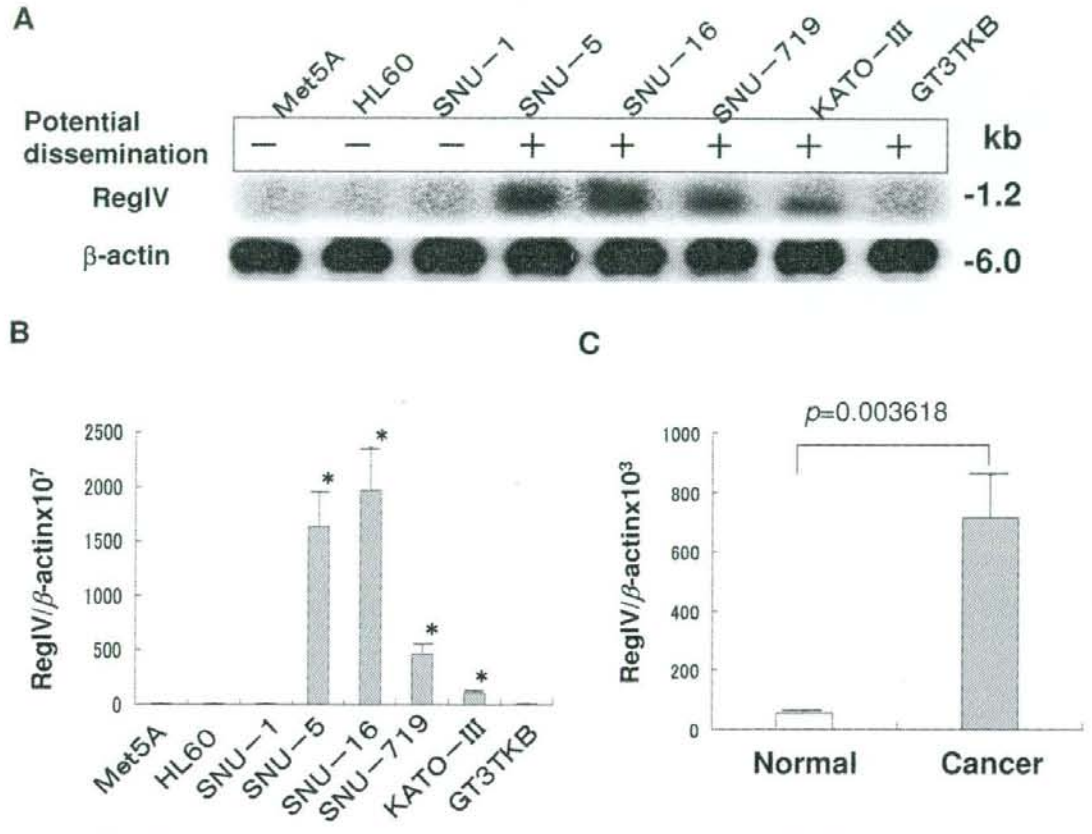

Figure 2. *RegIV mRNA expression in gastric cancer cell lines and primary gastric cancer specimens. A) RegIV mRNA expression in eight gastric cancer cell lines analyzed by Northern blot. RegIV was up-regulated in gastric cancer cells derived from malignant ascites compared to cells derived from a primary 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.

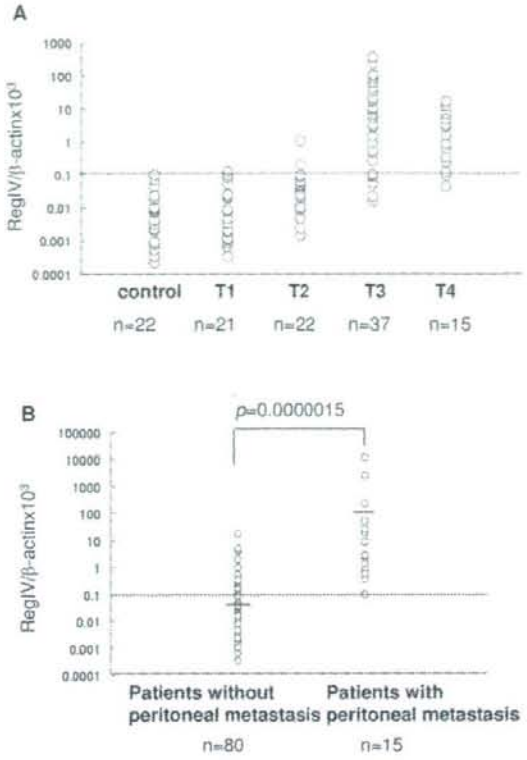

Figure 3. *RegIV/β-actin mRNA ratio in peritoneal washes from gastric cancer patients. A) RegIV/β-actin mRNA ratios in peritoneal washes from gastric cancer patients, grouped according to the depth of invasion (pT category). The broken line on the graph indicates the cut-off value for identifying RegIV+ 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*	Markers		
		Histology**	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).

