However, the sensitivity of this method is known to be low, especially for low-grade UC. Therefore, a more sensitive, non-invasive method for cancer detection is required.

We previously performed serial analysis of gene expression (SAGE) of four primary gastric cancers (6) and identified several gastric cancer-specific genes (7). 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. REG4 is a member of the REG gene family, which includes three other genes, and was originally identified by high-throughput sequence analysis of a large inflammatory bowel disease cDNA library (8). Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that ~50% of gastric cancers overexpress the REG4 gene (6) and Reg IV expression is associated with the intestinal and neuroendocrine differentiation (9). In addition to gastric cancer, overexpression of Reg IV in colorectal cancer (10), pancreatic cancer (11) and PCa (12) has been reported. In our previous immunohistochemical analysis (13), 14% of the PCa cases were positive for Reg IV and Reg IV positivity was associated with intestinal and neuroendocrine differentiation. Furthermore, Reg IV expression is an independent prognostic indicator of relapse after radical prostatectomy. However, histologically, gastric, colorectal, pancreatic and prostate cancers are adenocarcinomas, and expression of Reg IV except for adenocarcinomas, such as RCC or UC, has not yet been investigated.

Reg IV is a secreted protein which we have previously shown represents a novel biomarker for gastric cancer (14). The diagnostic sensitivity of serum Reg IV was superior to that of serum carcinoembryonic antigen or carbohydrate antigen 19-9. Serum Reg IV serves as a tumor marker for colorectal and pancreatic cancer (10,11). These data support the idea that Reg IV protein has potential as a novel serum tumor marker for a wide variety of malignancies; however, serum concentration of Reg IV in major urologic cancers including PCa, RCC or UC has not previously been measured.

In the present study, we examined the expression and distribution of Reg IV in human RCC and UC by immunohistochemistry. We have reported two Reg IV staining patterns (mucin-like and perinuclear staining) (9). Mucin-like Reg IV staining, observed in goblet cells and goblet cell-like vesicles of tumor cells, is associated with MUC2 (a goblet cell marker) positivity. Perinuclear Reg IV staining is detected in cells with neuroendocrine differentiation. Therefore, we also performed immunohistochemical analysis of MUC2, chromogranin A (a neuroendocrine cell marker) and synaptophysin (a neuroendocrine cell marker). Since Reg IV expression was frequently found in PCa, we also measured Reg IV levels in sera from patients with PCa by enzyme-linked immunosorbent assay (ELISA) to investigate the potential diagnostic utility of Reg IV measurement.

Materials and methods

Tissue samples. In total, 204 primary tumor samples and 78 serum samples were collected. Patients were treated at the Hiroshima University Hospital or an affiliated hospital.

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 196 patients who had undergone surgical excision for either RCC (n=101) or UC (n=95). All 101 patients with RCC were treated by radical nephrectomy, and all 95 patients with UC were treated by cystectomy. Tumor staging was performed according to the TNM classification system (15).

For Western blot analysis, 8 RCC samples and the corresponding non-neoplastic kidney samples were used. The samples were obtained during surgery at Hiroshima University Hospital or an affiliated hospital. We confirmed microscopically that the tumor specimens were predominantly RCC tissue (>80%). Samples were frozen immediately in liquid nitrogen and stored at -80°C until use.

All serum samples were collected before prostate biopsy and stored at -80°C. Seventy-eight consecutive men who visited the outpatient clinic of the Department of Urology, Hiroshima University Hospital due to elevated PSA levels (>4.0 ng/ml) served as the study population. The PCa population consisted of 38 men with evidence of cancer from a prostate biopsy (age range 50-93 years, mean 73 years), and the remaining 40 men without evidence of cancer from a prostate biopsy served as a control population (age range 47-90 years, mean 66 years). In accordance with the Ethics Guidelines for Human Genome/Gene Research enacted by the Japanese Government, tissue specimens were collected and used on the basis of approval from the Ethics Review Committee of the Hiroshima University School of Medicine and from the ethics review committees of collaborating organizations.

Immunohistochemistry. Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then immunohistochemically stained. Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 30 min for Reg IV, MUC2, chromogranin A and synaptophysin. 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 non-specific antibody binding. Sections were incubated with a primary antibody against Reg IV (rabbit polyclonal antibody, diluted 1:50; anti-Reg IV antibody was raised and characterized in our laboratory) (9), MUC2 (1:50; Novocastra, Newcastle, UK), chromogranin A (1:50; Novocastra), or synaptophysin (1:50; Dako Cytomation) for 1 h at room temperature, followed by incubation with peroxidase-labeled anti-rabbit or anti-mouse IgG for 1 h. Staining was completed with a 10-min incubation in a substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. The specificity of the Reg IV antibody has been characterized in detail (9). Staining of each antibody was considered positive if any tumor cells were stained. Intestinal differentiation was defined as positive staining for MUC2. Neuroendocrine differentiation was defined as positive staining for chromogranin A and/or synaptophysin.

Western blot analysis. Preparation of whole cell lysates from the RCC samples and Western blotting were performed as previously described (16). Protein concentrations were determined by Bradford protein assay (Bio-Rad, Richmond,

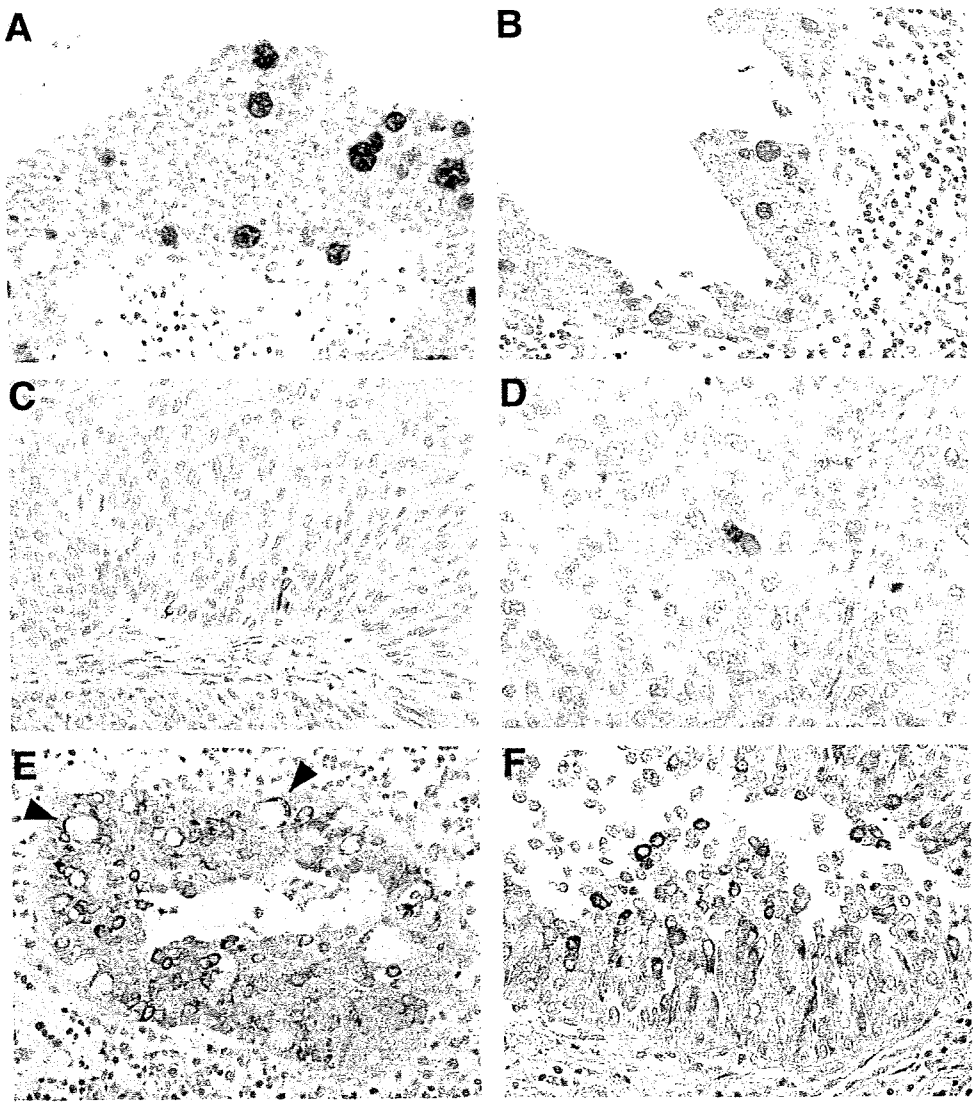


Figure 1. Immunohistochemical analysis of Reg IV expression in UC of the urinary bladder. (A) Immunostaining of Reg IV in UC. In case 37, mucin-like staining of Reg IV is present in goblet cell-like vesicles of tumor cells. (B) In case 37, PAS staining was observed in gland-like lumina. (C) Immunostaining of chromogranin A in UC. (D) Immunostaining of synaptophysin in UC. (E) Immunostaining of MUC2 in UC. In case 37, mucin-like and perinuclear staining are observed in tumor cells. Arrowhead indicates mucin-like staining of MUC2. (F) Immunostaining of MUC2 in UC. Perinuclear staining of MUC2 is present in tumor cells.

CA, USA) with BSA used as the standard. Protein (20 μg per lane) was electrophoresed on 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). Peroxidase conjugated anti-rabbit IgG was used in the secondary reaction. The immunocomplexes were visualized with an ECL Western blot detection system (Amersham Biosciences, Piscataway, NJ, USA). The quality and amounts of proteins on the gel were confirmed by detection with anti-ß-actin antibody (Sigma-Aldrich, St. Louis, MO, USA).

ELISA. For measurement of the serum Reg IV concentration, a sandwich ELISA method was developed as previously described (14). First, polystyrene microtiter plates were coated with mouse monoclonal anti-Reg IV antibody (R&D Systems, Abingdon, UK) by overnight incubation of 50 μl/125 ng per well of antibody diluted in Tris buffer (pH 7.4). The plates were then washed 3 times with wash buffer. After the plates were blocked with 1% milk in phosphate-buffered saline, 50 μl of recombinant Reg IV standard or sample was added to each well and incubated overnight at 4°C. After 3 washes, 50 μl of biotinylated goat polyclonal anti-Reg IV antibody (R&D Systems) in assay buffer [1% BSA, Tris buffer (pH 7.4) and 0.05% normal goat serum] was added to each well (75 ng antibody per well). The mixture was then incubated for 1 h with shaking at 37°C and washed 3 times with wash buffer. The plates were incubated with 50 μl per well alkaline phosphatase-conjugated streptavidin (Dako) diluted 1:2000 in diluent containing 1% BSA and Tris buffer (pH 7.4) for 1 h at 37°C and washed 3 times. Color development was performed with the addition of pNPP chromogenic substrate (Sigma-Aldrich) followed by incubation at 37°C for 1 h. Absorbance at 405 nm was measured with an ELISA

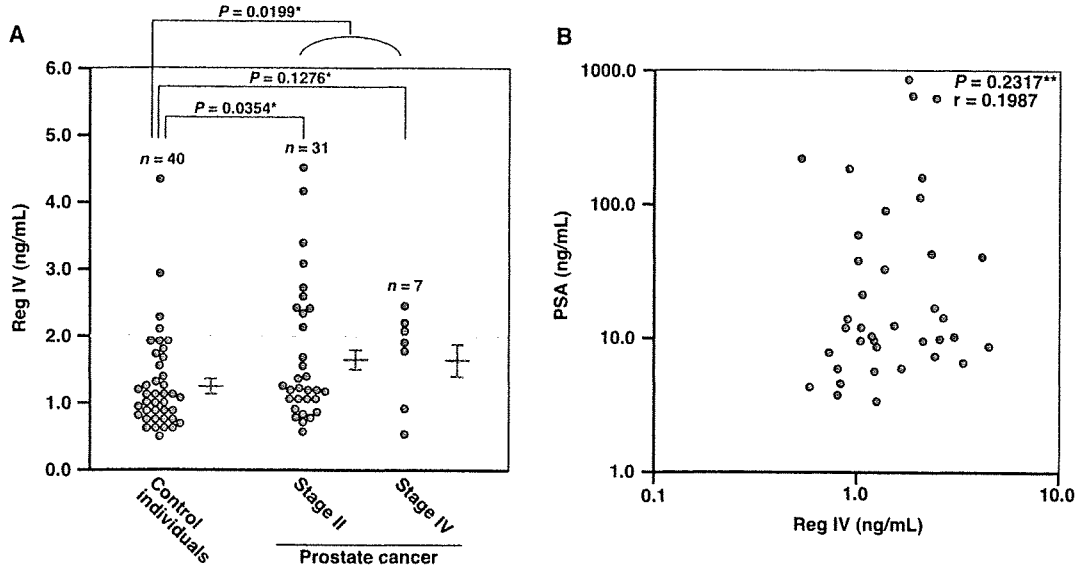


Figure 2. ELISA of serum samples from 40 control individuals and 38 patients with PCa. (A) Detection of Reg IV protein in serum samples by ELISA. Yellow bars indicate the cut-off levels defined on the basis of the previous study [2.00 ng/ml (14)]. Red bars indicate the mean ± SE. Differences in the serum concentration of Reg IV between two groups are tested by Mann-Whitney U test (•). (B) Relation between serum concentrations of Reg IV and PSA. Correlation is examined using Spearman's rank correlation (••).

plate reader. As a reference standard, known concentrations of human recombinant Reg IV (9) from 0 to 30 ng/ml were tested in triplicate.

