasiveness. To normalize the amount of RNA in each patient sample, β-actin mRNA was used as an internal control. The value for *RegIV* mRNA expression level was then determined as the ratio of *RegIV* mRNA to β-actin mRNA (*RegIV*/β-actin mRNA). When gastric cancer patients were grouped according to T score, the average *RegIV*/β-actin mRNA ratios (ratio x 10^3) in peritoneal washes were as follows (average±standard deviation): control, 0.014906±0.024916; T1, 0.027919±0.038021; T2, 0.086225±0.071765; T3, 38.01328±15.08207; T4, 3.286277±4.87313. Figure 3A shows a plot of *RegIV*/β-actin mRNA ratios (x10^3) from all patients, grouped according to T classification. When cases were further classified into cases positive (T3, T4) and

Table I. *Expression of RegIV mRNA and clinicopathological factors in gastric cancer patients.*

Variable	RegIV mRNA		*P*-value*
	Positive	Negative	
Gender			
Male	31	34	0.636174
Female	16	14	
Differentiation			
Differentiated	16	29	0.017843**
Undifferentiated	31	19	
Depth of invasion			
T1, T2	3	40	0.000001**
T3, T4	44	8	
Lymphatic invasion			
Negative	5	28	0.000023**
Positive	42	20	
Vascular invasion			
Negative	16	32	0.004893**
Positive	31	16	
Lymph node metastasis			
Negative	6	34	0.000001**
Positive	41	14	
Peritoneal dissemination			
Negative	33	47	0.000176**
Positive	14	1	

T classification: T1, mucosa to submucosa; T2, muscularis propria to subserosa; T3, serosa-exposed; T4, serosa-infiltrating. *Mann-Whitney test; **statistically significant.

Table II. *Clinicopathological features of recurrent gastric cancer patients with peritoneal dissemination and malignant ascites.*

Case No.	Stage*	Histology**	Markers		
			CEA	RegIV	CEA and RegIV
1	P1H0N2T3CY1 Stage IV	Por	+	+	+
2	P0H0N2T3CY0 Stage IIIB	Sig	-	+	+
3	P0H0N1T3CY1 Stage IV	Tub	+	+	+
4	P0H0N1T3CY0 Stage IV	Por	-	+	+
5	P1H0N2T3CY0 Stage IIIA	Sig	+	+	+
6	P0H1N1T4CY1 Stage IV	Sig	+	+	+
7	P0H1N3T3CY0 Stage IIIB	Tub	+	+	+
8	P1H1N2T3CY0 Stage IV	Por	-	+	+
9	P0H0N2T3CY1 Stage IV	Por	+	+	+
10	P1H0N1T4CY1 Stage IV	Sig	+	+	+
11	P0H0N2T3CY0 Stage IIIA	Tub	+	-	+
12	P0H0N3T3CY0 Stage IV	Por	+	+	+
13	P0H0N1T3CY0 Stage IIIB	Sig	-	+	+
14	P0H0N2T3CY1 Stage IV	Por	+	+	+
15	P0H0N2T3CY1 Stage IV	Por	+	+	+
	Sensitiviy for the detection of MM		73% (11/15)	93% (14/15)	100% (15/15)

*Clinical stage according to the Japanese Gastric Cancer Classification; **Histology of the primary lesion according to Japanese Gastric Cancer Classification. Por: poorly differentiated adenocarcinoma; Sig: signet ring cell carcinoma; Tub: tubular adenocarcinoma.

negative (T1, T2) for invasion of the serosa, there was a correlation between *RegIV*/β-actin mRNA ratio and the degree of wall invasiveness: The *RegIV*/β-actin mRNA ratio was significantly higher in cases that were positive for serosal invasion compared to those that were negative. *RegIV* mRNA values were also significantly higher in washes from metastasis-positive patients than in metastasis-negative patients (p=0.0000015, Figure 3B).

RegIV mRNA expression and clinicopathological factors. Among the 95 cases examined, 47 were *RegIV*+. Fifteen patients had positive cytology (CY+) or were observed to have peritoneal metastases. Fourteen out of these 15 patients with peritoneal metastases had *RegIV* values that were above the cut-off value (93% sensitivity). Of note, 3 of 43 T1 or T2 patients were *RegIV*+ (93% specificity). Differentiation, depth of invasion, lymphatic invasion, vascular invasion, lymph node invasion, and peritoneal dissemination showed statistically significant differences with respect to expression of *RegIV* (Table I).

Comparison of the sensitivity and specificity of RegIV and CEA expression as markers for peritoneal micro-metastasis of gastric cancer. CEA/β-actin mRNA ratios were also measured in all clinical samples. The average values of the *CEA*/β-actin mRNA ratios ($x10^3$) according to T classification were as follows (average±standard deviation): control, 3.4±1.7; T1, 5.3±3.5; T2, 8.4±6.8; T3, 71.1±22.8; and T4, 643.9±378.3. There was a correlation between the degree of wall invasion and *CEA*/β-actin mRNA ratio. When patients were classified according to serosal invasion as positive (T3, T4) or negative (T1, T2), we observed a significant difference between the two groups with respect to *CEA* expression with patients who were CEA-positive exhibiting significantly higher CEA expression than CEA-negative patients. As with *RegIV* mRNA, *CEA*/β-actin mRNA ratios from negative control patients (patients with benign disease) were determined and the highest value was set as the cut-off value. Cases with a value greater than the cut-off were regarded as *CEA*-positive. Four of the 43 T1 or T2 patients were *CEA*-positive (91% specificity, data not shown). Table II contains a list of patients who suffered from recurrent gastric cancer with peritoneal dissemination and ascites after surgery. Of the 15 who were CY+ or were observed intraoperatively to have peritoneal metastasis, 11 were *CEA*-positive. However, 4 *CEA*-negative patients had metastasis (Table II, 73% sensitivity).

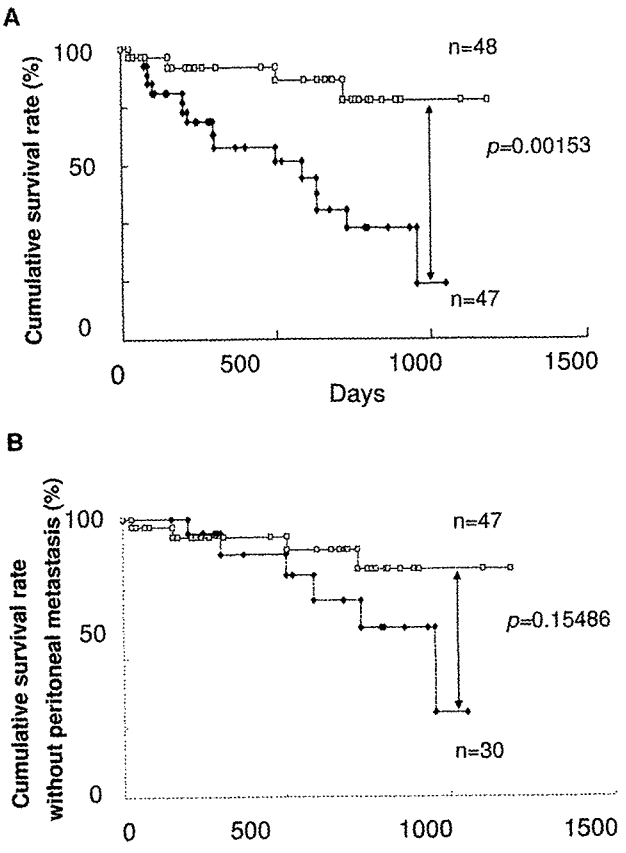

Figure 4. *Survival curves of gastric cancer patients. A) Survival curves of all 95 study patients with gastric carcinoma, grouped according to RegIV mRNA expression. RegIV+ cases (closed symbols) had a significantly worse prognosis than RegIV-negative cases (open symbols) (p=0.00153). B) Survival curves of 77 patients who underwent R0 resection, grouped according to RegIV mRNA expression. There was no significant difference in prognosis between RegIV+ (closed symbols) and RegIV-negative (open symbols) patients.*

Table III. *Multivariate analysis of RegIV mRNA and other known prognostic factors for 95 patients with gastric cancers.*

Covariate	Hazard ratio	95% Confidence interval	*P*-value
RegIV (cut-off value 0.1)			
Negative	1		
Positive	2.033659	1.059-1.132	0.0151
Vascular invasion			
Negative	1		
Positive	4.149176	1.461-14.940	0.0062
Lymph node metastasis			
Negative	1		
Positive	9.820896	1.660-190.220	0.0080

Although the 4 CEA-negative patients (cases 2, 4, 8 and 13 in Table II) had poorly differentiated adenocarcinoma, two of them had peritoneal metastases at an early stage. Three of these patients were RegIV+ (Table II). As shown in Table I, RegIV+ cases were more frequently observed in undifferentiated adenocarcinoma. These results suggested that *RegIV* may be a novel marker for more extensive disease even when a sample is negative for *CEA*. As shown in Table II, combining *CEA* and *RegIV* analysis improved the accuracy of diagnosis to 100%.