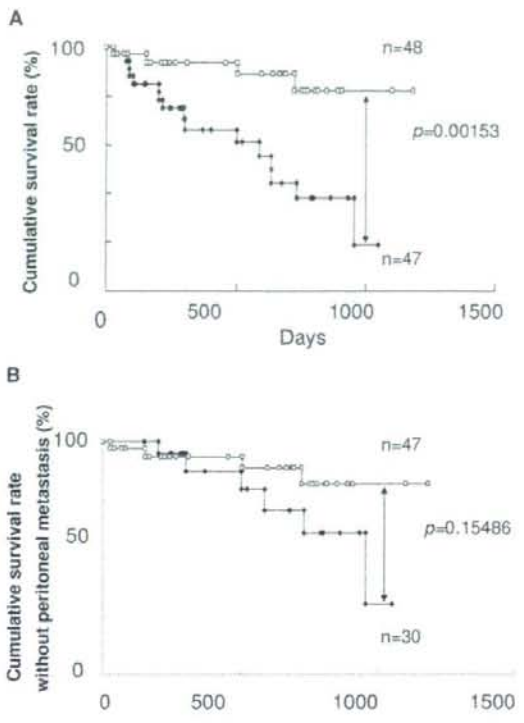

Figure 4. *Survival curves of gastric cancer patients. A) Survival curves of all 95 study patients with gastric carcinoma, grouped according to RegIV mRNA expression. RegIV+ cases (closed symbols) had a significantly worse prognosis than RegIV-negative cases (open symbols) (p=0.00153). B) Survival curves 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

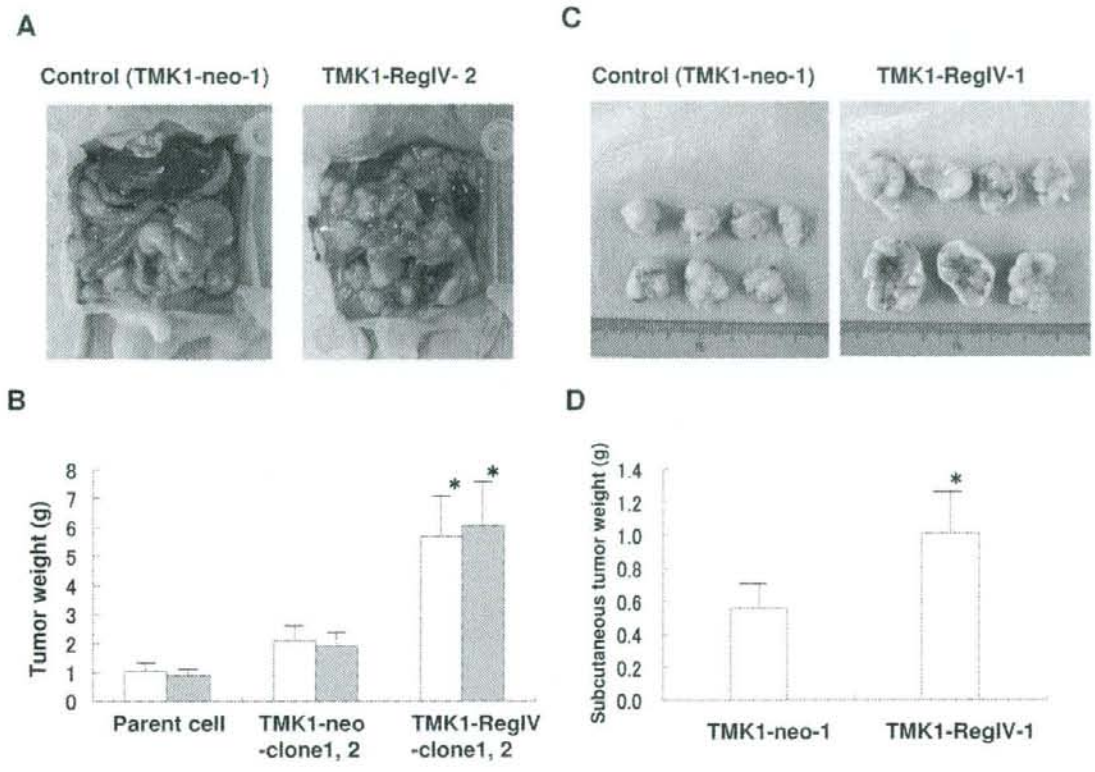

Figure 5. *Acceleration of peritoneal metastases of gastric cancer by RegIV expression. A) Macroscopic appearance of peritoneal disseminated metastatic nodules derived from TMK-1-RegIV-2 transfectants and control TMK-1 transfectants. B) Quantification of metastatic nodules in the mouse peritoneal cavity. 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

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

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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