Statistical methods. Association between clinicopathologic variables and Reg IV expression was analyzed by Fisher's exact test. Differences in the serum Reg IV concentration between the two groups were tested by the non-parametric Mann-Whitney U test. Correlations between the serum Reg IV concentration and the serum concentration of PSA were assessed by Spearman's rank correlation test. $P<0.05$ was considered statistically significant.

Results

Expression of Reg IV and neuroendocrine and intestinal differentiation in RCC. We performed immunohistochemical analysis of Reg IV in 101 RCC samples; however, no staining of Reg IV was found. In adjacent non-neoplastic kidney tissues, no Reg IV staining was found. Western blot analysis of Reg IV was also performed in 8 RCC samples (data not shown), in which no Reg IV expression was confirmed in the RCC samples nor in adjacent non-neoplastic kidney samples. We also performed immunohistochemical analysis of chromogranin A, synaptophysin and MUC2; however, no staining was detected.

Expression of Reg IV and neuroendocrine and intestinal differentiation in UC. We performed immunohistochemical analysis of Reg IV in 95 UC samples. In adjacent non-neoplastic urinary bladder tissues, no Reg IV staining was found. In UC tissues, Reg IV staining was observed in 1 (1%) of 95 UC cases. In this Reg IV-positive UC case (case 37), Reg IV-positive tumor cells were observed in 10% of tumor cells, and mucin-like staining (Fig. 1A) was observed. In case 37, Reg IV staining was observed in gland like lumina, and periodic acid-Schiff (PAS) staining was also observed in gland-like lumina (Fig. 1B), indicating that this Reg IV-positive case represents glandular differentiation. Analysis of the relationship between Reg IV staining and clinicopathologic characteristics showed that Reg IV did not correlate with gender, age, pT category, pN category or tumor stage (data not shown).

Immunostaining of chromogranin A and synaptophysin was also performed. Representative results of chromogranin A and synaptophysin immunostaining in UC are shown in Fig. 1C and D, respectively. Of the 95 UC cases, chromo-granin A-positive cases were observed in 3 cases (3%) and synaptophysin-positive cases were found in 11 cases (12%). In total, UC cases showing neuroendocrine differentiation were found in 13 cases (14%). However, case 37 (Reg IV-positive UC case) did not show neuroendocrine differentiation. Analysis of the relationship between neuroendocrine differentiation and clinicopathologic characteristics showed no correlation between neuroendocrine differentiation and gender, age, pT category, pN category or tumor stage (data not shown).

In UC tissues, MUC2 staining was observed in goblet cell-like vesicles (Fig. 1E) and perinuclear regions (Fig. 1F) of tumor cells. Of the 95 UC cases, MUC2-positive cases were observed in 33 cases (35%). Among the 33 UC cases showing positive results for MUC2, one case, case 37 (Reg IV-positive UC case), showed MUC2 staining in goblet cell-like vesicles and perinuclear regions. The remaining 32 cases showed only perinuclear MUC2 staining. Analysis of the relationship between MUC2 positivity and clinicopathologic characteristics showed no correlation between MUC2-positivity and gender, age, pT category, pN category or tumor stage (data not shown).

Serum Reg IV concentration in patients with PCa. As Reg IV expression was frequently found in PCa, we also measured

Reg IV levels in sera from patients with PCa by ELISA to investigate the potential diagnostic utility of Reg IV measurement. Serum Reg IV levels in 40 control individuals and 38 patients with PCa prior to biopsy are shown in Fig. 2A. The serum Reg IV concentration in PCa patients (n=38, 1.69±0.16 ng/ml, mean ± SE) was significantly higher than that in control individuals (n=40, 1.28±0.11 ng/ml, P=0.0199, Mann-Whitney U test), even at stage II (n=31, 1.69±0.18 ng/ml, P=0.0354, Mann-Whitney U test) (Fig. 2A). The mean of serum Reg IV concentration in PCa patients at stage IV (n=7, 1.68±0.26 ng/ml) was higher than that in control individuals (n=40, 1.28±0.11 ng/ml); however, statistical difference was not found (P=0.1276, Mann-Whitney U test). The Reg IV concentration in serum samples from patients with PCa showing a Gleason score of ≤7 (n=22, 1.75±0.21 ng/ml) was not significantly different from those showing a Gleason score of ≥8 (n=16, 1.60±0.24 ng/ml) (P=0.7448, Mann-Whitney U test). In our previous study, the cut-off level for Reg IV was set at 2.00 ng/ml (14). In the group showing serum PSA levels of >4 ng/ml, the sensitivity and specificity for detection of PCa were 34 (13/38) and 90% (36/40), respectively. Spearman's rank correlation test revealed that significant correlation was not found between serum Reg IV and PSA levels (r=0.1987, P=0.2317) (Fig. 2B).

Discussion

Previously, we showed that 14% of the PCa cases were positive for Reg IV, and Reg IV positivity was associated with intestinal and neuroendocrine differentiation (13). These PCa cases were all adenocarcinomas. In the present study, immunohistochemical analysis of Reg IV was performed in other major urologic cancers including RCC and UC, both of which are not adenocarcinomas. Immunohistochemical analysis of Reg IV revealed that Reg IV expression was not found in RCC, and only 1% of UC expressed Reg IV. These results and our previous immunohistochemical analysis of Reg IV in PCa indicate that Reg IV is expressed frequently in PCa among major urologic cancers. Furthermore, we showed that the serum Reg IV concentration in PCa patients was significantly higher than that in control individuals. The sensitivity of serum Reg IV concentration was 34%, and the specificity was 90%. It has been reported that the specificity of the PSA test is only 20% at a sensitivity of 80% (17), indicating that serum Reg IV concentration serve as a diagnostic tumor marker with high specificity. Since serum Reg IV concentration was measured in the group showing serum PSA levels of >4 ng/ml, the sensitivity and specificity calculated in the present study may differ from those in healthy individuals, and serum concentration of Reg IV should also be measured in the group showing serum PSA levels of <4 ng/ml.

In urologic cancers including PCa, RCC and UC, urine represents a particularly useful fluid in which to examine tumor markers because of its enhanced potential to contain higher concentrations of directly released tumor-derived products and the fact that collection is non-invasive. In the present study, Reg IV was detected in serum samples from patients with PCa. Reg IV expression was not found in RCC, and only 1% of UC expressed Reg IV. In adjacent non-neoplastic kidney and urinary bladder tissues, no Reg IV staining was found. These results suggest that Reg IV concentration in urine may represent a novel marker for PCa at high specificity.

In RCC, no Reg IV expression was detected. Although neuron-specific enolase (a neuroendocrine cell marker) is frequently expressed in RCC, chromogranin A expression is rare (18). It has been reported that staining of MUC2 is not found in 16 RCC cases (19). In the present study, we also observed that RCC showed neither neuroendocrine differentiation based on chromogranin A and synaptophysin staining nor MUC2 staining. As Reg IV expression is associated with neuroendocrine and intestinal differentiation, these results are accordance with immunonegativity of Reg IV in RCC.

In UC, only 1 (1%) UC case showed Reg IV staining. In the Reg IV-positive UC case, Reg IV staining was observed in gland-like lumina. Although this case was UC, PAS staining was also observed in gland-like lumina, indicating that this Reg IV-positive case possessed glandular differentiation. These results indicate that conventional UC is negative for Reg IV; however, UC with glandular differentiation may express Reg IV. A previous immunohistochemical study demonstrated that expression of chromogranin A is seen in 63.5% of UC cases (20). Yet, in the present study, only 14% of UC cases showed neuroendocrine differentiation and did not exhibit Reg IV expression. However, in gastric cancer, chromogranin A-positive tumor cells are not always positive for Reg IV (9). Furthermore, the one UC case with glandular differentiation was positive for MUC2 in goblet cell-like vesicles. Yet, Reg IV staining was not found in UC cases showing perinuclear MUC2 staining, nor was glandular differentiation observed in these cases. Based on this evidence, Reg IV expression is not likely involved in neuroendocrine differentiation of UC but is likely associated with glandular differentiation.

In conclusion, we showed that serum Reg IV concentration serves as a diagnostic tumor marker with high specificity for PCa. As the significantly high false-positive rate of PSA has lead to a tremendous increase in the number of unnecessary biopsies of the prostate (3), measurement of serum concentration of Reg IV may decrease the number of unnecessary prostate biopsies. Reg IV is correlated with relapse-free survival of patients with PCa (13), and hormone-refractory PCa has been reported to express high levels of REG4 mRNA (12). Therefore, serum concentration of Reg IV may predict relapse-free survival and resistance to androgen-deprivation therapy.

Acknowledgements

We thank Mrs Emiko Hisamoto for excellent technical assistance and advice. This work was supported, in part, by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan and from the Ministry of Health, Labor and Welfare of Japan. This work was carried out with the kind cooperation of the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. We thank the Analysis Center of Life Science, Hiroshima University, for the use of their facilities.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ: Cancer statistics. CA Cancer J Clin 57: 43-66, 2007.
2. Carter HB: Prostate cancers in men with low PSA levels - must we find them? N Engl J Med 350: 2292-2294, 2004.
3. Gambert SR: Prostate cancer. When to offer screening in the primary care setting. Geriatrics 56: 22-26, 2001.
4. Parkin DM, Bray F, Ferlay J and Pisani P: Estimating the world cancer burden: Globocan 2000. Int J Cancer 94: 153-156, 2001.
5. Pantuck AJ, Zisman A, Rauch MK and Belldegrun A: Incidental renal tumors. Urology 56: 190-196, 2000.
6. 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 64: 2397-2405, 2004.
7. Aung PP, Oue N, Mitani Y, *et al*: Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer. Oncogene 25: 2546-2557, 2006.
8. 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.
9. 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 207: 185-198, 2005.
10. Oue N, Kuniyasu H, Noguchi T, *et al*: Serum concentration of Reg IV in patients with colorectal cancer: overexpression and high serum levels of Reg IV are associated with liver metastasis. Oncology 72: 371-380, 2007.
11. Takehara A, Eguchi H, Ohigashi H, *et al*: Novel tumor marker REG4 detected in serum of patients with resectable pancreatic cancer and feasibility for antibody therapy targeting REG4. Cancer Sci 97: 1191-1197, 2006.
12. Gu Z, Rubin MA, Yang Y, *et al*: Reg IV: a promising marker of hormone refractory metastatic prostate cancer. Clin Cancer Res 11: 2237-2243, 2005.
13. Ohara S, Oue N, Matsubara A, *et al*: Reg IV is an independent prognostic factor for relapse in patients with clinically localized prostate cancer. Cancer Sci (In press).
14. 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 26: 4383-4393, 2007.
15. Sobin LH and Wittekind CH (eds): TNM Classification of Malignant Tumors, 6th edition. Wiley-Liss, New York, pp184-202, 2002.
16. Yasui W, Ayhan A, Kitadai Y, *et al*: Increased expression of p34cdc2 and its kinase activity in human gastric and colonic carcinomas. Int J Cancer 53: 36-41, 1993.
17. Catalona WJ, Hudson MA, Scardino PT, *et al*: Selection of optimal prostate specific antigen cutoffs for early detection of prostate cancer: receiver operating characteristic curves. J Urol 152: 2037-2042, 1994.
18. Rasmuson T, Grankvist K, Roos G and Ljungberg B: Neuro-endocrine differentiation in renal cell carcinoma-evaluation of chromogranin A and neuron-specific enolase. Acta Oncol 38: 623-628, 1999.
19. Lau SK, Weiss LM and Chu PG: Differential expression of MUC1, MUC2, and MUC5AC in carcinomas of various sites: an immunohistochemical study. Am J Clin Pathol 122: 61-69, 2004.
20. Mai KT, Perkins DG, Parks W, Rippstein P and Farmer J: Unusual immunostaining pattern of chromogranin in normal urothelium and in transitional cell neoplasms. Acta Histochem 96: 303-308, 1994.

Cell Prolif. 2009, **42**, 110–121 doi: 10.1111/j.1365-2184.2008.00577.x

Reg IV enhances peritoneal metastasis in gastric carcinomas

H. Kuniyasu*, N. Oue†, T. Sasahira*, L. Yi*, Y. Moriwaka*, T. Shimomoto*, K. Fujii*, H. Ohmori* and W. Yasui†

**Department of Molecular Pathology, Nara Medical University School of Medicine, Kashihara, Japan, †Department of Molecular Pathology, Hiroshima University Graduate School, Hiroshima, Japan*

Received 22 *April* 2008; *revision accepted* 4 *May* 2008

Abstract

Objectives: The role of Regenerating (Reg) IV on peritoneal metastasis was examined in gastric cancer using.