RegIV as an independent prognostic factor. Survival analysis was performed for all 95 gastric cancer patients. Univariate analysis of prognosis factors showed that RegIV+ cases (47 of 95) were significantly fewer than RegIV- cases (48 of 95, p=0.00153) (Figure 4A). We also performed survival analysis of 77 patients who underwent R0 resection. Eighteen patients treated with palliative resection, including 15 patients with synchronous peritoneal metastases, died within 490 days with peritoneal metastases. As shown in Figure 4B, there was no significant difference between the survival rate of *RegIV+* cases and *RegIV-* cases in this group of patients.

Multivariate analysis using the Cox proportional hazards model showed that a *RegIV*/β-actin mRNA ratio above a cut-off value of 0.1 was a significant independent factor, along with histological findings of lymph node metastasis and vascular invasion (Table III). In cases of R0 resection, we found a correlation between *RegIV* expression and prognosis; however, the results were not statistically significant (data not shown).

RegIV expression and tumorigenesis in nude mice. Following inoculation of nude mice with either TMK-1-neomycin or TMK-1-RegIV stable transfectants, we found far more peritoneal-disseminated metastatic lesions in TMK-1-RegIV-innoculated mice, as compared to the controls (Figure 5A). The metastatic nodules were found in the mesenterium as well as at the peritoneal wall. There was also an increase in ascites fluid in the peritoneal cavity. In addition to the number, the size of peritoneal metastases increased in TMK-1-RegIV-inoculated mice. Figure 5B shows the aggregate intraperitoneal tumor weight per animal (n=6 for each group, $p<0.01$). All mice that were injected with parental TMK-1 cells or TMK-1-neomycin transfectants (mock transfectants) died within 16 weeks (average lifespan 84 days). In contrast, all mice injected with TMK-1-RegIV transfectants died within 70 days (average lifespan 44 days). Figure 5C shows the tumors obtained from mice that were injected subcutaneously with the stable cell line TMK-1-RegIV-1, and Figure 5D shows the subcutanous tumor weight. The

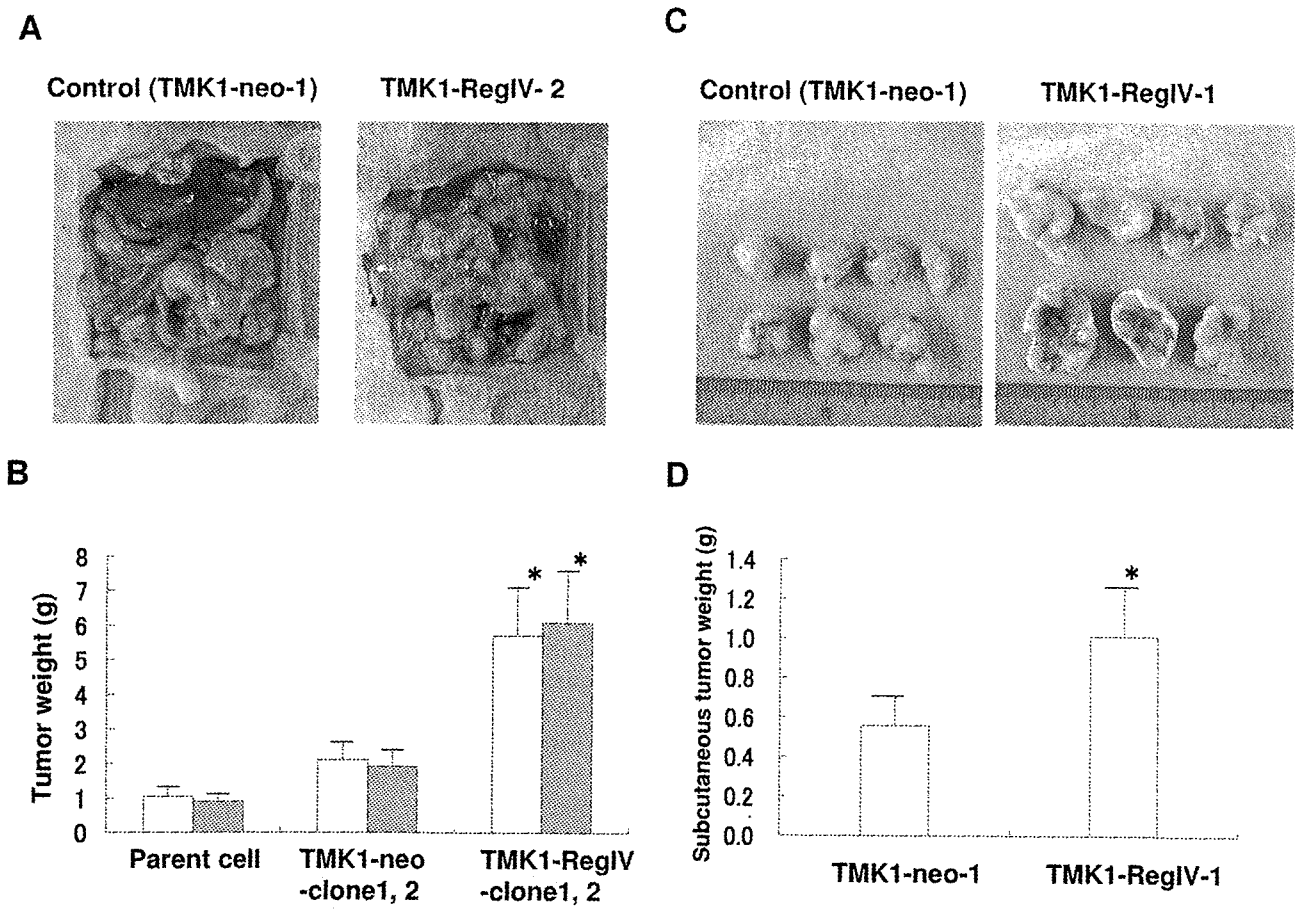

Figure 5. *Acceleration of peritoneal metastases of gastric cancer by RegIV expression. A) Macroscopic appearance of peritoneal disseminated metastatic nodules derived from TMK-1-RegIV-2 transfectants and control TMK-1 transfectants. B) Quantification of metastatic nodules in the mouse peritoneal cavity. Weight of tumors derived from RegIV transfectants were significantly higher than those derived from control cells (*p<0.01). C) Tumors obtained after subcutaneous injection of RegIV and control transfectants. Cells were injected subcutaneously to examine the growth stimulatory activity of RegIV transfectants and control cells. D, Subcutanous tumor weight in TMK-1-RegIV-1 and TMK-1-neomycin-inoculated mice. Subcutanous tumor weight increased in TMK-1-RegIV-1 inoculated mice (p<0.05).*

subcutaneous tumor weight was significantly higher in TMK-1-RegIV-1-inoculated mice compared to the neomycin controls ($p<0.05$).

Discussion

Peritoneal dissemination is the most important factor affecting the prognosis of individuals with gastric cancer (4). Previous reports have indicated that intraperitoneal chemotherapy improves the survival of these patients, but it can also be life-threatening because of the side-effects of the chemotherapeutic drugs. Recently we developed a novel technique to administer the anticancer agent mitomycin-C, in which the drug was adsorbed to activated carbon particles (MMC-CH) (25-28). Because MMC-CH particles are not actually absorbed through the capillary wall, a large amount of the anticancer agent was delivered through this route of adminstration. Particles are retained in the cavity, which remains closed for a long period of time; thus a high concentration of the anticancer agent is maintained. We previously reported on the efficacy of MMC-CH in the treatment of peritonitis carcinomatosis in gastric cancer (29). However, this therapeutic approach also had side-effects, such as ileus, fever and leukocytopenia, suggesting that this therapy should be limited to those most likely to benefit from it, in order to minimize these side-effects.