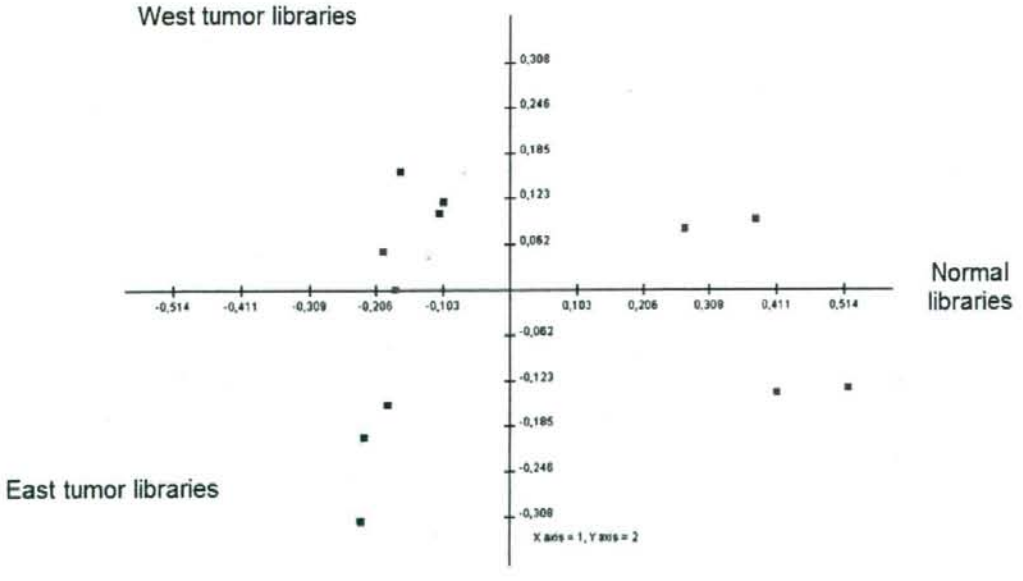

Figure 1
Correspondence Analysis of normal and tumor SAGE libraries of the stomach. A two-dimensional plot is shown where the green dots represent all the normal libraries, the blue dots are the East tumor libraries, and the red, orange and yellow dots are West tumor libraries, microdissected, xenograft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

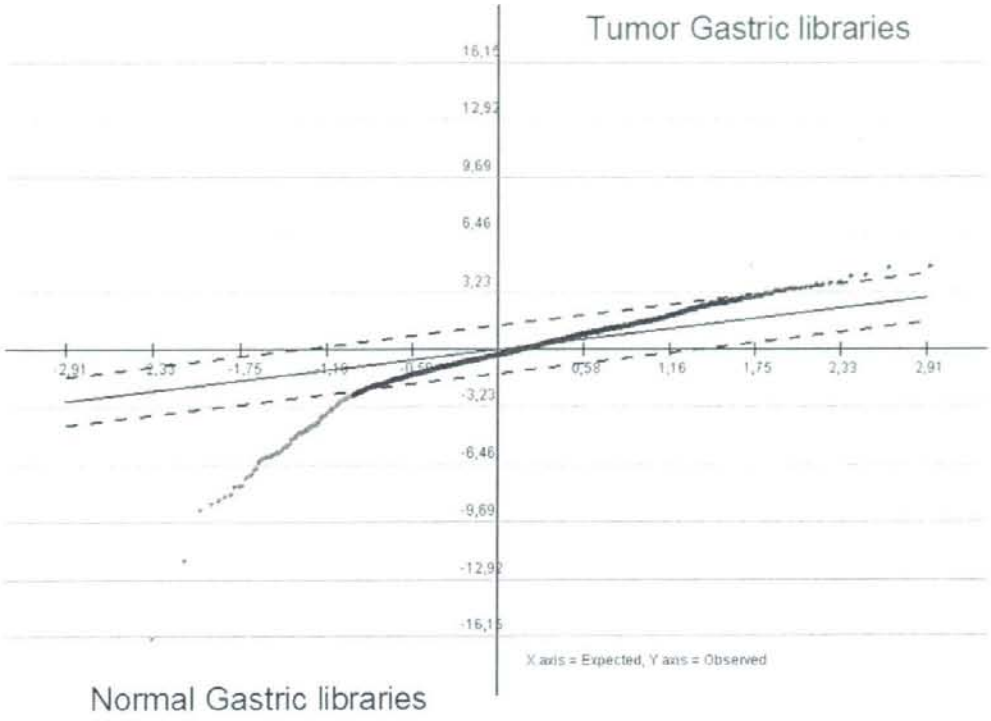

Figure 2
Serial Analysis for Microarray of normal and tumor SAGE libraries of the stomach. To the left and shown in green color, the significant tags with higher expression in the normal libraries; to the right and shown in red color, the significant tags with higher expression in the tumor libraries.

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

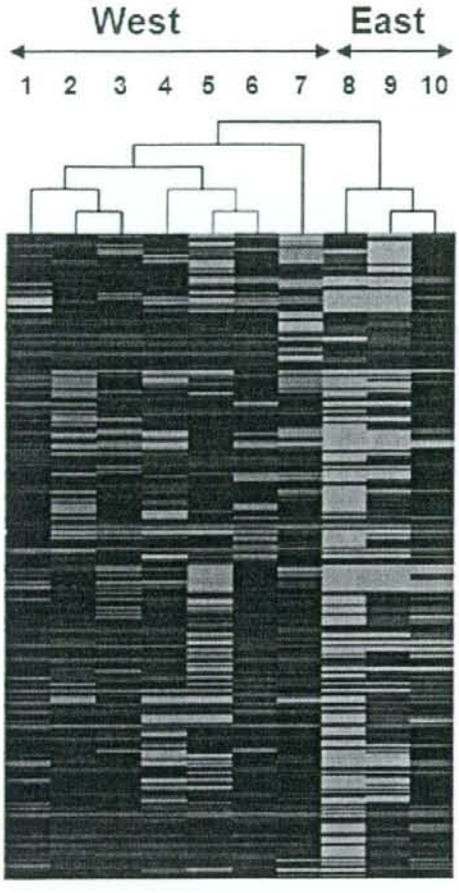

Figure 3
Support Tree of normal and tumor SAGE libraries of the stomach. Lanes 1–4 normal libraries (CGAP_MD_13S, GSM784, CGAP_MD_14S, GSM14780), lanes 5–11 West tumor libraries (CGAP_MD_HG7, CGAP_MD_HS29, CGAP_MD_G329, GSM757, GSM758, GSM14760, GSM2385) and lanes 12–14 East tumor libraries (GSM7800, 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-