Material and methods: *Reg IV*-transfected human gastric cancer cells (MKN28-R1, MKN28-R2, TMK1-R1), control transfectants (MKN28-R0, TMK1-R0), and *REG4*-knocked down MKN45 cells were examined in *in vitro* and in nude mice peritoneal metastasis models.

Results and Discussion: Increase of expression and secretion of Reg IV, and levels of BCL-2, BCL-XL, survivin, phosphorylated AKT, and phosphorylated EGFR, and decrease of nitric oxide-induced apoptosis were found in *Reg IV*-transfectants, whereas those were abrogated in the knockdown cells. In mice models, increased number and size of peritoneal tumors and decreased apoptosis were found in *Reg IV*-transfectants, whereas those were abrogated by the knockdown cells. Mice survivals were worsened in *Reg IV*-transfectants-inoculated mice, but were improved in *Reg IV*-knockdown cell-inoculated mice. Levels of Reg IV protein in peritoneal lavage fluids increased in *Reg IV*-transfectants inoculated mice, but decreased in *Reg IV*-knockdown cell inoculated mice. In metastasized human gastric cancers, Reg IV positivity in peritoneum-metastasis cases was higher than those in negative cases. Reg IV was detected in peritoneal lavage fluids from human gastric cancer patients, in whose lavages keratin mRNA was detected by reverse transcriptase-polymerase chain reaction. Collectively, Reg IV might accelerate peritoneal metastasis in gastric cancer. Reg IV in lavage fluids might be a good marker for peritoneal metastasis.

Correspondence: H. Kuniyasu, Department of Molecular Pathology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 734-8521, Japan. Tel.: +81 722 22 3015; Fax: +81 722 25 7308; E-mail: cooninh@zb4.so-net.ne.jp

Introduction

Gastric cancer is a leading cause of cancer death in the world, and is the second leading cause of deaths from cancer in Japan (1,2). Approximately 20% of gastric cancer patients show peritoneal and/or liver metastases at surgery (3), and 30% of who have died from gastric cancer suffered from peritoneal metastasis (4). Peritoneal metastasis causes the terminal stage of advanced gastric cancer, and diminishes the quality of patients' life by intestinal obstruction, ascites retention and subsequent malnutrition. Control of peritoneal metastasis is expected to improve patients' quality of life (5,6).

The molecular mechanism of peritoneal metastasis is an ongoing assignment of cancer research. We have identified loss of heterozygosity of chromosome 7q involving 7q35 locus as a peritoneal metastasis-associated event in gastric cancer (7). Overexpression of angiogenic factors, such as vascular endothelial growth factor (VEGF) and interleukin-8, is associated with peritoneal metastasis and ascites production in ovarian cancer (8). The truncated form of fibroblast growth factor/keratinocyte growth factor receptor 2 IIIb (K-*sam*) and c-*met* show gene amplification and/or overexpression in scirrhous-type gastric cancers, which frequently produces peritoneal metastasis (9–11). Gene expression profiling shows alteration in expression of several genes, such as up-regulation of trefoil factor 1, α-1-antitrypsin and galectin 4, and down-regulation of cytidine deaminase (12). Recently, we reported the importance of activation of peroxisome proliferator-activated receptor gamma (PPAR-γ) in suppressing peritoneal metastasis, which provides tumour growth inhibition and apoptosis induction in gastric and colon cancer cells (13,14). The importance of survival factors in peritoneal metastasis formation is emphasized in many reports; RUNX3, survivin, nuclear factor κB and Bcl-2/Bag are also associated with peritoneal metastasis (15–18).

The *Reg* (*regenerating*) gene family belongs to the calcium-dependent lectin superfamily (19,20). *Reg IV* is a new member family, and it is identified as a gene expressed in the gastrointestinal tract and pancreas (21,22). Human

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd.

Reg IV gene is located on chromosome 1, unlike other *Reg* family genes, which are located on 2p12 (23). *Reg IV* is expressed in Crohn's disease and ulcerative colitis (21,24) and is revealed to be associated with malignant potential of colorectal adenocarcinomas and malignant transformation of colorectal adenomas (25,26). Recently, Reg IV has been reported to activate epidermal growth factor receptor (EGFR), protein kinase B/Akt and activator protein-1 to accelerate colorectal cancer cell survival by increasing Bcl-2, Bcl-XL and survivin (27). The anti-apoptotic property of Reg IV is associated with colorectal cancer development and drug resistance in gastric cancer (28,29) and its expression is expected to be a marker for highly malignant potential (30–32).

We have identified *Reg IV* as a cancer-affiliated expressed gene, by a serial analysis of gene expression (SAGE) technique, in which results were deposited in the NCBI SAGE Library in the Web (http://www.ncbi.nlm.nih.gov/SAGE/). Reg IV protein is immunohistochemically detected in 36% of colorectal adenocarcinomas and this is associated with tumour stage, whereas Reg IV production is detected in 29% of gastric adenocarcinomas, and is associated with both the intestinal mucin phenotype and neuroendocrine differentiation but not with tumour stage or patient prognosis (33). Thus, the role of Reg IV in gastric cancer is still unclear.

In this study, we attempted to determine the relevance of Reg IV expression in peritoneal metastasis of gastric cancer using *Reg IV*-transfected human gastric cancer cells.

Materials and Methods

Cell culture

Human gastric cancer cell lines, MKN28 and TMK1, were transfected with the *Reg IV* expression vector (29,33). MKN28- and TMK1-*Reg IV* stable transfectants were selected by G418 (Sigma Chemical Co., St. Louis, MO, USA). Three transfectants with marked *Reg IV* expression were used in the present study, which were designated as MKN28-R1, MKN28-R2 and TMK1-R1. For the control, MKN28 and TMK1 cells were transfected with empty vector, which was designated as MKN28-R0 and TMK1-R0. MKN45 cells, which express Reg IV at a high level, was treated with *Reg IV* siRNA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or mixed siRNA for control. siRNA (50 nM for 2×10^5 cells) diluted with transfection solution (Santa Cruz Biotechnology) was used for treatment according to the manufacturer's instructions. The cells were routinely maintained in RPMI-1640 (Sigma Chemical) containing 10% foetal bovine serum (Sigma Chemical) and G418 (500 μg/ml) at 37 °C in a 5% CO_2/95% air atmosphere.

Tissue samples

Gastric cancer cases with metastasis were chosen from the patients operated on in Hiroshima University Hospital, Nara Medical University Hospital, and Miyoshi Central Hospital. Among the 85 cases, all showed lymph node metastasis, 8 cases showed liver metastasis, and 43 cases showed peritoneal metastasis at surgery. Of 42 cases without peritoneal metastasis (found via gross and cytological examinations) at the operation, 21 cases showed peritoneal recurrence. For immunohistochemistry, representative formalin-fixed, paraffin-embedded tissue samples were used, which contained the deepest invasive portion of the tumour.

Cell population growth

The cells were harvested from 80% confluent monolayer cultures by a brief trypsinization with 0.1% trypsin and 0.1% ethylenediaminetetraacetic acid (Sigma Chemical). Cells were seeded at a density of 10 000 cells per well in 24-well tissue culture plates and treated under the conditions mentioned in the Results section. Cell number was counted using an autocytometer (Sysmecs, Kobe, Japan) at 24, 48 and 72 h. The experiment was repeated three times. For sodium nitropusside (SNP) treatment, cell number was counted 48 h after the treatment.

In vitro *invasion assay*

A modified Boyden chamber assay was performed to examine *in vitro* invasion of MKN28 cells. Polycarbonate filters (pore size 3 μm; diameter 5 mm) were glued to collagen type IV inserts (Becton-Dickinson Labware, Bedford, MA, USA), which were placed in the wells of 24-well tissue culture plates. The cells were suspended in 500 μl of regular medium and placed in the upper part of the chamber. The lower part of the chamber was filled with regular medium. After 24-h incubation at 37 °C, filters were carefully removed from the inserts, stained with haematoxylin for 10 min and mounted on microscope slides. The number of stained cells was counted in whole inserts wide at ×100 magnification. Invasion activity was quantified by average number of cells per insert well. Mean values of invading cells were calculated from the results of three independent experiments.

Animal model

BALB/c nu-nu athymic mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were maintained according to the institutional guidelines approved by the Committee for Animal Experimentation of the Nara Medical University, in accordance with the current regulations and standards

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,

of the Ministry of Health, Labour and Welfare. The mice were used according to the institutional guidelines when they were 5 weeks old. Cells (as above) were briefly trypsinized and washed with Hanks' balanced saline solution (HBSS) three times. They were suspended in HBSS and were injected into the peritoneal cavity (1×10^7) of each mouse; eight or nine mice were injected per group. The mice were sacrificed to count numbers of metastatic foci in the peritoneal cavity. In another experimental set, survival of 10 mice was observed in each cell line until 16 weeks after the inoculation. Mice inoculated with MKN45 cells treated with siRNA were administrated siRNA encapsulated with liposome (34). siRNA (100 pmol) was encapsulated with 2 ml of kationic liposome (EL-C-01, Nippon-Oil_Fats Co., Tokyo, Japan), and 200 μl of the solution was administrated intraperitoneally to each mouse twice a week.

Immunohistochemistry

Consecutive 4-μm sections were cut from each block, and immunostaining was performed by immunoperoxidase technique following antigen retrieval with citrate buffer (pH 6.0) treatment for 10 min (three times). After endogenous peroxidase block by 3% hydrogen peroxide–methanol for 15 min, specimens were rinsed with 5% washing solution (BioGenex, San Ramon, CA, USA). Anti-Reg IV antibody established in our laboratory was used at 0.5 μg/ml primary antibody, and incubated at room temperature for 2 h (33). Specimens were rinsed with 5% washing solution and incubated at room temperature for 1 h with secondary antibody conjugated to peroxidase diluted at 0.5 μg/ml (anti-rabbit IgG, Medical & Biotechnological Laboratories Co., Ltd, Nagoya, Japan). All specimens were then rinsed with 5% washing solution and colour was developed by diaminobenzidine solution (Dako, Glostrup, Denmark). After washing with water, specimens were counterstained with Meyer's haematoxylin (Sigma Chemical). Immunostaining of all specimens was performed to ensure the same condition of antibody reaction and diaminobenzidine exposure.

Preparation of conditioned medium, peritoneal lavage and serum

Cells were cultured in RPMI-1640 containing 1% foetal bovine serum for 12 h. Then, the conditioned medium was filtered with 0.2-μm filter (Becton-Dickinson Labware). The peritoneal cavity of a sacrificed mouse was washed with 2 ml of phosphate-buffered saline and saved phosphate-buffered saline was filtered with 0.2-μm filter (Becton-Dickinson Labware). Mouse blood was obtained by cardiac puncture, mixed with heparin (5% v/v), and centrifuged at 500 ***g*** for 15 min at 4 °C. The supernatant serum was used for immunoblot analysis. For human peritoneal lavage, the entire peritoneal cavity was washed with 100 ml saline after opening of the peritoneal cavity. Of the peritoneal lavage fluid, 10 ml was centrifuged at 500 ***g*** for 15 min at 4 °C. The supernatant was used for immunoblotting and the pellet was used for reverse transcriptase–polymerase chain reaction (RT-PCR). Remnants of lavage fluid were used for cytological examination. Slot blotted lavage fluids and cultured media stained with Coomassie blue dye were served as the control for the sample loading.

Immunoblot analysis

Whole-cell lysates were prepared as described previously (35). The cultured medium and peritoneal lavage fluids were concentrated with the Protein Concentrate kit (TaKaRa Bio Inc., Shiga, Japan). Forty-microgram lysates were subjected to immunoblot analysis in 12.5% sodium dodecyl sulphate–polyacrylamide gels followed by electrotransfer on to nitrocellulose filters. Filters were incubated with primary antibodies and then with peroxidase-conjugated IgG antibodies (Medical and Biological Laboratories, Nagoya, Japan). α-tubulin or β-actin antibodies assessed levels of protein loaded per lane (Oncogene Research Products, Cambridge, MA, USA). The immune complex was visualized by CSA system (Dako) or ECL system (Amersham Biosciences Corp., Piscataway, NJ, USA). Antibodies for Reg IV (33), Bcl-2 (Dako), Bcl-XL, survivin (Santa Cruz Biotechnology), phosphorylated AKT (phospho-Ser473, Upstate Biotechnology Inc., Lake Placid, NY, USA), phosphorylated EGFR (phospho-Tyr992, Cell Signaling Technology, Beverly, MA, USA), and EGFR (Cell Signaling Technology) were used as primary antibodies.

Detection of cytokeratin in peritoneal lavage

Pellet from the peritoneal lavage fluids were used for RT-PCR, which was performed with iScript One-Step RT-PCR Kit (Bio-Rad Laboratories, Hercules, CA, USA). Primers for cytokeratin 20 mRNA were 5′-GAG GTT CAA CTA ACG GAG CT-3′ (forward) and 5′-TCT CTC TTC CAG GGT GCT TA-3′ (reverse) were referred to GenBank NM019010, which were synthesized by Sigma Genosys (Ishikari, Japan).