Peritoneal dissemination is observed in patients with negative cytological results, indicating that conventional cytological analysis lacks appropriate sensitivity. In contrast, RT-PCR has been shown to be of sufficient sensitivity to diagnose micrometastases on the basis of specific mRNA expression in tumor cells derived from the peripheral blood, bone marrow, lymph nodes and cerebrospinal fluid. Quantitative, rapid RT-PCR-based screening methods for the

detection of micrometastasis from clinical specimens has now become more widely used as a diagnostic tool (30-36). *CEA*, keratin 19 and alpha-fetoprotein (*AFP*) represent some of the conventional molecular markers that have been used to detect peritoneal micrometastases in RT-PCR-based assays of peritoneal washes from patients with gastric cancer (8, 35). Yonemura *et al.* increased the sensitivity of detection to 62% by using a combination of cytology and RT-PCR-based detection of matrix metalloproteinase (*MMP*)-7 mRNA (36). Using RT-PCR, Schuhmacher *et al.* demonstrated a relationship between the expression of an E-cadherin mutation and metastasis to the peritoneum (37). However, any assay of peritoneal washes is inferior in sensitivity and specificity to real-time RT-PCR in detecting *CEA* mRNA, as described by Nakanishi *et al.* (24). Because of this, *CEA* is currently the standard molecular marker for the detection of gastric cancer micrometastases. However, it is not always expressed in peritoneal metastases and is very weakly expressed in mesothelial cells, making it difficult to exclude completely both false-positive and false-negative results using *CEA* as a marker. To reduce the frequency of missed diagnosis, markers with greater sensitivity and specificity are needed.

When choosing a genetic marker for peritoneal dissemination, genes expressed more highly in cancer cells than in mesothelial cells should be chosen to to minimize false-positive or false-negative results. Previously, using cDNA microarray analysis of gastric cancer cell lines derived from either a primary tumor or from metastatic lesions in the peritoneal cavity, we identified *RegIV* as a candidate marker of peritoneal dissemination of gastric cancer. Thus, *RegIV* satisfies the conditions stated above. In the current study, using fluorescence-based, real-time RT-PCR, we examined *RegIV* mRNA expression in peritoneal washes from 95 patients with gastric cancer and compared it to *CEA* mRNA expression, as a diagnostic tool for predicting peritoneal recurrence. We demonstrated that *RegIV* and *CEA* expression correlated with wall penetration. Using data derived from negative control patients with benign disease to set a cut-off value for expression, we identified a group of MM+ patients and showed that the specificity of *RegIV* and *CEA* expression in this group was 93% and 91%, respectively. Among 15 patients with peritoneal dissemination, 7 of whom were CY+, 14 cases were *RegIV*-positive (93% sensitivity), while 4 cases appeared negative for CEA expression (73% sensitivity). *CEA*-specific RT-PCR failed to detect peritoneal dissemination of poorly differentiated adenocarcinoma, while *RegIV*-specific RT-PCR successfully detected these cancers (Table II). Taken together, quantitative RT-PCR of peritoneal washes to detect the novel marker *RegIV* yielded higher sensitivity and specificity than did similar analysis of *CEA*, particularly in patients with poorly differentiated adenocarcinoma. Our results also indicated that the combination of *CEA*- and *RegIV*-specific RT-PCR may improve the accuracy of diagnosis of peritoneal dissemination.

According to the survival analysis of patients with gastric cancer, *RegIV*-positive cases had a significantly worse prognosis than *RegIV*-negative cases. Moreover, multivariate analysis suggested that *RegIV* is an independent prognostic factor of survival. In view of the correlation between the results from RT-PCR analysis and prognosis, *RegIV* represents a potentially useful and effective marker of peritoneal recurrence of gastric cancer. A large-scale, long-term follow-up study is currently under way in our department to determine the rate of peritoneal recurrence in cytology-negative, PCR-positive patients, and to determine whether these patients remain disease-free.

Expression of *RegIV* in gastric cancer cells established from malignant ascites accelerated the rate of peritoneal metastases in a nude mouse model of gastric cancer. In addition, the tumorigenicity of the *RegIV*-expressing cells, when injected into the peritoneum, was significantly higher than either parental or mock-transfected cells. Given that Reg family members are involved in liver, pancreatic, gastric and intestinal cell proliferation or differentiation (14), our results suggest that *RegIV* is involved in gastric cancer cell proliferation and peritoneal metastasis.

In conclusion, we have presented evidence that *RegIV*-specific RT-PCR analysis of peritoneal washes may be more sensitive than conventional cytology or *CEA*-specific RT-PCR for predicting peritoneal recurrence in gastric cancer. While *RegIV* is overexpressed in gastric cancer peritoneal dissemination, the role of *RegIV* in this process remains the subject of future studies.

Acknowledgements

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare and from the Ministry of Education, Science and Culture.

References

1 Parkin DM, Pisani P and Ferlay J: Estimates of the worldwide incidence of 25 major cancers in 1990. Int J Cancer *80*: 827-841, 1999.

2 Yamazaki H, Oshima A, Murakami R, Endoh S and Ubukata T: A long-term follow-up study of patients with gastric cancer detected by mass screening. Cancer *63*: 613-617, 1989.

3 Kodera Y, Yamamura Y, Torii A, Uesaka K, Hirai T, Yasui K, Morimoto T, Kato T and Kito T: Postoperative staging of gastric carcinoma. A comparison between the UICC stage classification and the 12th edition of the Japanese General Rules for Gastric Cancer Study. Scand J Gastroenterol *31*: 476-480, 1996.

4 Baba H, Korenaga D, Okamura T, Saito A and Sugimachi K: Prognostic factors in gastric cancer with serosal invasion. Univariate and multivariate analyses. Arch Surg *124*: 1061-1064, 1989.

5 Abe S, Yoshimura H, Tabara H, Tachibana M, Monden N, Nakamura T and Nagaoka S: Curative resection of gastric cancer: limitation of peritoneal lavage cytology in predicting the outcome. J Surg Oncol *59*: 226-229, 1995.

6 Bonenkanmp JJ, Songun I, Hermans J and van de VELDE CJ: Prognostic value of positive cytology findings from abdominal washings in patients with gastric cancer. Br J Surg *83*: v672-674, 1996.

7 Boku T, Nakane Y, Minoura T, Takada H, Yamamura M, Hioki K and Yamamoto M: Prognostic significance of serosal invasion and free intraperitoneal cancer cells in gastric cancer. Br J Surg *77(4)*: 436-439, 1990.

8 Nakanishi H, Kodera Y, Torii A, Hirai T, Yamamura Y, Kato T, Kito T and Tatematsu M: Detection of carcinoembryonic antigen-expressing free tumor cells in peritoneal washes from patients with gastric carcinoma by polymerase chain reaction. Jpn J Cancer Res *88*: 687-692, 1997.

9 Sakakura C, Hagiwara A, Nakanishi M, Shimomura K, Takagi T, Yasuoka R, Fujita Y, Abe T, Ichikawa Y, Takahashi S, Ishikawa T, Nishizuka I, Morita T, Shimada H, Okazaki Y, Hayashizaki Y and Yamagishi H: Differential gene expression profiles of gastric cancer cells established from primary tumour and malignant ascites. Br J Cancer *87*: 1153-1161, 2002.

10 Hartupee JC, Zhang H, Bonaldo MF, Soares MB and Dieckgraefe BK: Isolation and characterization of a cDNA encoding a novel member of the human regenerating protein family: Reg IV. Biochim Biophys Acta *1518*: 287-293, 2001.

11 Broekaert D, Eyckerman S, Lavens D, Verhee A, Waelput W, Vandekerckhove J and Tavernier J: Comparison of leptin- and interleukin-6-regulated expression of the rPAP gene family: evidence for differential co-regulatory signals. Eur Cytokine Netw *13(1)*: 78-85, 2002.

12 Violette S, Festor E, Pandrea-Vasile I, Mitchell V, Adida C, Dussaulx E, Lacorte JM, Chambaz J, Lacasa M and Lesuffleur T: Reg IV, a new member of the regenerating gene family, is overexpressed in colorectal carcinomas. Int J Cancer *103*: 185-193, 2003.

13 Zhang H, Lai M, Lv B, Gu X, Wang H, Zhu Y, Zhu Y, Shao L and Wang G: Overexpression of Reg IV in colorectal adenoma. Cancer Lett *200*: 69-76, 2003.