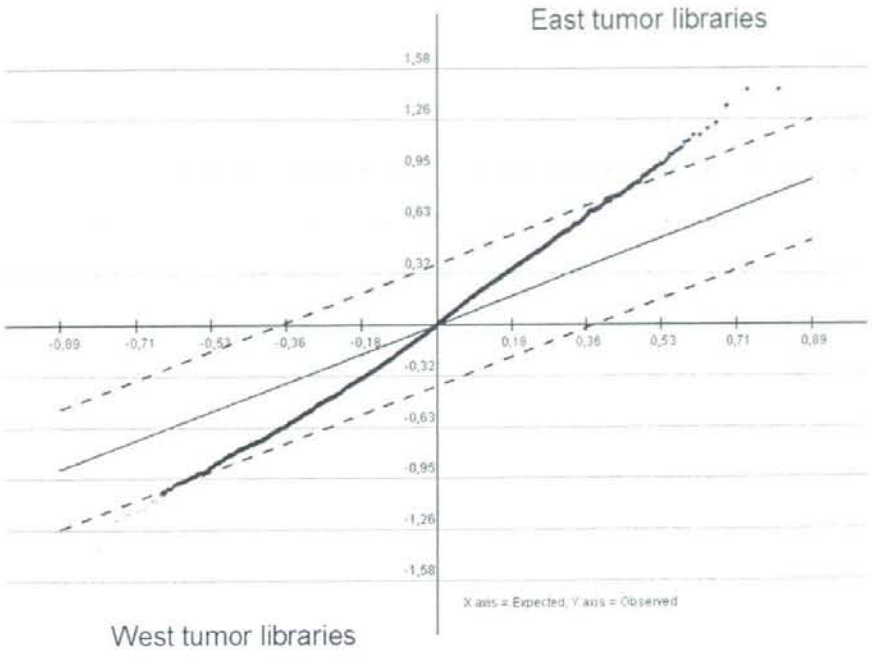

Figure 4
Serial Analysis for Microarray of East and West gastric carcinoma SAGE libraries. To the left and shown in orange color, the significant tags with higher expression in the West tumor libraries; to the right and shown in blue color, the significant tags with higher expression in the East tumor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

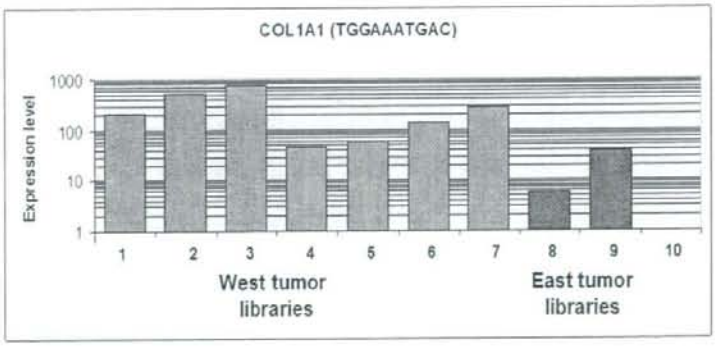

Figure 5
Expression levels of COL1A1 associated tag (TGGAAATGAC) in tumor libraries. Bars 1–7 correspond to all West tumor libraries (CGAP_MD_HG7, CGAP_MD_HS29, CGAP_MD_G329, GSM757, GSM758, GSM14760, GSM2385 and bars 8–10 correspond to all East tumor libraries (GSM7800, GSM8505, GSM8867). The tag normalized 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 over-expression 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.