Statistical analysis

Statistical significance was examined by two-tailed Fisher's exact test, two-tailed *chi*-squared test, and two-tailed, unpaired Mann–Whitney test by using InStat software (GraphPad Software, Los Angels, CA, USA). Survival

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,

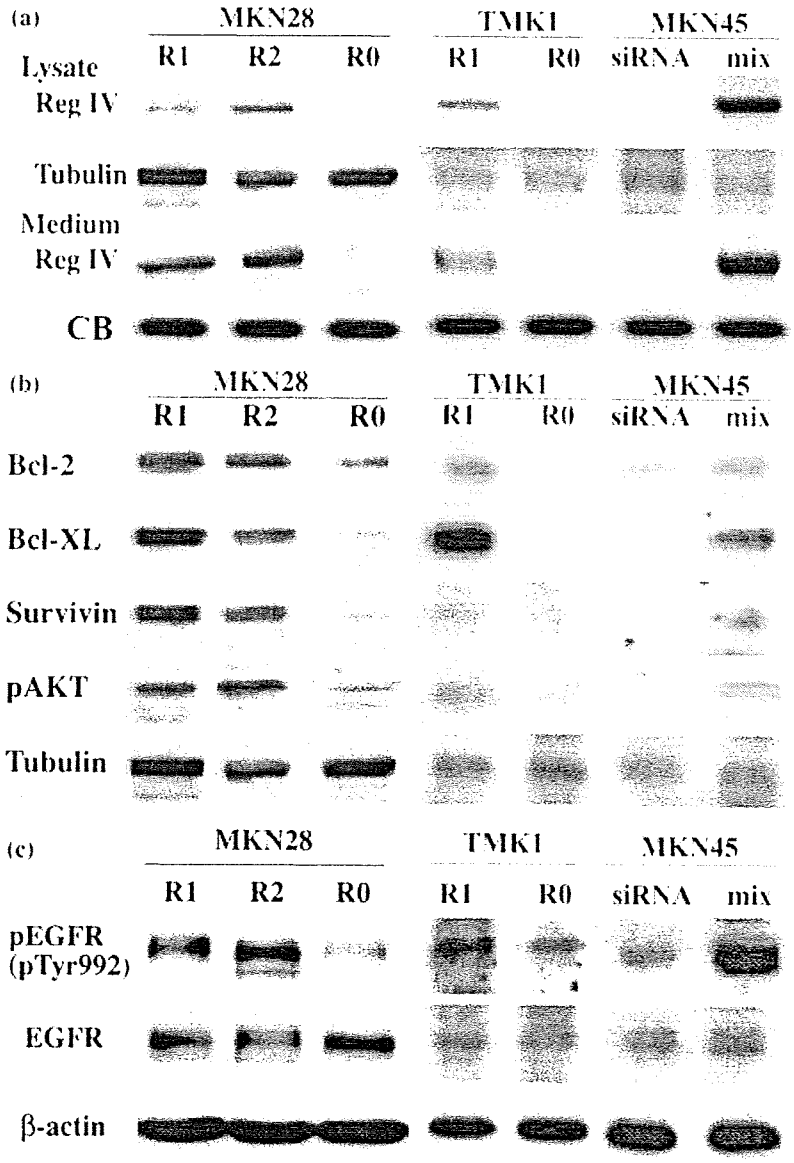


Figure 1. Production and secretion of Reg IV and survival-related proteins in gastric cancer cells. (a) Production and secretion of Reg IV protein were examined in lysates and culture media by immunoblotting. Tubulin and total loading protein detected by Coomassie blue staining (CB) served as loading controls. (b) Production of cell survival-related factors (Bcl-2, Bcl-XL, survivin and phophorylated AKT) was examined by immunoblotting. Tubulin served as loading control. (c) Phosphorylation levels of Tyr992 of EGFR were examined by immunoblotting. (a–c) MKN28-R1, MKN28-R2 and TMK1-R1: stable *Reg IV* transfectants. MKN28-R0 and TMK1-R0: empty vector-transfectant. MKN45-siRNA: *Reg IV* siRNA-treated MKN45 cells. MKN45-mix: siRNA mixture-treated MKN45 cells. β-actin or tubulin served as loading controls.

curves were calculated by Kaplan–Meier model (Statview 4.5, Abacus Concepts Inc., Berkeley, CA, USA). Difference of survivals was calculated by Cox proportional hazard model (Statview 4.5). Statistical significance was defined as a two-sided *P*-value of less than 0.05.

Results

Production of Reg IV protein and survival factors in gastric cancer cells

We first confirmed expression of Reg IV protein in *Reg IV*-transfected MKN28 and TMK1 cells and Reg IV siRNA-treated MKN45 cells (Fig. 1a). Three *Reg IV*-transfected cells (MKN28-R1, MKN28-R2 and TMK1-R1) and control vector-transfected cells (MKN28-R0 and TMK1-R0) were examined to detect Reg IV protein in the cell lysates and cultured media. MKN28-R0 and TMK1-R0 cells showed undetectable levels of Reg IV in the lysate and cultured media, whereas MKN28-R1, MKN28-R2 and TMK1-R1 cells produced Reg IV in the lysates and media. siRNA mixture-treated MKN45 cells expressed Reg IV at high level, whereas Reg IV siRNA-treated MKN45 cells did not express Reg IV. Second, levels of survival-related proteins in these cells were examined (Fig. 1b). Protein levels of Bcl-2, Bcl-XL, survivin and phorphorylated

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,

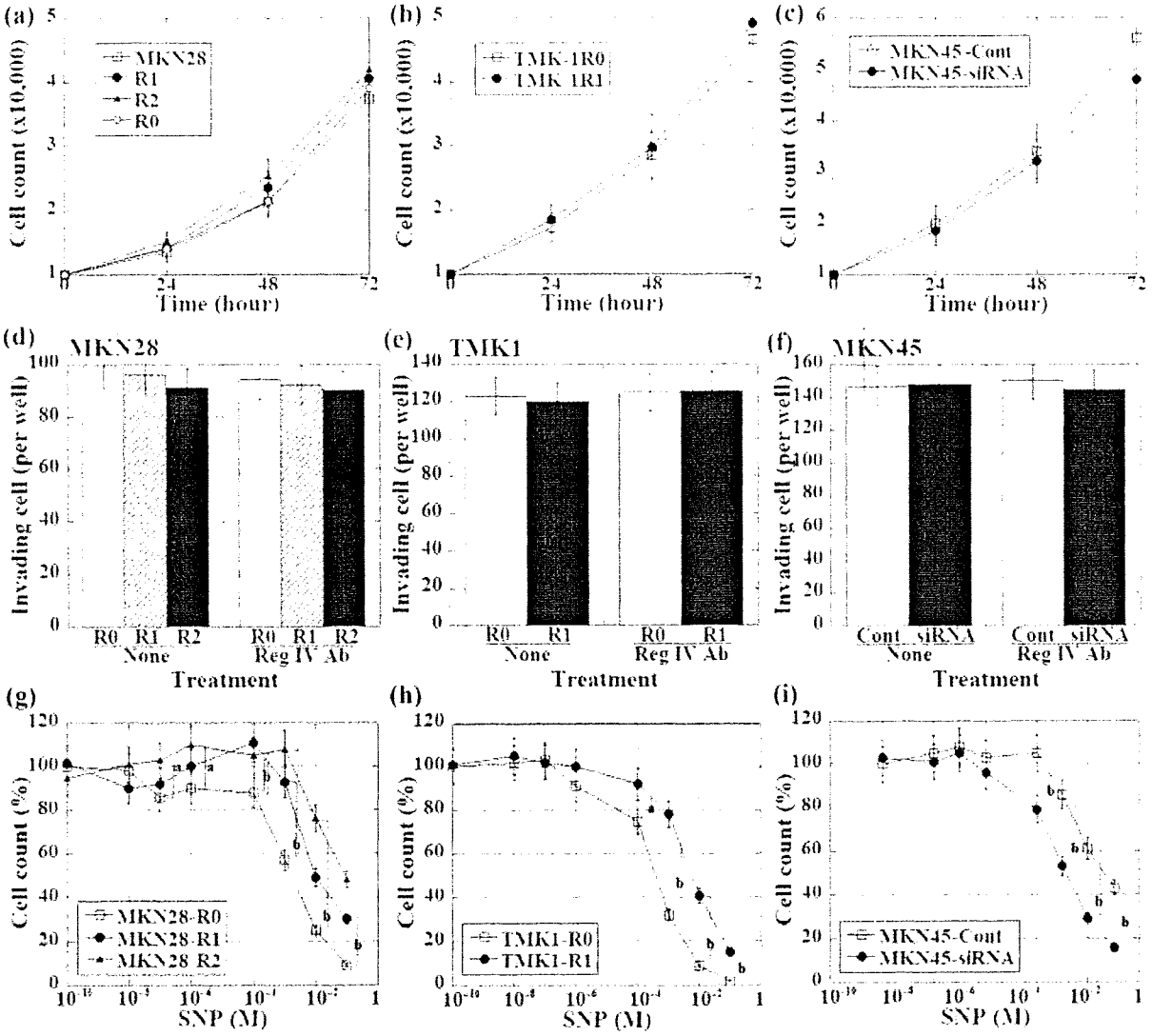


Figure 2. Effects of Reg IV transfection on cell population growth, *in vitro* invasion and nitric oxide-induced apoptosis in gastric cancer cells. (a–c) Cell growth of Reg IV transfectants and Reg IV siRNA-treated cells. (d–f) Invasion activity was examined by *in vitro* invasion assay with type IV collagen-coated insert. Anti-Reg IV polyclonal antibody was added to culture media for neutralizing secreted Reg IV at 0.5% v/v ('Reg IV Ab'). (g–i) Sensitivity to nitric oxide-induced cytotoxicity was examined. Sodium nitroprusside (SNP) was used as nitric oxide donor. (a) $P < 0.001$; (b) $P < 0.0001$. (a–c) MKN-R1, MKN-R2 and TMK1-R1: stable *Reg IV*-transfectants. MKN28-R0 and TMK1-R0: empty vector-transfectant. MKN45-siRNA: *Reg IV* siRNA-treated MKN45 cells. MKN45-Cont: siRNA mixture-treated MKN45 cells. Error bar: standard deviation.

AKT in MKN28-R1, MKN28-R2, TMK1-R1 and siRNA mixture-treated MKN45 cells were higher than those in MKN28-R0, TMK1-R0 and Reg IV siRNA-treated MKN45 cells. Third, phosphorylated form of EGFR (phosphotyrosin 992) in these cells was examined (Fig. 1c). pEGFR levels in MKN28-R1, MKN28-R2, TMK1-R1 and siRNA mixture-treated MKN45 cells were higher than those in MKN28-R0, TMK1-R0 and Reg IV siRNA-treated MKN45 cells.

Effect of Reg IV transfection on cell population growth, invasion and survival of gastric cancer cells

Next, biological effects of *Reg IV* transfection on MKN28 cells were examined (Fig. 2). As shown in Fig. 2(a), growth of MKN28-R1 and MKN28-R2 cells was not different from MKN28-R0 and MKN28 parental cells. Numbers of MKN28-R1 and MKN28-R2 cells invading into type IV collagen-coated membranes was not different

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,

Table 1. Peritoneal metastasis of *Reg IV* transfectants

Cells	Treatment	Metastasis[a]	Number	Size (mm)
MKN28				
R0	Vehicle	2/9[b]	1.5 ± 0.7[b]	1.0 ± 0.1[d]
R1	*Reg IV*	9/9[b]	5.3 ± 1.0[b]	4.2 ± 1.2[d]
R2	*Reg IV*	9/9[b]	5.6 ± 0.9[b]	5.2 ± 1.4[d]
TMK1				
R0	Vehicle	2/8[b]	0.7 ± 0.6[c]	0.6 ± 0.1[d]
R1	*Reg IV*	8/8[b]	9.8 ± 1.5[c]	4.6 ± 0.8[d]
MKN45				
Control	siRNA mix	8/8	14.4 ± 2.1[d]	3.5 ± 0.7[d]
siRNA	*Reg IV* siRNA	5/8	0.55 ± 0.7[d]	0.9 ± 0.5[d]

[a]Metastasis determined at 2 weeks post inoculation.
[b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.0001$.

from those of MKN28-R0 cells. Anti-Reg IV antibody added to the culture media to neutralize secreted Reg IV did not affect invasion of three types of MKN28 cells (Fig. 2d). In TMK1 and MKN45 cells, transfection or knockdown of *Reg IV* did not affect the cell growth or invasion (Fig. 2b,c,e,f). We next treated these cells with nitric oxide (NO) using SNP as an NO donor. NO cytotoxicity depends on NO concentration and sensitivity of the cells (36). MKN28-R0 cells were decreased by SNP treatment in a dose-dependent manner (Fig. 2g). In contrast, MKN28-R2 and MKN28-R1 cells attenuated cell decrease in SNP concentration higher than 10^{-7} M. At 10^{-1} M, the relative cell numbers compared to untreated MKN28 parental cells were 28 ± 3% and 57 ± 5% in MKN28-R1 and MKN28-R2 cells, respectively, which were higher than that in MKN28-R0 cells (13 ± 2%) (both $P < 0.0001$). TMK1-R1 cells also showed lower sensitivities to SNP-induced cytotoxicity than that in TMK1-R0 cells. In contrast, MKN45-siRNA cells showed higher SNP sensitivities than that in MKN45-Cont cells (Fig. 2h,i).