14 Zhang YW, Ding LS and Lai MD: Reg gene family and human disease. World J Gastroenterol *9*: 2635-2641, 2003.

15 Yonemura Y, Sakurai S, Yamamoto H, Endou Y, Kawamura T, Bandou E, Elnemr A, Sugiyama K, Sasaki T, Akiyama T, Takasawa S and Okamoto H: REG gene expression is associated with the infiltrating growth of gastric carcinoma. Cancer *98*: 1394-1400, 2003.

16 Oue N, Hamai Y, Mitani Y, Matsumura S, Oshimo Y, Aung PP, Kuraoka K, Nakayama H and Yasui W: Gene Expression Profile of Gastric Carcinoma. Cancer Res *64*: 2397-2405, 2004.

17 Oue N, Mitani Y, Aung PP, Sakakura C, Takeshima Y, Kaneko M, Noguchi T, Nakayama H and Yasui W: Expression and localization of Reg IV in human neoplastic and non-neoplastic tissues: Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma. J Pathol *207*: 185-198, 2005.

18 Park JG, Yang HK, Kim WH, Chung JK, Kang MS, Lee JH, Oh JH, Park HS, Yeo KS, Kang SH, Song SY, Kang YK, Bang YG, Kim YI and Kim JP: Establishment and characterization of human gastric carcinoma cell lines. Int J Cancer *70(4)*: 443-449, 1997.

19 Duncan EL, Whitaker NJ, Moy EL and Reddel RR: Assignment of SV40-immortalized cells to more than one complementation group for immortalization. Exp Cell Res *205*: 337-344, 1993.

20 Nakabayashi K, Ogimo H, Michishita E, Satoh N and Ayusawa D: Introduction of chromosome 7 suppresses telomerase with shortening of telomeres in a human mesothelial cell line. Exp Cell Res *252*: 376-382, 1999.

21 Sakakura C, Yamaguchi-Iwai Y, Satake M, Bae SC, Takahashi A, Ogawa E, Hagiwara A, Takahashi T, Murakami A, Makino K, Nakagawa T, Kamada N and Ito Y: Growth inhibition and induction of differentiation of t(8;21) acute myeloid leukemia cells by the DNA-binding domain of PEBP2 and the AML1/MTG8(ETO)-specific antisense oligonucleotide. Proc Natl Acad Sci USA *91*: 11723-11727, 1994.

22 Sakakura C, Sweeney EA, Shirahama T, Igarashi Y, Hakomori S, Nakatani H, Tsujimoto H, Imanishi T, Ohgaki M, Ohyama T, Yamazaki J, Hagiwara A, Yamaguchi T, Sawai K and Takahashi T: Overexpression of bax sensitizes human breast cancer MCF-7 cells to radiation-induced apoptosis. Int J Cancer *67*: 101-105, 1996.

23 Sakakura C, Hasegawa K, Miyagawa K, Nakashima S, Yoshikawa T, Kin S, Nakase Y, Yazumi S, Yamagishi H, Okanoue T, Chiba T and Hagiwara A: Possible involvement of RUNX3 silencing in the peritoneal metastases of gastric cancers. Clin Cancer Res *11*: 6479-6488, 2005.

24 Nakanishi H, Kodera Y, Yamamura Y, Ito S, Kato T, Ezaki T and Tatematsu M: Rapid quantitative detection of carcinoembryonic antigen-expressing free tumor cells in the peritoneal cavity of gastric-cancer patients with real-time RT-PCR on the lightcycler. Int J Cancer *89*: 411-417, 2000.

25 Hagiwara A, Takahashi T, Kojima O, Sawai K, Yamaguchi T, Yamane T, Taniguchi H, Kitamura K, Noguchi A, Seiki K and Sakakura C: Prophylaxis with carbon-adsorbed mitomycin against peritoneal recurrence of gastric cancer. Lancet *339*: 629-631, 1992.

26 Hagiwara A, Takahashi T, Kojima O, Kitamura K, Sakakura C, Shoubayashi S, Osaki K, Iwamoto A, Lee M and Fujita K: Endoscopic local injection of a new drug-delivery format of peplomycin for superficial esophageal cancer: a pilot study. Gastroenterology *104*: 1037-1043, 1993.

27 Hagiwara A, Togawa, T, Yamasaki J, Ohgaki M, Imanishi T, Shirasu M, Sakakura C, Yamaguchi T, Sawai K and Yamagishi H: Extensive gastrectomy and carbon-adsorbed mitomycin C for gastric cancer with peritoneal metastases. Case reports of survivors and their implications. Hepatogastroenterology *46*: 1673-1677, 1999.

28 Takahashi T, Hagiwar A, Shimotsuma M, Sawai K and Yamaguchi T: Prophylaxis and treatment of peritoneal carcinomatosis: intraperitoneal chemotherapy with mitomycin C bound to activated carbon particles. World J Surg *19*: 565-569, 1995.

29 Hagiwara A, Takahahi T, Sawai K, Taniguchi H, Shimotsuma M, Okano S, Sakakura C, Tsujimoto H, Osaki K, Sasaki S and Shirasu M: Milky spots as the implantation site for malignant cells in peritoneal dissemination in mice. Cancer Res *53*: 687-692, 1993.

30 Burchill SA, Bradbury MF, Pittman K, Southgate J, Smith B and Selby P: Detection of epithelial cancer cells in peripheral blood by reverse transcriptase-polymerase chain reaction. Br J Cancer *71*: 278-281, 1995.

31 Johnson PW, Burchill SA and Selby PJ: The molecular detection of circulating tumour cells. Br J Cancer *72*: 268-276, 1995.

32 Maehara Y, Yamamoto M, Oda S, Baba H, Kusumoto T, Ohno S, Ichiyoshi Y and Sugimachi K: Cytokeratin-positive cells in bone marrow for identifying distant micrometastasis of gastric cancer. Br J Cancer *73*: 83-87, 1996.
33 Mori M, Mimori K, Ueo H, Tsuji K, Shiraishi T, Barnard GF, Sugimachi K and Akiyoshi T: Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription-polymerase chain reaction in patients with gastrointestinal or breast carcinomas. J Clin Oncol *16*: 128-132, 1998.
34 Noguchi S, Hiratsuka M, Furukawa H, Aihara T, Kasugai T, Tamura S, Imaoka S, Koyama H and Iwanaga T: Detection of gastric cancer micrometastases in lymph nodes by amplification of keratin 19 mRNA with reverse transcriptase-polymerase chain reaction. Jpn J Cancer Res *87*: 650-654, 1996.
35 Schimidt P, Thiele M, Rudroff C, Vaz A, Schilli M, Friedrich K and Scheele J: Detection of tumor cells in peritoneal lavages from patients with gastrointestinal cancer by multiplex reverse transcriptase PCR. Hepatogastroenterology *48*: 1675-1679, 2001.
36 Yonemura Y, Fujimura T, Ninomiya I, Kim BS, Bandou E, Sawa T, Kinoshita K, Endo Y, Sugiyama K and Sasaki T: Prediction of peritoneal micrometastasis by peritoneal lavaged cytology and reverse transcriptase polymerase chain reaction by matrix metalloproteinase-7 mRNA. Clin Cancer Res *7*: 1647-1653, 2001.
37 Schuhmacher C, Becker KF, Reich U, Schenk U, Mueller J, Siewert JR and Hofler H: Rapid detection of mutated E-cadherin in peritoneal lavage specimens from patients with diffuse-type gastric carcinoma. Diagn Mol Pathol *8*: 66-70, 1999.