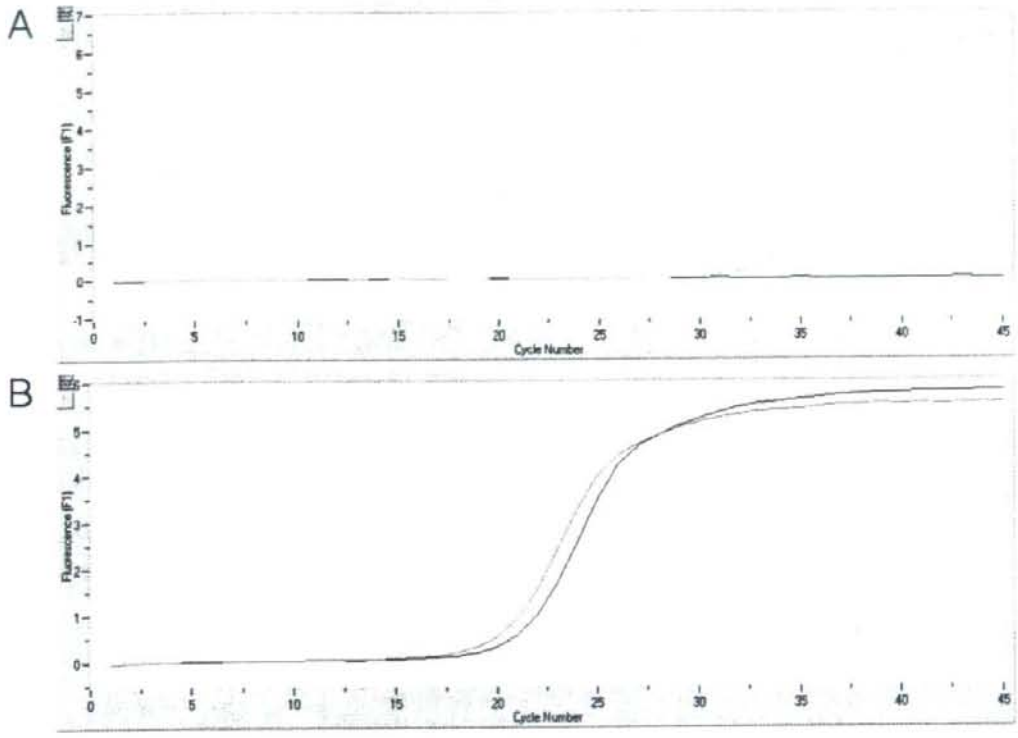

Figure 6
Amplification of PDGFRA (A) and RPL13 (B) mRNA by qRT-PCR. In (A) blue line is the East cell line (MKN45) and red line is the West cell line (N87). In (B) blue line is the East cell line (MKN45) and red line is the West cell line (AGS). Both genes are over-expressed in the East (MKN45) cell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

Additional File 2

Complete figure of Support Clustering Analysis of normal and tumor SAGE libraries of the stomach. The figure provided represent normal libraries CGAP_MD_13S, GSM784, CGAP_MD_14S, GSM14780 (lines 1–4), West tumor libraries CGAP_MD_HG7, CGAP_MD_HS29, CGAP_MD_G329, GSM757, GSM758, GSM14760, GSM2385 (lines 5–11) and East tumor libraries GSM7800, GSM8505, and GSM8867 (lanes 12–14).
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[http://www.biomedcentral.com/content/supplementary/1476-4598-7-22-S2.png]

Additional File 3

Complete figure of Support Clustering Analysis of West and East tumor SAGE libraries of the stomach. The figure provided represent West tumor libraries (CGAP_MD_HG7, CGAP_MD_HS29, CGAP_MD_G329, GSM757, GSM758, GSM14760, GSM2385) (lanes 1–7) and East tumor libraries (GSM7800, GSM8505, and GSM8867) (lanes 8–10).
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[http://www.biomedcentral.com/content/supplementary/1476-4598-7-22-S3.png]

Additional File 4

Table S1. The significant tags with higher expression in Normal by Significant Analysis for Microarray between Normal and 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.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1476-4598-7-22-S4.doc]

Additional File 5

Table S2. The significant tags with higher expression in Tumor by Significant Analysis for Microarray between Normal and 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.
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[http://www.biomedcentral.com/content/supplementary/1476-4598-7-22-S5.doc]

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Reg IV is an independent prognostic factor for relapse in patients with clinically localized prostate cancer

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Regenerating islet-derived family, member 4 (*REG4*, which encodes Reg IV) is a candidate marker for cancer and inflammatory bowel disease. We investigated the potential prognostic role of Reg IV immunostaining in clinically localized prostate cancer (PCa) after radical prostatectomy. Immunohistochemical staining of Reg IV was performed in 98 clinically localized PCa tumors obtained during curative radical prostatectomy. Intestinal and neuroendocrine differentiation was investigated by MUC2 and chromogranin A immunostaining, respectively. The prognostic significance of immunohistochemical staining for these factors on prostate-specific antigen (PSA)-associated recurrence was assessed by Kaplan–Meier analysis and a Cox regression model. Phosphorylation of the epidermal growth factor receptor (EGFR) by Reg IV was analyzed by Western blot. In total, 14 (14%) of the 98 PCa cases were positive for Reg IV staining. Reg IV positivity was observed frequently in association with MUC2 ($P = 0.0182$) and chromogranin A positivity ($P = 0.0012$). Univariate analysis revealed that Reg IV staining ($P = 0.0004$), chromogranin A staining ($P = 0.0494$), Gleason score ($P < 0.0001$) and preoperative PSA concentration ($P = 0.0167$) were significant prognostic factors for relapse-free survival. Multivariate analysis indicated that Reg IV staining ($P = 0.0312$), Gleason score ($P = 0.0014$) and preoperative PSA concentration ($P = 0.0357$) were independent predictors of relapse-free survival. In the LNCaP cell line, EGFR phosphorylation was induced by the addition of Reg IV-conditioned medium. These results suggest that Reg IV expression is an independent prognostic indicator of relapse after radical prostatectomy. (*Cancer Sci* 2008; 99: 1570–1577)