Peritoneal tumours of Reg IV-transfectants

Reg IV-transfected MKN28 cells were inoculated into the peritoneal cavities of nude mice. Peritoneal tumours of *Reg IV*-transfected MKN28 cells were compared to MKN28-R0 tumours (Table 1, Fig. 3). Tumorigenicity of MKN28-R1 and MKN28-R2 cells was significantly higher (both 9/9) than that of MKN-R0 cells (2/9) ($P = 0.0023$). Numbers of peritoneal tumours in MKN28-R1 and MKN28-R2 cells were higher (5.3 ± 1.0 and 5.6 ± 0.9 foci, respectively) than of MKN28-R0 cells (1.5 ± 0.7 foci) ($P = 0.0364$). Sizes of tumours of MKN28-R1 and MKN28-R2 cells were significantly larger (4.2 ± 1.2 mm and 5.2 ± 1.4 mm, respectively) than of MKN28-R0 cells (1.0 ± 0.1 mm) ($P = 0.0005$). MKN28-R2 cells formed larger tumours in the peritoneum than MKN28-R0 cells (Fig. 1a). *Reg IV*-transfected TMK1-R1 cells also showed higher tumorigenicity (8/8) than TMK1-R0 cells (2/8). Numbers and sizes of the peritoneal tumours were larger (9.8 ± 1.5 foci and 4.6 ± 0.8 mm, respectively) in TMK1-R1 cells than those in TMK1-R0 cells (0.7 ± 0.6 foci and 0.6 ± 0.1 mm, respectively) ($P < 0.001$ and $P < 0.0001$). In contrast, knockdown of *Reg IV* decreased tumourigenicity, tumour number and tumour growth in MKN45 cells. *Reg IV* siRNA-treated MKN45 cells showed lower tumorigenicity (5/8) than siRNA mixture-treated MKN45 cells (8/8). The number and sizes of the peritoneal tumours in *Reg IV* siRNA-treated MKN45 cells were smaller (0.55 ± 0.7 foci and 0.9 ± 0.5 mm, respectively) than those in siRNA mixture-treated MKN45 cells (14.4 ± 2.1 foci and 3.5 ± 0.7 mm, respectively) ($P < 0.0001$ and $P < 0.0001$).

Histologically, MKN28-R0 tumours showed large areas of necrosis, whereas no necrosis was found in the MKN28-R2 tumours (Fig. 3b). Production of Reg IV in the tumours was confirmed by immunohistochemistry (Fig. 3b, Table 2). MKN28-R0 tumours contained few Reg IV-positive cells, whereas MKN28-R2 tumours showed marked Reg IV immunoreactivity in the cytoplasm in all tumour cells (Allred's grade 8). As shown in Table 2, MKN28-R1, TMK1-R1 and siRNA mixture-treated MKN45 cells showed marked Reg IV expression (grades 7, 8 and 8, respectively), whereas MKN28-R0, TMK1-R0 and *Reg IV* siRNA-treated MKN45 cells showed no Reg IV expression (grade 0).

Cell proliferation and apoptotic properties of MKN28-R1 and -R2 tumours were compared to those of MKN28-R0 tumours (Fig. 3c, Table 2). Proliferating cell nuclear antigen (PCNA) indices in MKN28-R1 and MKN28-R2 tumours were 87 ± 5% and 83 ± 4%, respectively, which were similar to those in MKN28-R0 tumours (84 ± 5%). In contrast, TUNEL indices in MKN28-R1 and MKN28-R2 tumours were 0.8 ± 0.8% and 0.5 ± 0.7%, respectively, which were significantly lower than in MKN28-R0 tumours (6.2 ± 1.6%, $P < 0.0001$). We examined labelling indices of PCNA and TUNEL in TMK1-R1 and *Reg IV* siRNA-treated MKN45 tumours, which were compared to those in TMK1-R0 and siRNA mixture-treated MKN45 tumours. PCNA indices in TMK1-R0 and TMK1-R1 tumours were 58 ± 7 and 65 ± 7, respectively, which were similar to each other. In contrast, the TUNEL index in *Reg IV*-transfected TMK1-R1 tumours was significantly lower (2.3 ± 1.8) than that in TMK1-R0 tumours (8.6 ± 2.4) ($P < 0.01$). TUNEL-positive apoptotic cells were significantly increased by *Reg IV* knockdown in MKN45 cells. In contrast, PCNA indices were not affected by *Reg IV* knockdown.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,

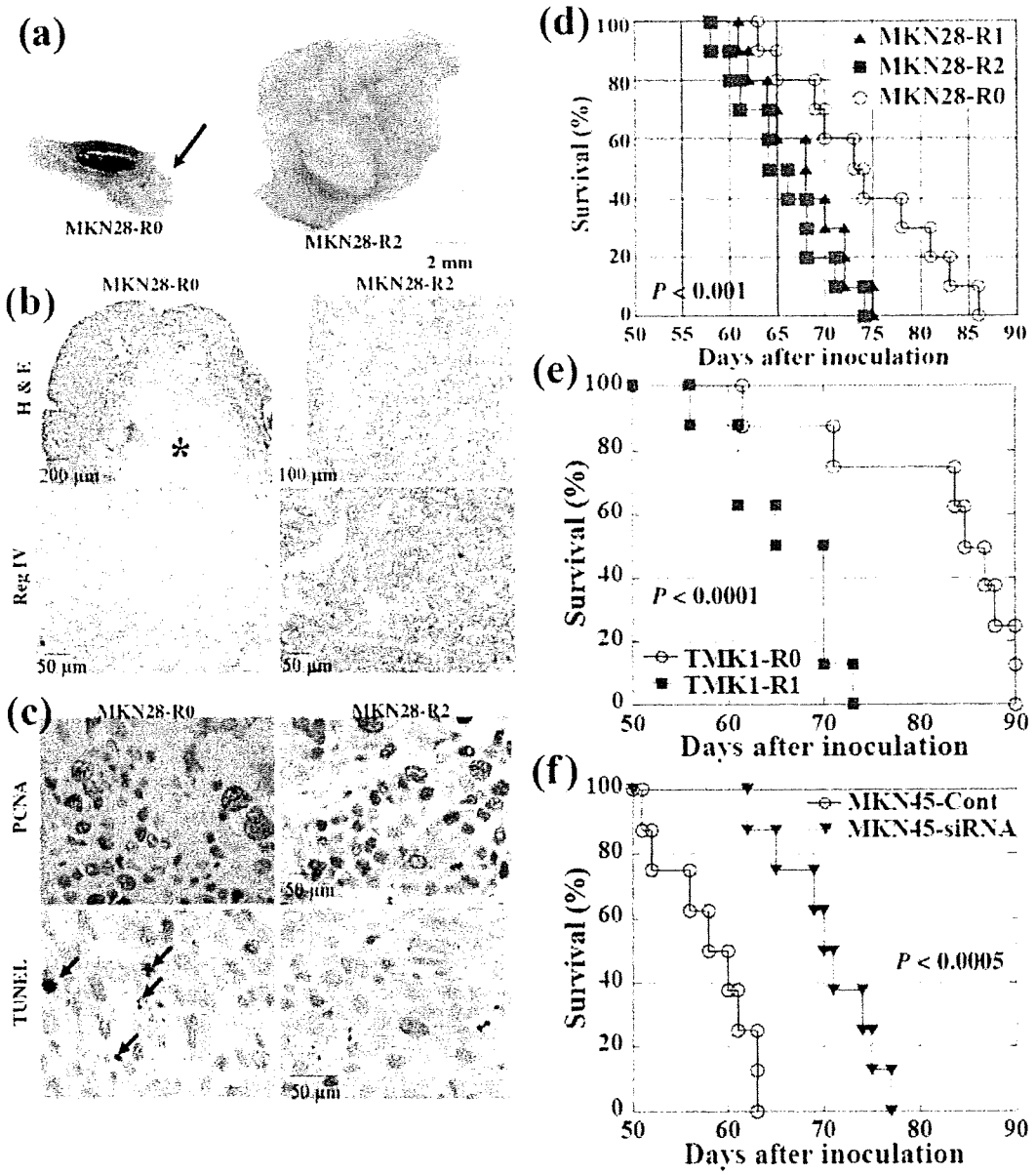


Figure 3. Peritoneal tumours of Reg IV transfectants. (a) Macroscopic appearance of MKN28-R0 cell tumour (at the mesocolon, arrow) and MKN28-R2 tumour (at the abdominal wall). (b) Histological findings using haematoxylin and eosin staining (upper panels). Necrotic area (asterisk). Immunostaining of Reg IV (lower panels). Immunoreactivity was observed in cytoplasm in MKN28-R2 cells. (c) Immunostaining of PCNA and TUNEL assay in MKN28-R2 and MKN28-R0 tumours. Arrow, TUNEL-positive apoptotic cells. (d–f) Survival of mice inoculated with MKN28-R1, MKN28-R2 and MKN28-R0 cells (d), TMK1-R0 and TMK1-R1 (e), and MKN45 treated with *Reg IV*-siRNA (MKN45-siRNA) or siRNA mixture (MIN45-Cont) (f) were calculated by Kaplan–Meier model and compared by Cox proportional hazard model. Survival of mice inoculated with MKN28-R1/MKN28-R2, TMK1-R1 and MKN45-Cont were significantly worse than those of mice inoculated with MKN28-R0, TMK-R0, MKN45-siRNA ($P < 0.001$, $P < 0.0001$, $P < 0.0005$, respectively).

Survival of mice burdened with peritoneal tumours of Reg IV-transfected gastric cancer cells

The survival of a further set of mice inoculated with *Reg IV*-transfected MKN28 and TMK1 cells or *Reg IV* siRNA-treated MKN45 cells into the peritoneal cavity was analysed (Fig. 3d–f). Mice inoculated with MKN45 cells were continuously administrated liposome-encapsulated siRNA into the peritoneal cavity. Survival of mice inoculated with MKN28-R1 and MKN28-R2 cells, or

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,

Table 2. PCNA and TUNEL indices in peritoneal metastasis of *Reg IV* transfectants in mice

Cell line	Immunohistochemistry		
	Reg IV grade[a]	PCNA (%)	TUNEL (%)
MKN28			
R0	0	84 ± 5	6.2 ± 1.6[b,c]
R1	7	87 ± 5	0.8 ± 0.8[b]
R2	8	83 ± 4	0.5 ± 0.7[c]
TMK1			
R0	0	58 ± 7	8.6 ± 2.4[d]
R1	8	65 ± 7	2.3 ± 1.8[d]
MKN45			
Control	8	76 ± 8	3.7 ± 0.5[d]
siRNA	0	72 ± 9	10.6 ± 2.3[d]

[a]According to Allred grading. Grade 0, no staining; Grade 7, intermediates immunoreactivity was found in all cells; Grade 8, strong immunoreactivity was found in all cells.
[b,c]$P < 0.0001$, [d]$P < 0.01$.

TMK1-R1 cells was significantly worse than of those of mice inoculated with MKN28-R0 and TMK-R0 cells ($P < 0.001$ and $P < 0.0001$, respectively). In contrast, mice inoculated with *Reg IV* siRNA-treated MKN45 cells showed significantly better survival than those inoculated with siRNA mixture-treated MKN45 cells ($P < 0.0005$). All mice died from extended peritoneal tumours which lead to malnutrition.

Reg IV levels in peritoneal lavage fluid and serum of Reg IV transfectants-inoculated nude mice and human gastric cancer patients

Next, we detected Reg IV protein in peritoneal lavage fluid and serum from *Reg IV*-transfected MKN28 and TMK1 cells or siRNA-treated MKN45 cells (Fig. 4a,b). Reg IV protein levels in peritoneal lavage fluid from mice inoculated with MKN28-R1, MKN28-R2 and TMK1-R1 cells increased 12.3, 19.6 and 1.5 times, respectively, higher than that from mice inoculated with MKN28-R0 or TMK1-R0 cells. In contrast, Reg IV protein levels in peritoneal lavage fluid from mice inoculated with *Reg IV* siRNA-treated MKN45 cells were 9% of that in mice inoculated with siRNA mixture-treated MKN45 cells. Reg IV protein levels in serum from mice inoculated with MKN28-R1, MKN28-R2 and TMK1-R1 increased 2, 3.4 and 12 times, respectively, higher than that in mice inoculated with MKN28-R0 or TMK1-R0 cells. In contrast, Reg IV protein levels in serum from mice inoculated with *Reg IV* siRNA-treated MKN45 cells were 0.6% of that in mice inoculated with siRNA mixture-treated MKN45 cells.

Table 3. Reg IV expression in metastatic gastric cancer

	n	Reg IV expression	
		Positive	Negative
At operation			
Peritoneal metastasis (+)	43	29[a]	14
Peritoneal metastasis (–)	21	1[a,b]	20
Peritoneal recurrence	21	18[b]	3
Total	85	48	37

[a,b]$P < 0.0001$ (Fisher's exact test).