Received June 26, 2007
Revised December 12, 2007
Accepted December 31, 2007

Molecular Cancer

BioMed Central

Research

Open Access

In Silico analysis of Gastric carcinoma Serial Analysis of Gene Expression libraries reveals different profiles associated with ethnicity

Francisco J Ossandon[1], Cynthia Villarroel[1], Francisco Aguayo[1], Eudocia Santibanez[1], Naohide Oue[2], Wataru Yasui[2] and Alejandro H Corvalan*[1,3]

Address: [1]Laboratory of Molecular Pathology and Epidemiology, Centro Investigaciones Medicas, Pontificia Universidad Catolica de Chile, Santiago, Chile, [2]Department of Molecular Pathology, Hiroshima University, Graduate School of Biomedical Sciences, Hiroshima, Japan and [3]Department of Pathology, University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, USA

Email: Francisco J Ossandon - fossandon@vtr.net; Cynthia Villarroel - cjvillar@gmail.com; Francisco Aguayo - faguayo@med.puc.cl; Eudocia Santibanez - eudociaj@yahoo.com; Naohide Oue - noue@hiroshima-u.ac.jp; Wataru Yasui - wyasui@hiroshima-u.ac.jp; Alejandro H Corvalan* - acorvala@mdanderson.org

* Corresponding author

Published: 27 February 2008

Molecular Cancer 2008, **7**:22 doi:10.1186/1476-4598-7-22

Received: 4 October 2007
Accepted: 27 February 2008

This article is available from: http://www.molecular-cancer.com/content/7/1/22

Abstract

Worldwide gastric carcinoma has marked geographical variations and worse outcome in patients from the West compared to the East. Although these differences has been explained by better diagnostic criteria, improved staging methods and more radical surgery, emerging evidence supports the concept that gene expression differences associated to ethnicity might contribute to this disparate outcome. Here, we collected datasets from 4 normal and 11 gastric carcinoma Serial Gene Expression Analysis (SAGE) libraries from two different ethnicities. All normal SAGE libraries as well as 7 tumor libraries were from the West and 4 tumor libraries were from the East. These datasets we compare by Correspondence Analysis and Support Tree analysis and specific differences in tags expression were identified by Significance Analysis for Microarray. Tags to gene assignments were performed by CGAP-SAGE Genie or TAGmapper. The analysis of global transcriptome shows a clear separation between normal and tumor libraries with 90 tags differentially expressed. A clear separation was also found between the West and the East tumor libraries with 54 tags differentially expressed. Tags to gene assignments identified 15 genes, 5 of them with significant higher expression in the West libraries in comparison to the East libraries. qRT-PCR in cell lines from west and east origin confirmed these differences. Interestingly, two of these genes have been associated to aggressiveness (COL1A1 and KLK10). In conclusion we found that in silico analysis of SAGE libraries from two different ethnicities reveal differences in gene expression profile. These expression differences might contribute to explain the disparate outcome between the West and the East.

Introduction

Gastric carcinoma is the second leading cause of cancer-related death worldwide and has marked geographical variations [1-3]. The observed advantage in 5-year survival rate from patients from the East than from the West may reflect differences in diagnostic criteria, better staging methods and more radical surgery [4]. However emerging evidence supports the concept that ethnicity might contribute to the disparate gastric carcinoma outcomes between the East and the West [4,5]. Serial Analysis of Gene Expression (SAGE) is a comprehensive profiling method that allows for global, unbiased and quantitative characterization of transcriptomes [6]. A major advantage of SAGE is that once normalized is possible to directly compare the levels of tags generated by a single experiment with any other available [7]. To gain an insight of the differences between gastric carcinoma transcriptomes that might explain the disparate outcomes between the East and the West here we compare datasets of fifteen SAGE libraries derived from normal and gastric tumor tissues from Japanese and American gastric cancer patients by Correspondence Analysis, Support Tree and Significance Analysis for Microarray for significative tags and gene selection. We found specific genes differentially expressed between normal and tumor SAGE libraries as well as tumor libraries from the East and the West. These differentially expressed genes could explain the worse survival rate in the West in comparison to the East.

Methods

Serial Analyses of Gene Expression data

Fifteen gastric SAGE libraries (4 normal and 11 tumor) from Cancer Genome Anatomy Project (CGAP) [7] were combined for the analysis. Only libraries with 10 bp tags and the same cutting enzymes (BsmFI and NlaIII) were included in this study. Normal libraries consist of a tissue pool (GSM784 and GSM14780) or microdissected samples (CGAP_MD_13S and CGAP_MD_14S) and were produced by El-Rifai et al [8] in Virginia, USA. Gastric tumor libraries consist of five libraries, three microdissected (CGAP_MD_HG7, CGAP_MD_HS29, CGAP_MD_G329), two primary tumors (GSM757 and GSM2385) and two xenografts (GSM758 and GSM14760) all from western patients and produced by El-Rifai et al [8] also in Virginia, USA ("West tumor libraries") and 4 libraries (GSM7800, GSM8505, GSM8867 and GSM9103) all from japanese patients produced by Oue et al [9] in Hiroshima, Japan ("East tumor libraries"). A database containing 121,409 different tags was generated from libraries which have between 9,000 and 34,000 unique tags. Thus, only library GSM9103 was removed because its unique tag count was too low (around 6,000 unique tags). The frequency of each tag was normalized by dividing it with the total tag number of the corresponding library and multiplying by 200,000 tags (CGAP normalization format). A selection process to reduce noise from an enormous amount of tags collected was performed. This selection criterion was i) "tags found in *all normal libraries*" vs. "tags found in *all tumor libraries*" and ii) "tags found in *all West tumors libraries*" vs. "tags found in *all East tumors libraries*". The Institute for Genomic Research software MultiExperiment Viewer [10] was used to perform the following analysis: i) Correspondence Analysis (COA) to explore associations between samples that tend to have similar profiles ii) Support Tree to shows the statistical support after repeating at least 1000 times the analysis by resampling with replacement (Bootstrap method) for samples with similar profiles and iii) Significance Analysis for Microarray (SAM) to select tags whose expression was significantly different between samples. The association of tags to genes was perform by SAGE Genie [11] or TAGmapper [12] when no association was found by SAGE Genie. To predict functional classes of annotated genes the FatiGO+ tool of Babelomics [13,14] was applied. The unadjusted p-value given by Babelomics was used because the small number of genes analyzed made it more appropriate than the adjusted-False Discovery Rate (FDR) value.

Quantitative Real-Time Reverse-Transcription PCR

Quantitative real-time reverse-transcription PCR (qRT-PCR) was performed on two western cell lines (AGS, N87) and one eastern cell line (MKN45). Total RNA was extracted using Trizol (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations. RNA concentration was determined by measuring absorbance at 260 nm, and quality was verified by the integrity of 28S and 18S rRNA after ethidium bromide staining of total RNA samples subjected to 0.8% agarose gel electrophoresis. Total cDNA was synthesized with MMLV (Moloney Murine Leukemia Virus) reverse transcriptase (ThermoScript RT; Invitrogen Life Technologies, Carlsbad, CA). Reverse transcription-PCR was performed using 1 ug of total cellular RNA to generate cDNA. qRT-PCR was performed using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany). We designed gene-specific primers for human PDFGR (5' AGCTGATCCGTGCTAAGGAA 3' and 5' CGACCAAGTCCAGAATGGAT 3') and RPL13 (5' GAGGAGGCGGAACAAGTCC 3' and 5' TCAGCAGAACTGTCTCCCTTC 3') and conditions of amplification are available upon request. A single-melt curve peak was observed for each product, thus confirming the purity of all amplified cDNA products. The qRT-PCR results were normalized to GADPH (5' CGGGAAGCTTGTCATCAATGG 3' and 5' CATGGTTCACACCCATGACG 3'), which had minimal variation in all cell lines tested. Analysis was performing by LightCycler software 3.0. Crossing points (beginning of the PCR exponential phase) were

assessed by the second derivated maximum method and plotted against the concentrations of the standards.

Results

Tags with consistent expression in normal and tumor SAGE libraries

The selection process to find SAGE tags that were consistently expressed in "*all normal libraries*" vs. "*all tumor libraries*" resulted in 2,437 tags. As shown in Fig. 1, COA shows clear separation between normal libraries and East and West tumor libraries. The same COA in a three-dimensional plot (accounting for 56% of the total inertia) shows more details in the position of each library (see Additional File 1). These results were confirmed by a Support Tree using the Pearson Correlation and Average Linkage (see Additional File 2). Next, to identify SAGE tags differentially expressed between normal and tumor samples, we performed SAM, with a delta value of 1.38 calculated to maintain the FDR near to 0 (probability to find significant tags merely by chance), 1001 unique permutations and a fold change = 10. This approach revealed 90 tags differentially expressed between normal and tumor libraries with a similar behavior for both tumor groups (Fig. 2). Among these 90 tags, 78 were down-regulated and 12 tags were up-regulated.