Prostate cancer (PCa) is one of the most common cancers and the second leading cause of cancer death in men in the USA.[1] PCa screening by assessing serum prostate-specific antigen (PSA) level has led to increased detection of early stage PCa that can be cured by radical prostatectomy or radiation therapy. Nonetheless, a substantial fraction of patients with clinically localized PCa who undergo curative radical prostatectomy will eventually recur with metastatic disease. These patients would benefit most from the discovery of a prognostic factor that can identify individuals for whom adjuvant therapy would be advantageous. Treatment decisions are based mainly on known prognostic factors. High risk of relapse is defined according to preoperative PSA level (>20 ng/mL), biopsy Gleason score (≥8), and the 1992 American Joint Committee on Cancer (AJCC) clinical T stage (≥T2c).[2] These factors are helpful but far from perfect due to significant clinical heterogeneity. Clearly, new biological markers are needed to accurately predict the risk of relapse.

We previously performed serial analysis of gene expression of four primary gastric cancers,[3] and identified several gastric cancer-specific genes.[4] Of these genes, regenerating islet-derived family, member 4 (*REG4*, which encodes Reg IV) is a candidate gene for cancer-specific expression, at least in patients with gastric cancer. Although various normal tissues express *REG4*, the levels of expression are much lower in normal tissues than in cancerous tissues.[3] Our previous immunohistochemical analysis revealed that Reg IV was expressed in 30% of gastric cancers and was associated with both intestinal mucin phenotype and neuroendocrine differentiation.[5] Reg IV is a secreted protein, and we also showed that serum Reg IV represents a novel biomarker for gastric cancer.[6]

Understanding of the genetic and epigenetic pathways involved in the pathogenesis of PCa is essential for the development of improved diagnostic and treatment modalities. A variety of genetic and epigenetic alterations is associated with PCa.[7,8] In addition to gastric, colorectal,[9] and pancreatic[10] cancers, overexpression of *REG4* mRNA in PCa has been reported by *in situ* hybridization.[11] The majority of localized PCa tumors expressed a low level of *REG4* mRNA, whereas the majority of metastatic PCa tumors expressed a high level of *REG4* mRNA. Although the biological function of Reg IV is poorly understood, it has been reported that Reg IV is a potent activator of the epidermal growth factor receptor (EGFR)/Akt/activator protein-1 (AP-1) signaling pathway in colon cancer cells and increases expression of Bcl-2, Bcl-xl and surviving, proteins associated with the inhibition of apoptosis.[12] We have also reported that forced expression of Reg IV induces phosphorylation of the EGFR and inhibits 5-fluorouracil-induced apoptosis in gastric cancer cells.[5] Taken together, these findings suggest that Reg IV may also participate in tumor cell growth in PCa and may be a new prognostic marker for relapse in patients with PCa. However, the expression and distribution of Reg IV protein and the biological significance of Reg IV in PCa has not been investigated.

In the present retrospective study, we examined the expression and distribution of Reg IV in 98 clinically localized PCa tumors by immunohistochemistry. The relation between staining for Reg IV and clinicopathological characteristics was also examined. We have reported two Reg IV staining patterns (mucin-like staining and perinuclear staining).[5] Mucin-like staining, observed in goblet cells and goblet cell-like vesicles of tumor cells, is associated with MUC2 (a marker of goblet cells) positivity. Perinuclear staining is detected in cells with neuroendocrine differentiation and that show chromogranin A (a marker of neuroendocrine cells) positivity. Therefore, we examined staining for Reg IV and chromogranin A or MUC2 by double-immunofluorescence. Because Reg IV activates the EGFR, we also performed an immunohistochemical analysis of Reg IV and EGFR expression.

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Materials and Methods

Tissue samples. Ninety-eight primary tumors were collected from patients diagnosed with PCa who underwent surgery during 2000–2002 at the Department of Urology, Hiroshima University Hospital (Hiroshima, Japan). All 98 patients were treated by radical prostatectomy and bilateral lymphadenectomy for clinically localized PCa and were confirmed to be node negative by pathological examination. None of the patients were treated preoperatively with hormonal or radiation therapy, and none had secondary cancer. All 98 specimens were archival, formalin-fixed, paraffin-embedded tissues. Tumor staging was performed according to the AJCC classification system. After prostatectomy, the serum PSA level was measured by *E*-test Tosoh II Assay (Tosoh, Tokyo, Japan). Patients were followed up by PSA measurement monthly during the first 6 months after prostatectomy and then every 3 months thereafter. Biochemical relapse was defined as a PSA level of 0.2 ng/mL or greater. Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Immunohistochemistry. Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized and stained with hematoxylin–eosin to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistochemically. Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 30 min for Reg IV, MUC2, chromogranin A, EGFR, phospho-EGFR (Tyr^{1068}) and transforming growth factor (TGF)-α. Peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, and sections were then incubated with normal goat serum (Dako Cytomation, Carpinteria, CA, USA) for 20 min to block nonspecific antibody binding. Sections were incubated with primary antibody against Reg IV (rabbit polyclonal antibody, diluted 1:50; anti-Reg IV antibody was raised in our laboratory),[5] MUC2 (1:50; Novocastra, Newcastle, UK), chromogranin A (1:50; Novocastra), EGFR (1:50; Dako Cytomation), phospho-EGFR (Tyr^{1068}) (1:50; Cell Signaling Technology, Beverly, MA, USA) or TGF-α (Calbiochem, San Diego, CA, USA) for 1 h at room temperature, followed by incubation with peroxidase-labeled antirabbit or antimouse IgG for 1 h. Staining was completed with a 10-min incubation in substrate–chromogen solution. The sections were counterstained with 0.1% hematoxylin. The specificity of the Reg IV antibody has been characterized in detail elsewhere.[5]

Double-immunofluorescence staining was performed as described previously.[5] Alexa Fluor 546-conjugated antirabbit immunoglobulin (Ig)G (Molecular Probes, Eugene, OR, USA) and Alexa Fluor 488-conjugated antimouse IgG (Molecular Probes) were used as secondary antibodies.