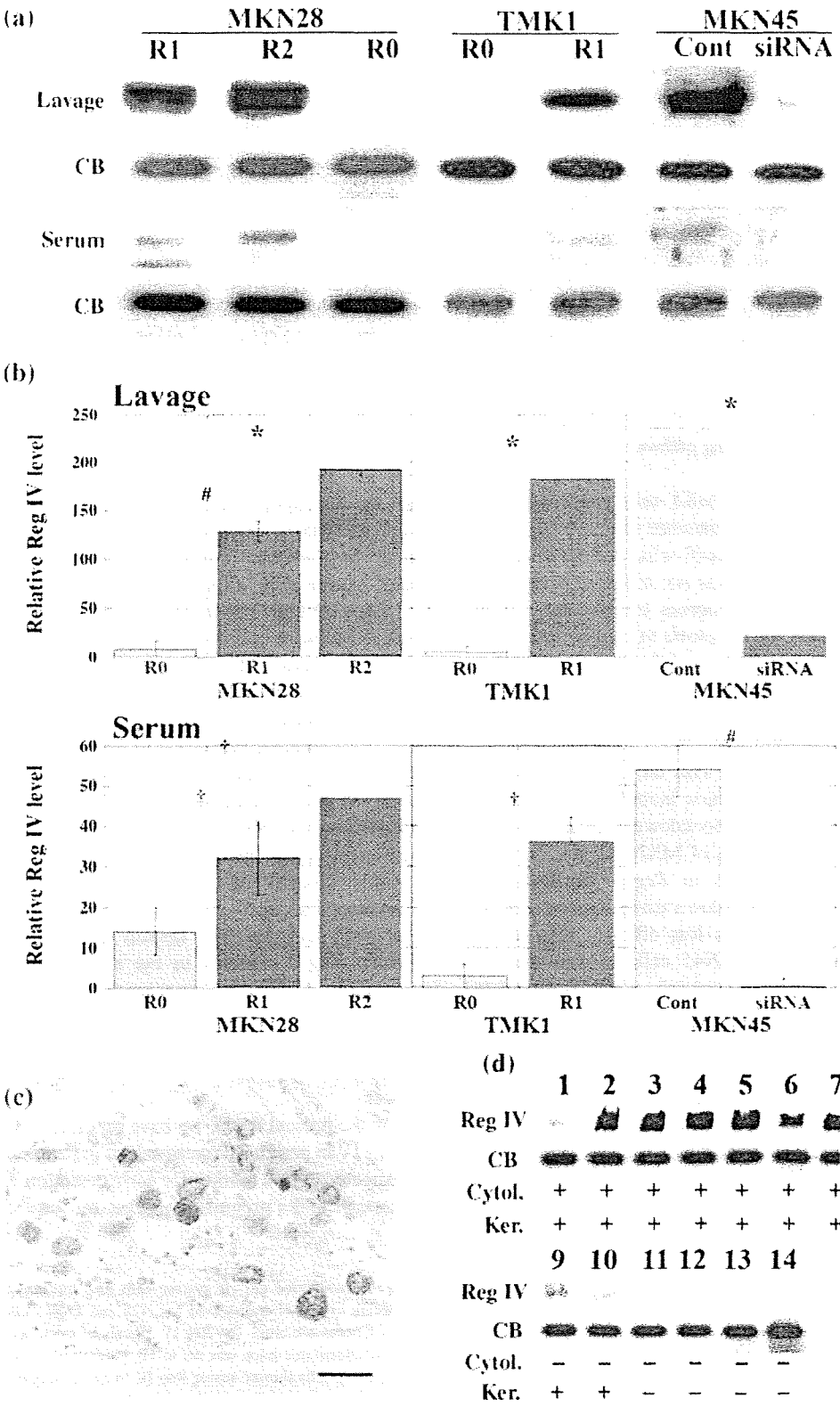
We next examined expression of Reg IV in 85 human gastric cancer patients with metastasis to the lymph nodes, liver or peritoneum (Table 3, Fig. 4c). Reg IV expression was detected in 30 of 64 (47%) gastric cancers at the time of surgery. In these cases, Reg IV was detected in 29 of 43 (67%) peritoneal metastasis-positive cases at the operation, whereas 1 of 21 (5%) peritoneal metastasis-negative cases showed Reg IV expression ($P < 0.0001$). In 21 peritoneal recurrent cases, 18 (86%) were positive for Reg IV. In 48 Reg IV-positive cases, 29 showed peritoneal metastasis at the time of operation. In contrast, the 18 out of the 48 cases showed peritoneal recurrence after the operation despite no peritoneal metastasis at the operation. It suggested that Reg IV-positive peritoneal lavage might be a marker for peritoneal recurrence.

We further detected Reg IV protein in peritoneal lavage fluid from 14 gastric cancer cases, which were found to be invading into the serosa (Fig. 4c). In 8 out of the 14 cases, Reg IV protein, keratin mRNA, and cancer cells were detected in the peritoneal lavage fluids (Fig. 4d). PCR examination of epithelial cell-specific keratin was also positive in the above 8 cases, which supported the evidence that cancer cells existed in the lavage fluid. In 6 cytology-negative cases, Reg IV protein was detected in 2 cases in which keratin was detected. The other 4 cases were negative for Reg IV protein, cytology and keratin.

Discussion

Our data have shown that Reg IV increased expression levels of anti-apoptotic BCL-2, BCL-XL and survivin, and phosphorylation levels of AKT in *Reg IV*-transfected MKN28 and TMK1 gastric cancer cells. Moreover, *Reg IV* knockdown decreased these apoptotic factors in *Reg IV*-expressing MKN45 cells; Reg IV protein levels paralleled apoptotic factors in these cells. Although NO is a strong inducer of apoptosis, increments of anti-apoptotic factors reduced NO-induced cytotoxicity in these cells (36). Anti-apoptotic property of Reg IV has been reported in several studies, and this gives cancer cells a

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,



© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,

survival advantages for progression and metastasis (24,27,29,37).

We have previously confirmed that EGF and the receptor formed autocrine and paracrine loops in MKN28 and TMK1 cells (38). MKN28 and TMK1 cells showed high phosphorylation levels of EGFR and Reg IV is reported to be associated with phosphorylation of EGFR (27). Our data confirmed increased phophorylation levels of EGFR in *Reg IV*-transfected MKN28 and TMK1 cells, whereas cell population growth and invasive capacity were not enhanced in the transfectants. Reg IV enhanced phosphorylation of EGFR, however, downstream signals might be preferentially associated with cell survival but not with growth and invasion in the *Reg IV*-transfected MKN28 and TMK1 cells. *Reg IV* knockdown inversely suppressed EGFR phosphorylation in MKN45 cells but the intracellular signalling pathway of Reg IV needs to be elucidated.

Reg IV-transfected and TMK1 cells produced peritoneal metastasis with larger diameter tumours and higher multiplicity than those of control cells. Proliferative activity of transfectant tumours was not different from that of the control cell tumours, whereas transfectant tumours had reduced necrosis and apoptosis in comparison to control tumours. These findings suggest that anti-apoptotic property of Reg IV renders more pronounced potential for peritoneal metastasis of MKN28 and TMK1 cells. Moreover, remarkable progression of metastatic tumours worsened survival of mice inoculated with the *Reg IV*-transfected MKN28 cells than that of mice inoculated with control cells. In contrast, *Reg IV* knockdown significantly suppressed peritoneal metastasis of MKN45 cells. Tumourigenicity was still sustained in *Reg IV* siRNA-treated MKN45 cells. *In vivo* knockdown using liposome encapsulation of siRNA might be less efficient than that of *in vitro* treatment (34). MKN45 cells express c-*met* at high levels with gene amplification, which is associated with the scirrhous phenotype and peritoneal metastasis (7,9,10).

Our previous report shows that Reg IV expression is not associated with peritoneal metastasis in the overall gastric cancer cases (33). However, examination of metastatic gastric cancer shows that Reg IV expression is significantly associated with peritoneal metastasis and peritoneal recurrence. In establishment of peritoneal metastasis, several mechanisms are proposed. Cell-to-cell adhesion between cancer cells and peritoneal mesothelial cells is an initial step of peritoneal metastasis, expression of CD44 and β1 integrin and intercellular adhesion molecule-1 playing a role in cancer cell adhesion to mesothelial cells (39,40). However, CD44 expression is silenced in MKN28 cells and also in the *Reg IV*-transfected MKN28 cells (data not shown) (41). The anti-apoptotic property of cancer cells is emphasized in formation of peritoneal metastasis (15–18). In our data, *Reg IV* transfectants showed up-regulation of several anti-apoptotic proteins: Bcl-2, Bcl-XL, survivin, and phosphorylated AKT. *Reg IV* transfectants acquired resistance to NO-induced apoptosis. TMK1 cells, which are sensitive to various apoptotic inducers, show the anti-apoptotic phenotype after *Reg IV* transfection (35). Reg IV is associated with anti-apoptotic phenotype in MKN45 cells, which carry wild-type p53 differently from MKN28 and TMK1 cells (41) and the anti-apoptotic property is not specific to peritoneal metastasis; however, enhanced survival potential might be a relevant advantage for peritoneally disseminated cancer cells to form metastatic foci.

Because Reg IV is a small secretory protein, its detection in ascites might be expected as a marker for peritoneal metastasis (22). We examined Reg IV protein in peritoneal lavage of gastric cancer cell-inoculated mice. Reg IV in peritoneal lavage fluid was at higher levels in *Reg IV*-transfected MKN28 and TMK1 cells than in control cells. In contrast, lavage Reg IV was significantly lower in mice inoculated with *Reg IV*-knocked down MKN45 cells. We then examined Reg IV protein in peritoneal lavage fluid of gastric cancer patients at the operation. Reg IV protein was detected in all cases with macroscopical and cytological peritoneal metastasis. Moreover, all cases with keratin mRNA-positive ascites showed Reg IV protein in the ascites. Ascites keratin detected by RT-PCR is a sensitive marker for scanty cancer cells in ascites in cytologically metastasis-negative cases (42). These findings suggest that ascites Reg IV might be a sensitive marker for peritoneal metastasis of gastric cancer.

In the present study, we have reported the pivotal role of Reg IV in peritoneal metastasis of gastric cancer. Reg IV is expected to be a marker for early detection of peritoneal metastasis and a prognostic marker for gastric cancer.

Figure 4. Reg IV protein levels in peritoneal lavage fluids and serum of Reg IV-transfected gastric cancer cells and human gastric cancer cases. (a) Reg IV protein levels were examined in peritoneal lavage fluids and serum of *Reg IV*-transfected MKN28 and TMK1 cells and *Reg IV* siRNA-treated MKN45 cells by immunoblotting (CB: loaded protein detected by Coomassie blue). (b) Reg IV signals of mice lavage fluids and serum were semiquantified. Reg IV signal of peritoneal lavage fluid of MKB28-R0 inoculated mice was set to 10. Error bar: standard deviation. (c) Immunohistochemistry of Reg IV in serosa-invading human gastric cancer. Signet ring cells showed strong Reg IV immunoreactivity. Bar: 50 μm. (d) Reg IV protein levels were examined in peritoneal lavage fluids from human gastric cancers by immunoblotting. Cytol., cytological examination of the lavage fluid; +, cancer cell positive; –, cancer cell negative; Ker., PCR examination of keratin in the lavage fluid.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,

Acknowledgement

This study was supported by Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare, Japan.