Selection of discriminatory tags between East and West SAGE libraries

Since the tumor side of the COA shows 2 groups, one containing all the East libraries and the other containing all the West libraries, we searched for discriminatory elements between both tumors libraries. Thus, a new selection process to find tags that were consistently expressed in "*all East tumors*" vs. "*all West tumors*" resulted in 3,952 tags. Another Support Tree using the Pearson Correlation and Average Linkage was performed. As shown in Fig. 3, the tree shows an organized structure with a high confidence degree in their branches (90%–100% support), given by the great number of discriminatory elements (tags) with distinctive families and subfamilies (the Additional File 3 shows the full dendrogram). There are two main clusters, one contains all West libraries and the other contains all East libraries. The West cluster contains two distinctive subclusters, the first contains the 3 microdissected libraries (CGAP_MD_HG7, CGAP_MD_HS29 and CGAP_MD_G329) and the second includes primary

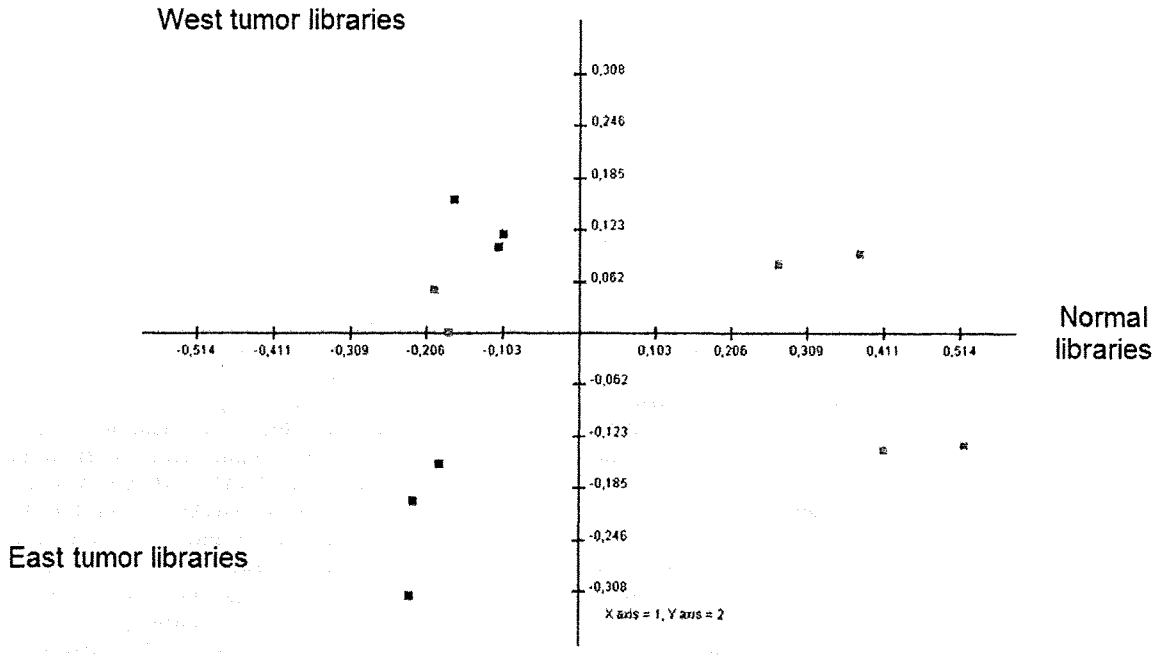

Figure 1
Correspondence Analysis of normal and tumor SAGE libraries of the stomach. A two-dimensional plot is shown where the green dots represent all the normal libraries, the blue dots are the East tumor libraries, and the red, orange and yellow dots are West tumor libraries, microdissected, xenograft and bulk respectively.

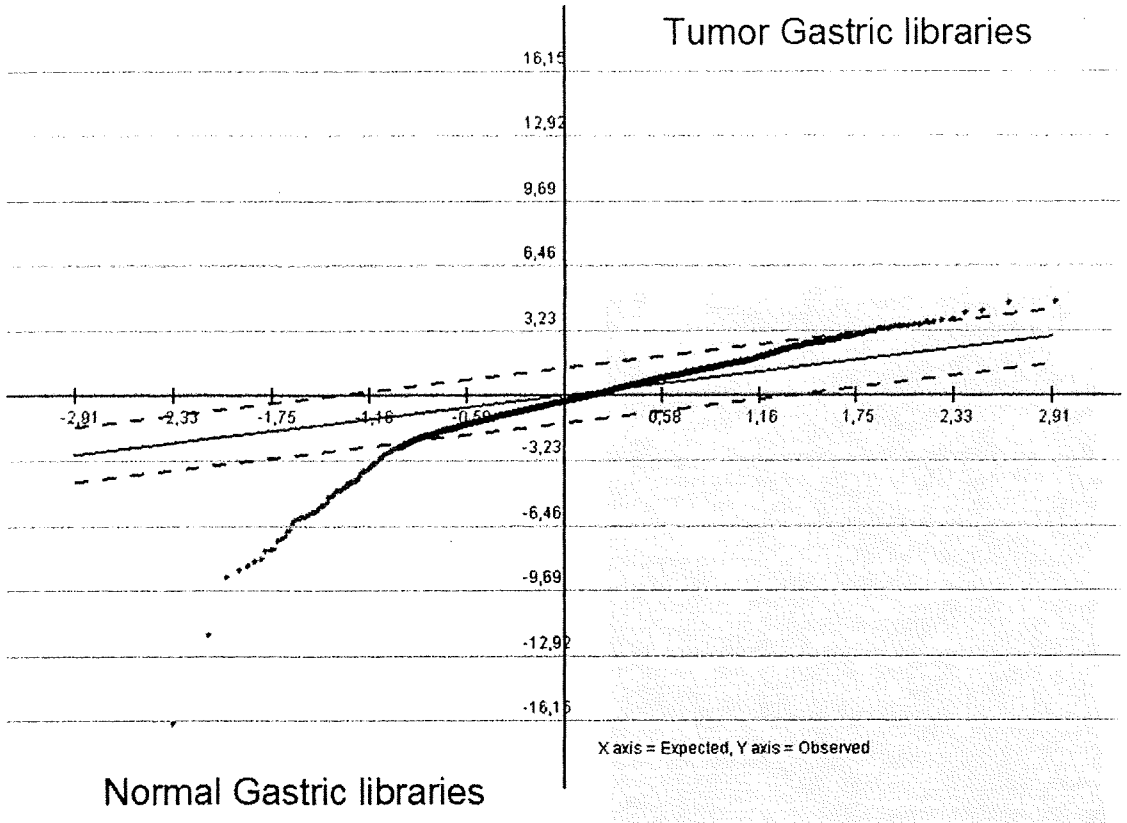

Figure 2
Serial Analysis for Microarray of normal and tumor SAGE libraries of the stomach. To the left and shown in green color, the significant tags with higher expression in the normal libraries; to the right and shown in red color, the significant tags with higher expression in the tumor libraries.

tumors (GSM757 and GSM2385) and xenografts (GSM758 and GSM14760). The East cluster contains a central pair (GSM8505 and GSM8867 libraries) that comes from histological well differentiated tumors and a third library (GSM7800) that comes from a histological poorly differentiated tumor. Next, to identify SAGE tags differentially expressed between the West and the East tumor libraries, we performed a SAM using the same criteria mentioned above. This approach revealed 54 tags differentially expressed (Fig. 4). Among these, 8 tags were up-regulated in the West tumors and 46 tags were up-regulated in the East tumors.

Mapping SAGE tags to genes

For mapping differentially expressed SAGE tags to genes we used the CGAP-SAGE Genie and/or TAGmapper resources. Among the 90 tags differentially expressed between normal and tumor libraries, only 53 tags were successfully assignment to specific genes (Table S1 and Table S2 [Additional files 4 &5]). Genes like GIF, CPA2, DRD5, CLIC6, ATP4A, LIPF, GKN1 and PGA5 appear among the most repressed genes, while TRAPPC5, KRT7, MTHFD1, TMBIM1, PDIA3 and PPGB genes appear among the overexpressed genes. On the other side, among the 54 tags differentially expressed between the West and the East tumor libraries only 15 tags where successfully associated to specific genes (Table 1). FatiGO+ analysis showed that tumor libraries had significantly more expressed genes related to "cell organization and biogenesis" (GO:0016043), KRT7, PDIA3, PPGB and TRAPPC5 ($p = 0.005$); and "ligase activity" (GO:0016874), UBE2S and MTHFD1 ($p = 0.028$) than normal libraries,. The same comparison revealed significantly less expressed genes related to "integral to membrane" (GO:0016021), ADORA1, UGT2B15, DRD5, SYNE2, ATP5J2, KCNE2, ATP4A, KDR, PTGER3 and PPAP2B ($p = 0.016$). On the