The specificity of immunohistochemical detection for Reg IV was verified by triple-immunofluorescence staining with different antibodies against Reg IV (goat polyclonal, rabbit polyclonal and mouse monoclonal). Goat polyclonal and mouse monoclonal anti-Reg IV antibodies were purchased from R&D Systems (Abingdon, UK). Alexa Fluor 405-conjugated antigoat IgG (Molecular Probes), Alexa Fluor 488-conjugated antimouse IgG (Molecular Probes) and Alexa Fluor 546-conjugated antirabbit IgG (Molecular Probes) were used as secondary antibodies.

Cell culture and conditioned medium production. Colon cancer cell line, colo320, and human prostate cell line, LNCaP, were maintained in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Whittaker, Walkersville, MD, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Reg IV-conditioned medium (Reg IV-CM) and colo320 control medium were prepared as follows. Colo320 cells stably transfected with a Reg IV cDNA were grown to 80–90% confluence in RPMI-1640 medium containing 10% FBS. The medium was removed and the cells were washed twice with phosphate-buffered saline (PBS). Cells were incubated for 48 h in 20 mL serum-free RPMI-1640 medium. After 48 h, the medium was collected and filtered (0.22-μm pores; Becton Dickinson Labware, Bedford, MA, USA). Control medium from colo320 cells stably transfected with the pcDNA3.1 vector alone was prepared under the same conditions. Conditioned medium was then normalized for DNA content between samples by adding RPMI-1640 medium. Levels of EGF and TGF-α in Reg IV-CM and control medium were assessed by sandwich-type enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

Western blot analysis. Western blot analysis was performed as described previously.[13] To examine whether Reg IV activates phosphorylation of the EGFR, cells were serum starved for 24 h and treated with Reg IV-CM or control medium for 3 min. To examine whether EGF or TGF-α induce Reg IV expression, cells were serum starved for 24 h and treated with EGF (100 nM, Sigma, Saint Louis, MO, USA) or TGF-α (10 nM, Sigma) for 1, 2 and 3 days. Cells were scraped in PBS supplemented with 1 mM Na_3VO_4, centrifuged, and lysed in ice-cold RIPA buffer (20 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM ethyleneglycotetraacetic acid [EGTA], 1 μg/mL leupeptin, 2 μg/mL aprotinin and 10 μg/mL pepstatin). Supernatant protein concentration was measured with a the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). Protein (20 μg/lane) was electrophoresed on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose filters. Filters were incubated for 1 h at room temperature with anti-Reg IV antibody (rabbit polyclonal antibody raised in our laboratory),[5] anti-EGFR antibody (Cell Signaling Technology), antiphospho-EGFR (Tyr^{992}) antibody (Cell Signaling Technology) or antiphospho-EGFR (Tyr^{1068}) antibody (Cell Signaling Technology).

Statistical methods. Association between clinicopathological variables and Reg IV expression was analyzed by Fisher's exact test. Kaplan–Meier survival curves were constructed for Reg IV-positive and Reg IV-negative patients. Survival rates were compared between Reg IV-positive and Reg IV-negative groups. Differences in survival between groups were tested for statistical significance by log–rank test.[14] The Cox proportional hazards multivariate model was used to examine the association of clinical and pathological factors and Reg IV and chromogranin A staining with survival. $P < 0.05$ was considered statistically significant.

Results

Immunohistochemical analysis of Reg IV in PCa tissues. We performed an immunohistochemical analysis of Reg IV expression in 98 clinically localized PCa cases. In adjacent non-neoplastic prostate tissue, focal Reg IV staining was found in five (5%) of 98 cases. Periodic luminal epithelial cells stained for Reg IV, but stromal cells showed no staining for Reg IV (Fig. 1a). We confirmed that Reg IV-positive cells were not stained by 34βE12 (a marker of basal cells) (Fig. 1b). Although the specificity of the Reg IV antibody has been characterized in detail,[5] the specificity of immunohistochemical detection for Reg IV was further verified by triple-immunofluorescence staining with different antibodies against Reg IV (goat polyclonal, rabbit polyclonal and mouse monoclonal). Cells stained with anti-Reg IV goat polyclonal antibody also stained with anti-Reg IV rabbit polyclonal antibody and anti-Reg IV mouse monoclonal antibody, indicating that these anti-Reg IV antibodies specifically