References

1 Jamal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ (2003) Cancer statistics, 2003. *CA Cancer J. Clin.* **53**, 5–26.
2 National Cancer Center, Cancer Information Service. (2008) in: Kato H, ed. *Cancer Statistics in Japan*, 2008 [WWW document]. http://ganjoho.ncc.go.jp/public/statistics/backnumber/2008_en.html [accessed 11.05.2008]
3 Maehara Y, Moriguchi S, Kakeji Y, Kohnoe S, Korenaga D, Haraguchi M, Sugimachi K (1991) Pertinent risk factors and gastric carcinoma with synchronous peritoneal dissemination or liver metastasis. *Surgery* **110**, 820–823.
4 Moriguchi S, Maehara Y, Korenaga D, Sugimachi K, Nose Y (1992) Risk factors which predict pattern of recurrence after curative surgery for patients with advanced gastric cancer. *Surg. Oncol.* **1**, 341–346.
5 Sugarbaker PH, Yu W, Yonemura Y (2003) Gastrectomy, peritonectomy, and perioperative intraperitoneal chemotherapy: the evolution of treatment strategies for advanced gastric cancer. *Semin. Surg. Oncol.* **21**, 233–248.
6 Vogel I, Kalthoff H (2001) Disseminated tumour cells. Their detection and significance for prognosis of gastrointestinal and pancreatic carcinomas. *Virchows Arch.* **439**, 109–117.
7 Kuniyasu H, Yasui W, Yokozaki H, Akagi M, Akama Y, Kitahara K, Fujii K, Tahara E (1994) Frequent loss of heterozygosity of the long arm of chromosome 7 is closely associated with progression of human gastric carcinomas. *Int. J. Cancer* **59**, 597–600.
8 Yoneda J, Kuniyasu H, Crispens MA, Price JE, Bucana CD, Fidler IJ (1998) Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. *J. Natl. Cancer Inst.* **90**, 447–454.
9 Kuniyasu H, Yasui W, Kitadai Y, Yokozaki H, Ito H, Tahara E (1992) Frequent amplification of the c-*met* gene in scirrhous type stomach cancer. *Biochem. Biophys. Res. Commun* **189**, 227–232.
10 Kuniyasu H, Yasui W, Yokozaki H, Kitadai Y, Tahara E (1993) Aberrant expression of c-*met* mRNA in human gastric carcinomas. *Int. J. Cancer* **55**, 72–75.
11 Takeda M, Arao T, Yokote H, Komatsu T, Yanagihara K, Sasaki H, Yamada Y, Tamura T, Fukuoka K, Kimura H, Saijo N, Nishio K (2007) AZD2171 shows potent antitumor activity against gastric cancer over-expressing fibroblast growth factor receptor 2/keratinocyte growth factor receptor. *Clin. Cancer Res.* **13**, 3051–3057.
12 Hippo Y, Yashiro M, Ishii M, Taniguchi H, Tsutsumi S, Hirakawa K, Kodama T, Aburatani H (2001) Differential gene expression profiles of scirrhous gastric cancer cells with high metastatic potential to peritoneum or lymph nodes. *Cancer Res.* **61**, 889–895.
13 Kuniyasu H, Yoshida K, Sasaki T, Sasahira T, Fujii K, Ohmori H (2006) Conjugated linoleic acid inhibits peritoneal metastasis in human gastrointestinal cancer cells. *Int. J. Cancer* **118**, 571–576.
14 Sasaki T, Fujii K, Yoshida K, Shimura H, Sasahira T, Ohmori H, Kuniyasu H (2005a) Peritoneal metastasis inhibition by linoleic acid with activation of PPARγ in human gastrointestinal cancer cells. *Virchows Arch.* **448**, 422–427.
15 Sakakura C, Hasegawa K, Miyagawa K, Nakashima S, Yoshikawa T, Kin S, Nakase Y, Yazumi S, Yamagishi H, Okanoue T, Chiba T, Hagiwara A (2005) Possible involvement of RUNX3 silencing in the peritoneal metastases of gastric cancers. *Clin. Cancer Res.* **11**, 6479–6488.
16 Tsuburaya A, Noguchi Y, Yoshikawa T, Saito A, Doi C, Okamoto T, Fukuzawa K (2002) An anti-apoptosis gene, survivin and telomerase expression in gastric cancer. *Hepatogastroenterology* **49**, 1150–1152.
17 Sasaki N, Morisaki T, Hashizume K, Yao T, Tsuneyoshi M, Noshiro H, Nakamura K, Yamanaka T, Uchiyama A, Tanaka M, Katano M (2001) Nuclear factor-κB p65 (RelA) transcription factor is constitutively activated in human gastric carcinoma tissue. *Clin. Cancer Res.* **7**, 4136–4142.
18 Yawata A, Adachi M, Okuda H, Naishiro Y, Takamura T, Hareyama M, Takayama S, Reed JC, Imai K (1998) Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells. *Oncogene* **16**, 2681–2686.
19 Chakraborty C, Katsumata N, Myal Y, Schroedter IC, Brazeau P, Murphy LJ, Shiu RP, Friesen HG (1995) Age-related changes in peptide-23/pancreatitis-associated protein and pancreatic stone protein/reg gene expression in the rat and regulation by growth hormone-releasing hormone. *Endocrinology* **136**, 1843–1849.
20 Lasserre C, Simon MT, Ishikawa H, Diriong S, Nguyen VC, Christa L, Vernier P, Brechot C (1994) Structural organization and chromosomal localization of a human gene (HIP/PAP) encoding a C-type lectin overexpressed in primary liver cancer. *Eur. J. Biochem.* **224**, 29–38.
21 Hartupee JC, Zhang H, Bonaldo MF, Soares MB, Dieckgraefe BK (2001) Isolation and characterization of a cDNA encoding a novel member of the human regenerating protein family: Reg IV. *Biochim. Biophys. Acta* **1518**, 287–293.
22 Li A, Crimmins DL, Luo Q, Hartupee J, Landt Y, Ladenson JH, Wilson D, Anant S, Dieckgraefe BK (2003) Expression of a novel regenerating gene product, Reg IV, by high density fermentation in *Pichia pastoris*: production, purification, and characterization. *Protein Expr. Purif.* **31**, 197–206.
23 Nata K, Liu Y, Xu L, Ikeda T, Akiyama T, Noguchi N, Kawaguchi S, Yamauchi A, Takahashi I, Shervani NJ, Onogawa T, Takasawa S, Okamoto H (2004) Molecular cloning, expression and chromosomal localization of a novel human REG family gene, REG III. *Gene* **340**, 161–170.
24 Nanakin A, Fukui H, Fujii S, Sekikawa A, Kanda N, Hisatsune H, Seno H, Konda Y, Fujimori T, Chiba T (2007) Expression of the REG IV gene in ulcerative colitis. *Lab. Invest.* **87**, 304–314.
25 Violette S, Festor E, Pandrea-Vasile I, Mitchell V, Adida C, Dussaulx E, Lacorte JM, Chambaz. J, Lacasa M, Lesuffleur T (2003) Reg IV, a new member of the regenerating gene family, is overexpressed in colorectal carcinomas. *Int. J. Cancer* **103**, 185–193.
26 Zhang Y, Lai M, Lv B, Gu X, Wang H, Zhu Y, Shao L, Wang G (2003a) Overexpression of Reg IV in colorectal adenoma. *Cancer Lett.* **200**, 69–76.
27 Bishnupuri KS, Luo Q, Murmu N, Houchen CW, Anant S, Dieckgraefe BK (2006b) Reg IV activates the epidermal growth factor receptor/Akt/AP-1 signaling pathway in colon adenocarcinomas. *Gastroenterology* **130**, 137–149.
28 Bishnupuri KS, Luo Q, Korzenik JR, Henderson JO, Houchen CW, Anant S, Dieckgraefe BK (2006a) Dysregulation of Reg gene expression occurs early in gastrointestinal tumorigenesis and regulates anti-apoptotic genes. *Cancer Biol. Ther* **5**, 1714–1720.
29 Mitani Y, Oue N, Matsumura S, Yoshida K, Noguchi T, Ito M, Tanaka S, Kuniyasu H, Kamata N, Yasui W (2007) Reg IV is a serum biomarker for gastric cancer patients and predicts response to 5-fluorouracil-based chemotherapy. *Oncogene* **26**, 4383–4393.
30 Gu Z, Rubin MA, Yang Y, Deprimo SE, Zhao H, Horvath S, Brooks JD, Loda M, Reiter RE (2005) Reg IV: a promising marker of hormone refractory metastatic prostate cancer. *Clin. Cancer Res.* **11**, 2237–2243.
31 Heiskala K, Giles-Komar J, Heiskala M, Andersson LC (2006) High expression of RELP (Reg IV) in neoplastic goblet cells of

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,

appendiceal mucinous cystadenoma and pseudomyxoma peritonei. *Virchows Arch.* **448**, 295–300.
32 Pankova-Kholmyansky I, Arber N (2007) Reg IV can serve for early diagnosis and therapy. *Cancer Biol. Ther.* **6**, 123–124.
33 Oue N, Mitani Y, Aung PP, Sakakura C, Takeshima Y, Kaneko M, Noguchi T, Nakayama H, Yasui W (2005) 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.
34 Chihara Y, Fujimoto K, Kondo H, Moriwaka Y, Sasahira T, Hirao Y, Kuniyasu H (2007) Anti-tumor effects of liposome-encapsulated titanium dioxide in nude mice. *Pathobiology* **74**, 353–358.
35 Kuniyasu H, Yasui W, Kitahara K, Naka K, Yokozaki H, Akama Y, Hamamoto T, Tahara H, Tahara E (1997) Growth inhibitory effect of interferon-β is associated with the induction of cyclin-dependent kinase inhibitor p27Kip1 in a human gastric carcinoma cell line. *Cell Growth Differ.* **8**, 47–52.
36 Sasaki T, Yoshida K, Kondo H, Ohmori H, Kuniyasu H (2005b) Heme oxygenase-1 accelerates protumoral effects of nitric oxide in cancer cells. *Virchows Arch.* **446**, 525–531.
37 Zhang YW, Ding LS, Lai MD (2003b) Reg gene family and human diseases. *World J. Gastroenterol.* **9**, 2635–2641.
38 Takekura N, Yasui W, Kyo E, Yoshida K, Kameda T, Kitadai Y, Abe K, Umezawa K, Tahara E (1991) Effects of tyrosine kinase inhibitor, erbstatin, on cell growth and growth-factor/receptor gene expression in human gastric carcinoma cells. *Int. J. Cancer* **47**, 938–942.
39 Alkhamesi NA, Ziprin P, Pfistermuller K, Peck DH, Darzi AW (2005) ICAM-1 mediated peritoneal carcinomatosis, a target for therapeutic intervention. *Clin. Exp. Metastasis* **22**, 449–459.
40 Skubitz AP (2002) Adhesion molecules. *Cancer Treat. Res.* **107**, 305–329.
41 Yokozaki H (2000) Molecular characteristics of eight gastric cancer cell lines established in Japan. *Pathol. Int.* **50**, 767–777.
42 Mori K, Aoyagi K, Ueda T, Danjoh I, Tsubosa Y, Yanagihara K, Matsuno Y, Sasako M, Sakamoto H, Mafune K, Kaminishi M, Yoshida T, Terada M, Sasaki H (2004) Highly specific marker genes for detecting minimal gastric cancer cells in cytology negative peritoneal washings. *Biochem. Biophys. Res. Commun.* **313**, 931–937.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd,

THYROID
Volume 20, Number 1, 2010
© Mary Ann Liebert, Inc.
DOI: 10.1089/thy.2009.0098

Improved Method for Analysis of RNA Present in Long-Term Preserved Thyroid Cancer Tissue of Atomic Bomb Survivors

Kiyohiro Hamatani,[1] Hidetaka Eguchi,[1,2] Mayumi Mukai,[1] Kazuaki Koyama,[1] Masataka Taga,[1] Reiko Ito,[1] Yuzo Hayashi,[3] and Kei Nakachi[1]

Background: Since many thyroid cancer tissue samples from atomic bomb (A-bomb) survivors have been preserved for several decades as unbuffered formalin-fixed, paraffin-embedded specimens, molecular oncological analysis of such archival specimens is indispensable for clarifying the mechanisms of thyroid carcinogenesis in A-bomb survivors. Although *RET* gene rearrangements are the most important targets, it is a difficult task to examine all of the 13 known types of *RET* gene rearrangements with the use of the limited quantity of RNA that has been extracted from invaluable paraffin-embedded tissue specimens of A-bomb survivors. In this study, we established an improved 5′ rapid amplification of cDNA ends (RACE) method using a small amount of RNA extracted from archival thyroid cancer tissue specimens.
Methods: Three archival thyroid cancer tissue specimens from three different patients were used as in-house controls to determine the conditions for an improved switching mechanism at 5′ end of RNA transcript (SMART™) RACE method; one tissue specimen with *RET/PTC1* rearrangement and one with *RET/PTC3* rearrangement were used as positive samples. One other specimen, used as a negative sample, revealed no detectable expression of the *RET* gene tyrosine kinase domain.
Results: We established a 5′ RACE method using an amount of RNA as small as 10 ng extracted from long-term preserved, unbuffered formalin-fixed, paraffin-embedded thyroid cancer tissue by application of SMART technology. This improved SMART RACE method not only identified common *RET* gene rearrangements, but also isolated a clone containing a 93-bp insert of rare *RTE/PTC8* in RNA extracted from formalin-fixed, paraffin-embedded thyroid cancer specimens from one A-bomb survivor who had been exposed to a high radiation dose. In addition, in the papillary thyroid cancer of another high-dose A-bomb survivor, this method detected one novel type of *RET* gene rearrangement whose partner gene is acyl coenzyme A binding domain 5, located on chromosome 10p.
Conclusion: We conclude that our improved SMART RACE method is expected to prove useful in molecular analyses using archival formalin-fixed, paraffin-embedded tissue samples of limited quantity.

Background

Molecular oncological analyses of archival tissue specimens are indispensable, especially for studies of rare cancers or cancers associated with uncommon past events, such as radiation exposures in Thorotrast treatment, nuclear power plant accidents, and atomic bombings. In recent years, application of new molecular techniques, including polymerase chain reaction (PCR) in the use of archival tissue samples, is anticipated to advance the understanding of the molecular mechanisms of these cancers (1).