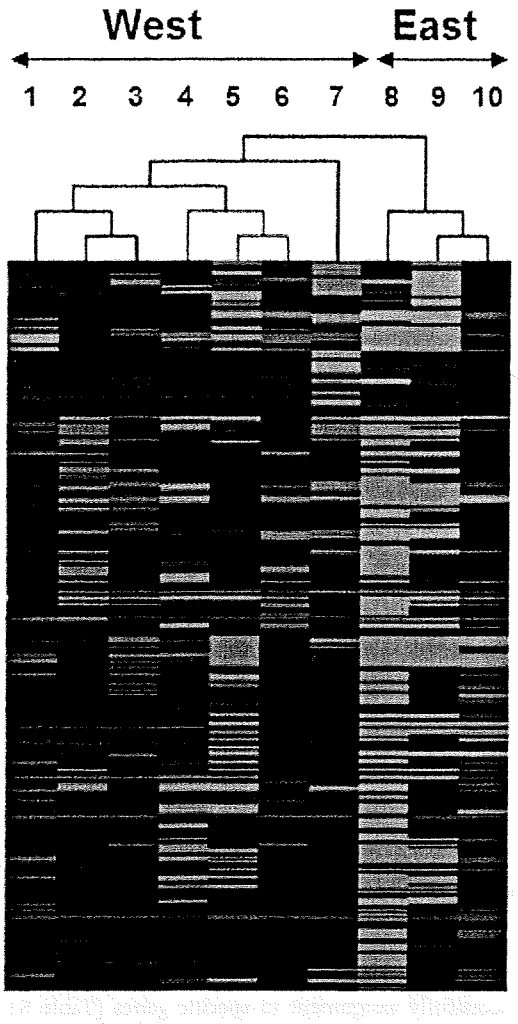

Figure 3
Support Tree of normal and tumor SAGE libraries of the stomach. Lanes 1–4 normal libraries (CGAP_MD_13S, GSM784, CGAP_MD_14S, GSM14780), lanes 5–11 West tumor libraries (CGAP_MD_HG7, CGAP_MD_HS29, CGAP_MD_G329, GSM757, GSM758, GSM14760, GSM2385) and lanes 12–14 East tumor libraries (GSM7800, GSM8505, and GSM8867). Only the top of the dendrogram is shown here. The full dendrogram appear in Additional File 3.

other hand, comparison of genes differentially expressed between the West and the East tumor libraries showed that the West tumors had significantly more expressed genes related to "ectoderm development" (GO:0007398) (COL1A1 shown on Fig. 5, also KLK10, KRT17, EMP1, and CCDC12) (p = 0.018). However, the East tumors had near significant more expressed genes related to "cellular metabolism" (GO: 0044237) PDGFRA, MAPK13, MECR, AKR1C2, RPL13, HLX1 and ADH4 (p = 0.066). Since at least two of these "ectoderm development" genes (COL1A1 and KLK10) have been found up-regulated in advanced gastric carcinoma [9,15] our findings might suggest more aggressiveness of the West tumors.

Validation of genes differentially expressed between East and West tumor SAGE libraries

To validated our SAGE data analysis two genes significantly more expressed in the East tumors (PDGFRA and RPL13) were further studied in three cell lines, two from the West (AGS and N87) and one from the East (MKN45). qRT-PCR shows a ratio of 825 for PDFGR (MKN45/N87) and 4.68 for RPL13 (MKN45/AGS) (Fig. 6). Thus, these data confirms the observed difference in gene expression in SAGE tumor libraries. Interestingly, the magnitudes of gene expression differences in cell lines were similar to that of in SAGE tumor libraries.

Discussion

Our results, based on two non-supervised analyses, COA and Support Tree, are highly suggestive of a different expression profile of tumor SAGE libraries, along with differences between normal and tumor samples. These differences in expression levels might have an influence on the recognized better survival of the East patients in comparison to the West. Both, COA and Support Tree show two clusters (microdissected and non-microdissected samples) mixed indistinctly, suggesting that the heterogeneity of a normal sample is not reduced by the microdissection. This might be explained by multiple cell activities of the normal cells compared with tumor cells [16]. However among tumor libraries, a tight grouping of microdissected tumors was found. These findings suggest that the increase of the purity of the sample improves the homogeneity of the results. The neighborhood of the xenografts also points to an increase in homogeneity but differ from the microdissected tumor samples since they group in different subclusters. This difference is probably due to subtle changes in the transcriptomes given by a different genetic environment, such as the microenvironment given by surrounding animal tissue [17]. On the other hand, the non-microdissected libraries were found more scattered in the COA analysis, probably because of sample contamination and heterogeneity.

The FatiGO+ results show that the tumor cells are characterized by up-regulation of genes related to cell organization, biogenesis and cell proliferation, and a down-regulation of genes related to cell-to-cell communication. After searching for specific differences between the West and the East tumor libraries, we found that the most sig-

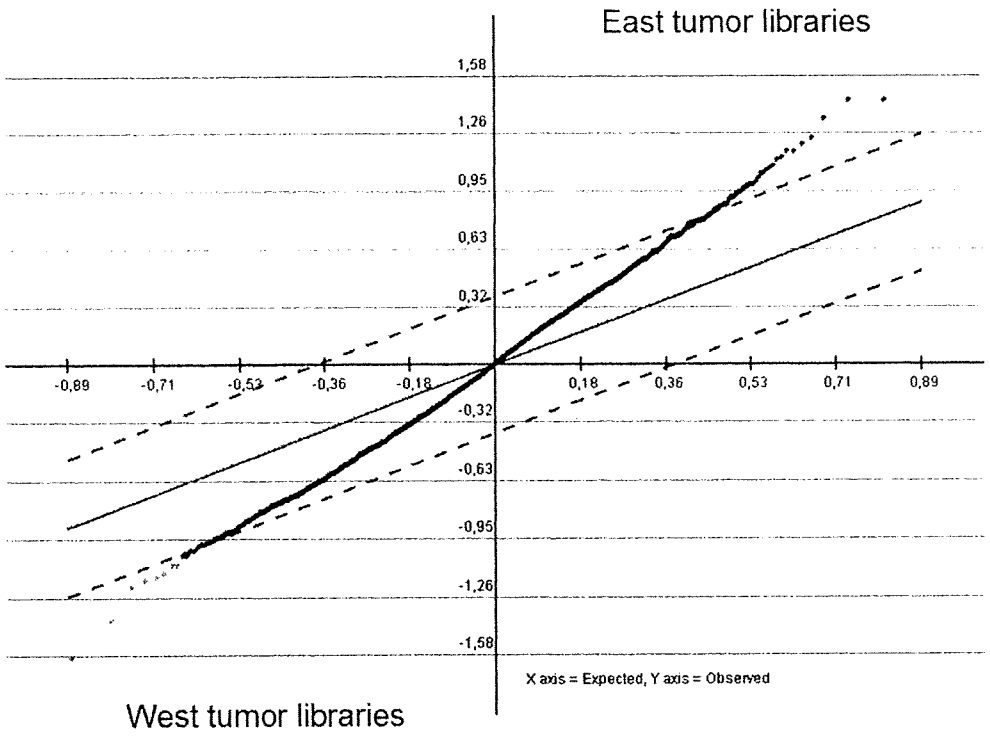

Figure 4
Serial Analysis for Microarray of East and West gastric carcinoma SAGE libraries. To the left and shown in orange color, the significant tags with higher expression in the West tumor libraries; to the right and shown in blue color, the significant tags with higher expression in the East tumor libraries.

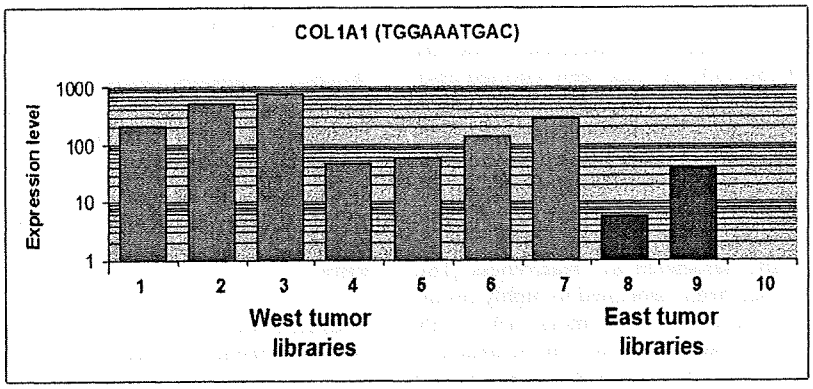

Figure 5
Expression levels of COL1A1 associated tag (TGGAAATGAC) in tumor libraries. Bars 1–7 correspond to all West tumor libraries (CGAP_MD_HG7, CGAP_MD_HS29, CGAP_MD_G329, GSM757, GSM758, GSM14760, GSM2385 and bars 8–10 correspond to all East tumor libraries (GSM7800, GSM8505, GSM8867). The tag normalized expression level appears in the CGAP format value (Tags per 200,000) plotted in a logarithmic scale.