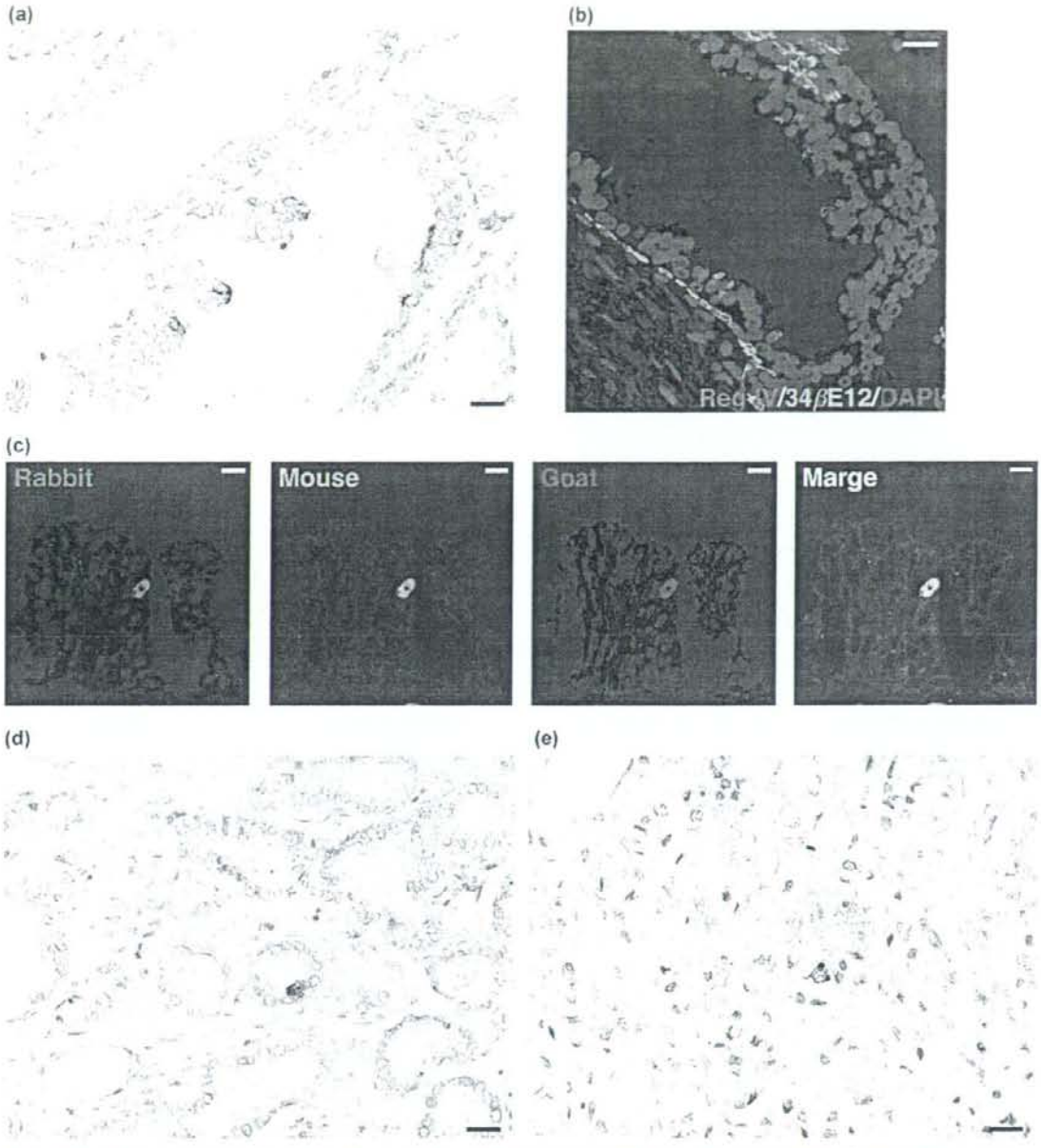

Fig. 1. Immunohistochemical analysis of Reg IV expression in clinically localized prostate cancer. (a) Immunostaining of Reg IV in adjacent non-neoplastic prostate tissue. Several luminal epithelial cells show Reg IV staining. Scale line, 25 μm. (b) Double-immunostaining of Reg IV (red) and 34βE12 (a marker for basal cells; green). Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale line, 25 μm. (c) Triple-immunostaining of rabbit polyclonal anti-Reg IV (red), mouse monoclonal anti-Reg IV (green), and goat polyclonal anti-Reg IV (blue). Scale line, 13 μm. (d) Immunostaining of Reg IV in prostate cancer (PCa). Mucin-like staining of Reg IV is present in goblet cell-like vesicles of tumor cells. Scale line, 25 μm. (e) Immunostaining of Reg IV in PCa. Perinuclear staining of Reg IV is present in tumor cells. Scale line, 25 μm.

recognize Reg IV protein (Fig. 1c). In PCa tissues, Reg IV staining was observed in 14 (14%) of 98 PCa cases. In all 14 PCa cases, few tumor cells (1–10%) showed Reg IV staining. Stromal cells showed no Reg IV staining. Reg IV staining was considered positive if any tumor cells were stained. In each Reg IV-positive case, both mucin-like staining (Fig. 1d) and perinuclear staining (Fig. 1e) were observed. We analyzed relations between Reg IV staining and clinicopathological

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