Since many thyroid cancer tissue samples from atomic bomb (A-bomb) survivors have been preserved for a long time as unbuffered formalin-fixed, paraffin-embedded specimens, analysis of such archival specimens is indispensable for clarifying the characteristics of thyroid carcinogenesis in A-bomb survivors. *RET* gene rearrangements are thought to play an initial and critical role in papillary thyroid cancer development, although wide variations in the prevalence of such rearrangements, ranging from 3% to 70%, have been observed in different geographic areas (2–4). To date, at least 13 rearranged forms of the *RET* gene (*RET/PTC 1–9, PCM1-RET,*

[1]Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation, Hiroshima, Japan.
[2]Translational Research Center, Saitama Medical University, International Medical Center, Saitama, Hidaka, Japan.
[3]Geriatric Health Service Facility Hidamari, Hiroshima, Japan.

ELKS-RET, *ΔRFP-RET*, and *HOOK3-RET*) have been identified, of which *RET/PTC1* and *RET/PTC3* are by far the most frequent, from papillary thyroid cancers in patients both with and without radiation-exposure history (3,5–7). Analysis of *RET* gene rearrangements is one of the most important issues in molecular thyroid cancer research among A-bomb survivors. However, it is a difficult task to examine all types of *RET* rearrangements because of the limited quantity of paraffin-embedded tissue specimens from A-bomb survivors; namely, it is almost impossible to investigate all 13 rearranged forms of the *RET* gene using such limited amounts of RNA.

Clontech's (Mountain View, CA) switching mechanism at 5′ end of RNA transcript (SMART™) technology is based on the terminal transferase activity of reverse transcriptase, which adds a few additional nucleotides, primarily deoxycytidine, to the 3′ end of cDNA, creating an extended template at the 5′ end in combination with a SMART oligonucleotide (8). Therefore, PCR amplification of cDNA using a set of adaptor-specific primer and gene-specific primer will produce the cDNA that contains the complete 5′ end of mRNA (9).

We improved and established the 5′ rapid amplification of cDNA ends (RACE) method (10) using a very small amount of RNA extracted from unbuffered paraffin-embedded thyroid cancer tissue by application of SMART technology. In addition to the analysis of common *RET* gene rearrangements, this method succeeded in detecting not only rare *RET* gene rearrangements but also one *RET* gene rearrangement that has not yet been reported.

Materials and Methods

Tissue

All cancer tissue specimens used for this study were prepared from archival unbuffered formalin-fixed, paraffin-embedded blocks. Three thyroid cancer tissue specimens from three different patients were used as in-house controls to determine the conditions for an improved SMART RACE method; one tissue specimen with *RET/PTC1* rearrangement and one with *RET/PTC3* rearrangement were used as positive samples. One other specimen, used as a negative sample, revealed no detectable expression of the *RET* gene tyrosine kinase (TK) domain. The control samples had been preserved at room temperature for 19–21 years. Papillary thyroid cancer specimens from A-bomb survivors, preserved at room temperature for 20–50 years, were collected after approval from the Human Investigation Committee and the Ethics Committee for Genome Research at the Radiation Effects Research Foundation (RERF). After deparaffinization of 5 μm sections by Hemo-De (Fujisawa Yakuhin Kogyo, Osaka, Japan) and staining with methyl green (Sigma-Aldrich, St. Louis, MO), cancerous regions were isolated using disposable scalpels. All cancerous regions from two to four successive tissue sections were combined for RNA extraction.

RNA extraction

RNA was isolated from dissected tissue using the High Pure RNA Paraffin kit according to the manufacture's instructions (Roche Diagnostics, Manheim, Germany), with some modifications. Briefly, dissected tissue was digested with proteinase K at 55°C overnight, followed by DNase I treatment. After the lysate was purified by High Pure filter, RNA was eluted twice with 100 μL of RNase-free water. RNA was then precipitated by ethanol in the presence of 2 μL of ethachinmate (Nippon Gene, Tokyo, Japan) as a carrier and resuspended in 30 μL of RNase-free water.

cDNA synthesis

To enhance template activity of RNA extracted from archival unbuffered formalin-fixed, paraffin-embedded tissue specimens, total RNA was first heated in 10 mM of citrate buffer (pH 4.0) at 70°C for 45 minutes and then precipitated by ethanol (11). Then, 100 ng of total RNA and 50 pmol/μL of random primers (9 mer) were heated in 11 μL of RNase-free water at 65°C for 10 minutes and chilled in ice water. A mixture consisting of 4 μL of 5× reverse transcription (RT) buffer, 2 μL of 20 mM DTT, 1 μL of 10 mM dNTPs, and 1 μL of RNase Inhibitor (20 U/μL; TaKaRa, Tokyo, Japan) was added to the RNA solution and incubated at room temperature for 5 minutes. After addition of 1 μL of Rever Tra Ace (100 U/μL; Toyobo, Osaka, Japan), the reaction mixture was incubated at 42°C for 45 minutes.

Improved SMART RACE

After 1 μL of various concentrations of SMART adaptor (SMART II A oligonucleotide; Clontech) was added to 20 μL of cDNA solution, the mixture was further incubated at 42°C for 60 minutes and heated at 70°C for 15 minutes to inactivate reverse transcriptase. SMART RACE-PCR was performed in a 25 μL volume containing 1×PCR buffer; 200 μM each of dATP, dCTP, dGTP, and dTTP; 3 mM of $MgCl_2$; 0.4 μM of each primer (SMART adaptor-specific primer, S-RACE 1, 5′-AAGCAGTGGTAACAACGCAGAGTA-3′; exon 12 of *RET* gene-specific primer, *RET*-Ex12PR9, 5′-TCCGAGGGAATTC CCACTTT-3′); 0.5 U of FastStart Taq DNA polymerase containing a thermostable proof-reading protein (Roche Diagnostics); and 2 μL of SMART adaptor-treated cDNA. PCR was carried out on a DNA Engine using the following cycle conditions: at 95°C for 3 minutes; 45 cycles at 95°C for 30 seconds, at 60°C for 30 seconds, and at 72°C for 45 seconds; and a final extension at 72°C for 5 minutes. The first PCR products were diluted at 1000- to 10,000-fold in water, and 1 μL of diluted samples was used as a template for semi-nested SMART RACE-PCR with SMART adaptor and nested primer (*RET* gene-specific primer, *RET*-Ex12A4; Table 1). The second PCR conditions were as described in the first PCR, with the exception of annealing temperature (66°C instead of 60°C) and number of cycles (25 cycles instead of 45 cycles).

Cloning and sequencing of cDNA fragments

Five microliters of second SMART RACE-PCR products was electrophoresed on an 8% acrylamide gel and viewed with ethidium bromide. Target candidate bands were determined by *Bam*HI (TaKaRa) digestion of aliquots of second SMART RACE-PCR products, because the fragments amplified by SMART RACE-PCR included a *Bam*HI recognition site located at the 5′ end of exon 12 of the *RET* gene. Target candidate cDNA fragments derived from 50 μL of second SMART RACE-PCR products were eluted from 8% acrylamide gel and cloned into *Hinc*II-digested and dephosphorylated pUC118 vector using TaKaRa Blunting Kination Ligation kit (TaKaRa). Plasmid DNA containing a longer insert than 70 bp was

TABLE 1. PRIMERS AND OLIGONUCLEOTIDES USED FOR DETECTION OF mRNA EXPRESSION BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

	Primer name	*Sequence*	*Annealing temperature*
BCR	BCR-S2	5′-GGTGGGCGACCTCTTCCAGA-3′ (20 mer)	58°C
	BCR-A2	5′-TCCACGAAGGCCCGGTACAC-3′ (20 mer)	
Kinase domain of *RET*	RET-Ex12S5	5′-GAGCAGGGTACACCACGGTGG-3′ (21 mer)	60°C
	RET-Ex13A2	5′-CTCACTCGGGGAGGCGTTCT-3′ (20 mer)	
RET/PTC1	PTC1S14	5′-AACCGCGACCTGCGCAAAGC-3′ (20 mer)	58°C
	RET-Ex12A	5′-GAGGGAATTCCCACTTTGGA-3′ (20 mer)	
RET/PTC3	PTC3/4–12	5′-ACCCAAAAGCAGACCTTGGA-3′ (20 mer)	56°C
	RET-Ex12A4	5′-GAGGGAATTCCCACTTTGGA-3′ (20 mer)	
RET/PTC8	PTC8S1	5′-GAGTGATCTTTCTAGCAAAACACA-3′ (24 mer)	58°C
	RET-Ex12A4	5′-GAGGGAATTCCCACTTTGGA-3′ (20 mer)	
	PTC8-oligo77	5′-AAGAGAGGGAGAGTGATCTTTCTAGCAAAA CACAGCTGTTACAGGAGGATCCAAAGT GGGAATTCCCTCAAGCCGAA-3′ (77 mer)	
ACBD5-RET	ACBD5S1	5′-ACATTGCAGACTGCTCCTCA-3′ (20 mer)	58°C
	RET-Ex12A4	5′-GAGGGAATTCCCACTTTGGA-3′ (20 mer)	
	ACBD5-RET-oligo77	5′-TCAACATCAACATTGCAGACTGCTCCTCAG CCCACCTCACAGGAGGATCCAAAGTGGG AATTCCCTCGGAAGAACTT-3′ (77 mer)	

sequenced using DNA sequencer CEQ8000 (Beckman Coulter, Fullerton, CA), because the total length of the SMART adaptor and the 5′ portion of exon 12 of *RET* was 55 bp.

Detection of RET/PTC8 *expression in papillary thyroid cancer from A-bomb survivors by RT-PCR*

Amplification of the *BCR* gene as an internal control by RT-PCR was at first conducted for examination of availability of RNA extracted from archival tissue samples. The cDNA derived from 10 ng of total RNA was used as a template for RT-PCR. RT-PCR for detection of *RET/PTC8* and *ACBD5-RET* was performed in a 25 μL volume of solution containing 1×PCR buffer; 200 μM each of dATP, dCTP, dGTP, and dTTP; $MgSO_4$ (3.5 mM for *RET/PTC8* and 3.0 mM for *ACBD5-RET*); 0.4 μM of each primer (PTC8S1, RET-Ex12A4 for *RET/PTC8*; ACBD5S1, RET-Ex12A4 for *ACBD5-RET*); and 0.5 U of Platinum Taq DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA). PCR conditions consisted of initial denaturation (95°C for 3 minutes), followed by 40 cycles (denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 68°C for 45 seconds), and final extension at 68°C for 5 minutes. To detect expression of *BCR*, the TK domain of the *RET* gene, *RET/PTC1*, and *RET/PTC3*, RT-PCR was conducted using Platinum Taq DNA polymerase (Invitrogen) in the presence of 1×PCR buffer; 200 μM each of dATP, dCTP, dGTP, and dTTP; 2.5 mM $MgCl_2$ (2.0 mM for *RET/PTC3*); and 0.4 μM of each primer at 40 cycles for *BCR*, *RET/PTC1*, and *RET/PTC3*, and 36 cycles for the TK domain. Reaction conditions were as described in *RET/PTC8*, except for extension temperature (72°C instead of 68°C). Primer sets and annealing temperatures are summarized in Table 1. RT-PCR products of *RET/PTC8* and *ACBD5-RET* were confirmed to be actual products by sequencing after cloning of corresponding bands into a cloning vector.

RT-PCR products other than *RET/PTC8* and *ACBD5-RET* were confirmed to be actual products by digestion of restriction enzymes, *Bam*HI for *RET/PTC1* and *RET/PTC3*, *Alu*I for *BCR* (TaKaRa), and *Hae*III (New England Biolabs, Beverly, MA) for the *RET* gene TK domain, which existed within each amplified target fragment. For positive control of RT-PCR on the *BCR* gene, the *RET* gene TK domain and *RET/PTC1*, cDNA derived from a human thyroid cell line (TPC1) with *RET/PTC1* rearrangement was used as a template. For positive control of RT-PCR on *RET/PTC8* and *ACBD5-RET*, a mixture of 77 base-synthesized nucleotides (PTC8-oligo77 for *RET/PTC8* and ACBD5-RET-oligo77 for *ACBD5-RET*, respectively) and genomic DNA at a molar ratio of 1:1 was used as a template. For negative control, H_2O was used as a template. No amplification of RNA without RT was observed.

Results

Concentration of SMART adaptor

All cDNA syntheses in this study were performed with random primer (9 mer), since RNA extracted from archival formalin-fixed, paraffin-embedded thyroid cancer tissue specimens was invariably degraded to some degree. Using RNA prepared from each of two in-house controls harboring *RET/PTC1* and *RET/PTC3* rearrangements, first we examined the effects of concentration of SMART adaptor on the amplification efficiency by SMART RACE-PCR with gene-specific and adaptor-specific primers. One microliter of various concentrations of SMART adaptor was added to the reaction mixture immediately after cDNA synthesis had been completed. The mixture was further incubated at 42°C for 60 minutes in the presence of SMART adaptor. Since the total length of the SMART adaptor and the 5′ portion of exon 12 of *RET* is 55 bp, the length of amplified target fragments requires more than 70 bp to identify the counterpart gene. In addition, it is important to conduct cloning of obvious and intense fragments for successful isolation and identification of the counterpart gene. Therefore, among clear-cut fragments of