Table 1: The significant tags with higher expression by Significant Analysis for Microarray between the West and the East tumor SAGE libraries. Only the tags that were successfully associated with a specific gene are shown. The tags are sorted in a significance descending order, first the tags highly expressed in the East and then those highly expressed in the West.

Tags	Gene Symbol	Protein Name	N° of West libraries where present	West tumor average (Tags per 200,000)	N° of East libraries where present	East tumor average (Tags per 200,000)
TGATTGGTGG	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	3	1.88	3	115.05
GGCTGGGTTT	HLX1	H2.0-like homeo box 1 (Drosophila)	2	1.04	3	59.13
TCCGTCCGGA	RPL13	Ribosomal protein L13	3	1.36	3	39.56
ATCTGGAGCA	ADH1C	Alcohol dehydrogenase 1C (class I), gamma polypeptide	3	5.99	3	294.91
TGCTCCTACC	FCGBP	Fc fragment of IgG binding protein	4	4.91	3	111.10
TACCCTGGAA	ADH4	Alcohol dehydrogenase 4 (class II), pi polypeptide	3	3.35	3	56.30
AGGTCTGCCA	AKR1C2	Aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	3	1.53	3	38.50
GCACCACCGG	MAPK13	Mitogen-activated protein kinase 13	0	0	3	10.62
GGAGGGGAGG	MECR	Mitochondrial trans-2-enoyl-CoA reductase	1	0.55	3	15.72
CTTCCTTGCC	KRT17	Keratin 17	7	220.64	0	0
TAATTTGCAT	EMP1	Epithelial membrane protein 1	7	43.26	0	0
TAAGGCTTAA	KLK10	Kallikrein 10	7	20.35	0	0
TGGAAATGAC	COL1A1	Collagen, type I, alpha 1	7	294.99	2	14.36
TGGATGTACA	CCDC12	Coiled-coil domain containing 12	7	21.69	0	0

nificantly different tags have a higher expression in the East compared with the West tumors. Thus, it seems that the average expression level of the West samples falls more than the East samples, probably because of a wider gene repression.

Of the 5 genes identified with significant higher expression in the West libraries at least two (COL1A1 and KLK10) have been associated with invasiveness and disease progression [9,15]. COL1A1 has been reported associated with more advanced tumor stage in 46 gastric carcinoma cases [9]. KLK10 has been reported up-regulated in gastric as well as colorectal carcinomas and associated with invasion and more advanced clinical stage for both types of tumors [15]. In addition KRT17 has been found up-regulated in human esophageal squamous cell carcinoma (ESCC) and associated to invasiveness [18]. Another gene, EMP1 has been associated to highly proliferative cell types in mouse brain tumors [19]. Only CCDC12 gene does not have available clinical data and also lacks GO annotations. The qRT-PCR analysis on cell lines confirmed the SAGE results and validated the overexpression of PDFGR and RPL13 in the East tumor libraries.

In summary here we report that the predominant up-regulation of invasive and metastatic genes in the West tumor libraries might result in a more malignant disease with a poorer survival. Taken together these findings might suggest that that differentially expressed genes might contribute to explain the observed differences observed in the outcome of gastric carcinoma between the East and the West. Finally, our analysis is an example of how computational biology can effectively assist biomedical researchers in identifying the molecular mechanisms of disease [6].

Authors' contributions

FJO carried out the *in silico* analysis of SAGE databases, performed the bioinformatics analysis and drafted the manuscript.

CV participated in cell cultures, RNA extraction and carried out the RealTime PCR assays and drafted the manuscript.

FA carried out the cell culture, performed RNA extractions for the RealTime PCR assays and drafted the manuscript.

ES carried out the RealTime PCR assays.

NO participated in SAGE construction and SAGE database analysis.

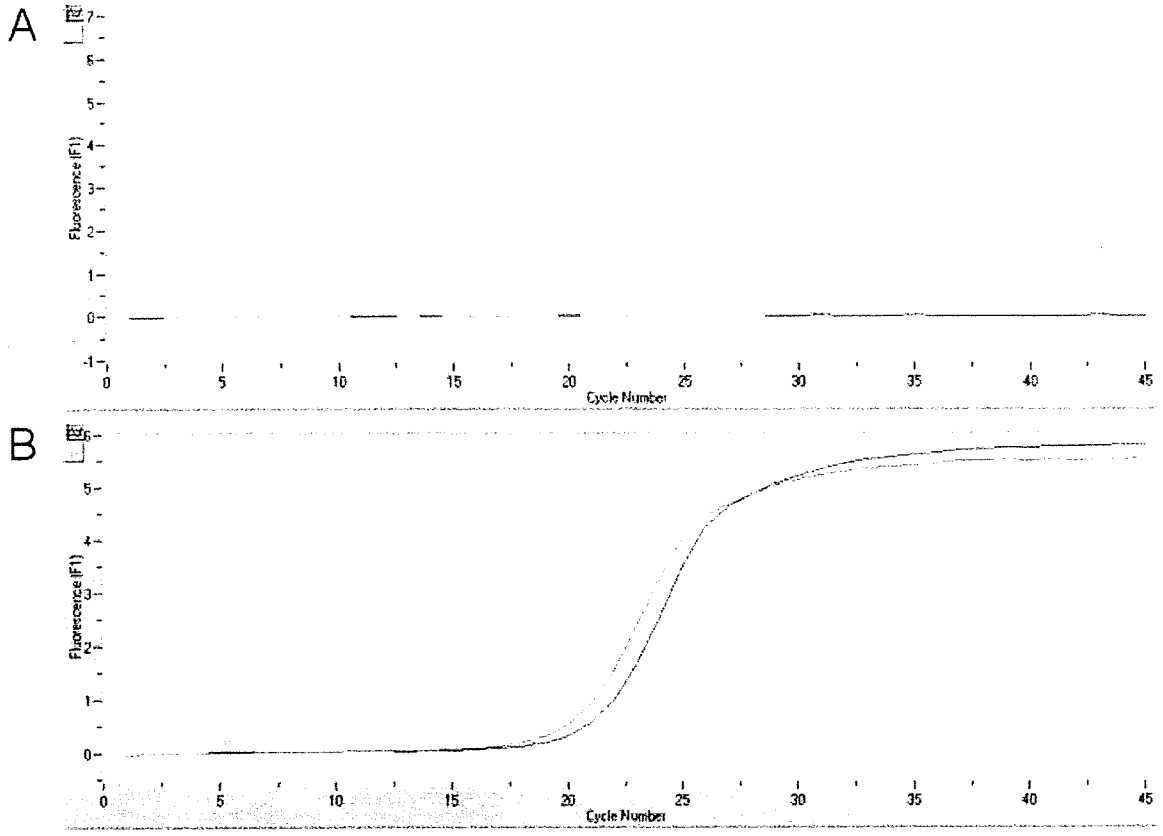

Figure 6
Amplification of PDGFRA (A) and RPL13 (B) mRNA by qRT-PCR. In (A) blue line is the East cell line (MKN45) and red line is the West cell line (N87). In (B) blue line is the East cell line (MKN45) and red line is the West cell line (AGS). Both genes are over-expressed in the East (MKN45) cell line.

WY participated in SAGE construction and SAGE database analysis and drafted the manuscript.

AHC conceived the study, participated in its design, performed the evaluation of results and drafted the manuscript.

All authors read and approved the final manuscript.

Additional material

Additional File 1

Correspondence Analysis of normal and tumor SAGE libraries of the stomach in 3 dimensions. The data provided represent the three-dimensional plot where the green dots represent all the normal libraries, the blue dots are the East tumor libraries, and the red, orange and yellow dots are West tumor libraries, microdissected, xenograft and bulk respectively. The X-axis is grey, the Y-axis is blue, and the Z-axis is pink. The figure is slightly rotated to the right and down to better show the tumor libraries position in the plot 3-D space.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1476-4598-7-22-S1.png]