Reg IV is a serum biomarker for gastric cancer patients and predicts response to 5-fluorouracil-based chemotherapy

Yoshitsugu Mitani 1,2, Naohide Oue 1, Shunji Matsumura 1, Kazuhiro Yoshida 3, Tsuyoshi Noguchi 4, Masanori Ito 5, Shinji Tanaka 5, Kuniyasu Hiroki 6, Nobuyuki Kamata 2 and and Wataru Yasui 1

[1]Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan
[2]Department of Oral and Maxillofacial Surgery, Division of Cervico-Gnathostomatology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan
[3]Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan
[4]Department of Oncological Science (Surgery II), Oita University Faculty of Medicine, Oita, Japan
[5]Department of Medicine and Molecular Science, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan
[6]Department of Molecular Pathology, Nara Medical University, Kashihara, Japan

To whom correspondence should be addressed. E-mail: wyasui@hiroshima-u.ac.jp

Abstract

Regenerating gene family, member 4 (Reg IV), a secreted protein, is overexpressed in several cancers, including gastric cancer (GC). In the present study, we measured Reg IV levels in sera from patients with GC by enzyme-linked immunosorbent assay. We also examined the effect of forced Reg IV expression on the apoptotic susceptibility to 5-fluorouracil (5-FU). Forced expression of Reg IV inhibited 5-FU-induced apoptosis. Induction of Bcl-2 and dihydropyrimidine dehydrogenase was involved in inhibition of apoptosis. Among 36 GC patients treated with a combination chemotherapy of low-dose 5-FU and cisplatin, all 14 Reg IV-positive patients showed no change or disease progression. The serum Reg IV concentration was similar between healthy individuals (means.e., 0.520.05 ng/ml) and patients with chronic-active gastritis (0.360.09 ng/ml). However, the serum Reg IV concentration in presurgical GC patients was significantly elevated (1.960.17 ng/ml), even at stage I. The diagnostic sensitivity of serum Reg IV (36.1%) was superior to that of serum carcinoembryonic antigen (11.5%) or carbohydrate antigen 19-9 (13.1%). These results indicate that expression of Reg IV is a marker for prediction of resistance to 5-FU-based chemotherapy in patients with GC. Serum Reg IV represents a novel biomarker for GC.

Keywords: Reg IV, apoptosis, 5-fluorouracil, serum tumor marker, SAGE, gastric cancer

Introduction

Gastric cancer (GC) is one of the most common human cancers. Early detection remains the most promising approach to improve long-term survival of patients with GC. Assessment of tumor markers in serum may be useful for detection of GC. There are two available tumor markers for GC, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). However, CEA and CA19-9 are not suitable for early screening because preoperative positivity for these markers depends on the tumor stage at the time of detection (Kochi *et al.*, 2000). Therefore, there is an urgent need for new biomarkers for GC. Genes encoding transmembrane/secretory proteins expressed specifically in cancers may be ideal diagnostic biomarkers (Buckhaults *et al.*, 2001). Moreover, if the gene product functions in the neoplastic process, the gene is not just a biomarker but may also be a therapeutic target (Yasui *et al.*, 2004).

Despite improvements in cancer diagnosis and therapy, many patients are still diagnosed at the late stages of the disease, and the disease often recurs even after curative surgery. 5-fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents for breast cancer, colorectal cancer (CRC), and GC (Longley *et al.*, 2003). Unfortunately, some patients showed a poor response, possibly owing to inefficiency of the chemotherapy. For effective treatment, identification of the patients who will respond well to a specific chemotherapy may be important. Therefore, it is also important to look for biomarker to predict patients' response to 5-FU in GC.

We previously performed serial analysis of gene expression (SAGE) of four primary GCs (Oue *et al.*, 2004) and identified several GC-specific genes (Aung *et al.*, 2006). Of these genes, Regenerating gene family (REG), member 4 (*REG4*, which encodes Reg IV) is a candidate gene for cancer-specific expression, at least in patients with GC. Reg IV, a member of the REG gene family, was originally identified by high-throughput sequencing of a complementary DNA (cDNA) library derived from inflammatory bowel disease patient (Hartupee *et al.*, 2001). Quantitative reverse transcription–polymerase chain reaction (PCR) analysis revealed that approximately 50% of GCs overexpress the *REG4* gene (Oue *et al.*, 2004). Although various normal tissues express *REG4* (Hartupee *et al.*, 2001), the levels of expression are much lower in

normal tissues than in cancerous tissues (Oue *et al.*, 2005). We reported previously that Reg IV is expressed in GC cells but not stromal cells. Reg IV was expressed in 30% of GC tissues and was associated with both the intestinal mucin phenotype and neuroendocrine differentiation. In CRC, expression of Reg IV was observed in 36% of cases and was associated with tumor stage (Oue *et al.*, 2005). Furthermore, because it is a secreted protein, Reg IV may be a serum biomarker for GC; however, the concentration of Reg IV in serum has not been investigated.

The biologic function of Reg IV is poorly understood. Involvement of *REG4* in drug resistance has been suggested, but the detailed mechanism remains unclear (Violette *et al.*, 2003). A more recent study revealed that Reg IV is a potent activator of the epidermal growth factor receptor (EGFR)/Akt/activator protein-1 (AP-1) signaling pathway and those colon cancer cell lines treated with recombinant Reg IV showed increased expression of Bcl-2, Bcl-xl and survivin, which are proteins associated with inhibition of apoptosis (Bishnupuri *et al.*, 2006). EGFR activation modulates apoptotic susceptibility (reviewed by Kari *et al.*, 2003), and we have shown that EGFR is overexpressed in GC (Yasui *et al.*, 1988). Taken together, Reg IV may be a marker for prediction of resistance to 5-FU-based chemotherapy; however, modulation of apoptotic susceptibility by Reg IV has not been investigated.

In the present study, we show that forced expression of Reg IV inhibits apoptosis induced by 5-FU. Several molecules associated with resistance to 5-FU have been identified (reviewed by Longley *et al.*, 2003). We investigated expression of molecules associated with resistance to 5-FU in Reg IV-overexpressing cells. Because Reg IV activates EGFR, we also performed immunohistochemical analysis of Reg IV and EGFR expression in 161 cases of GC. We measured Reg IV levels in sera from patients with GC by enzyme-linked immunosorbent assay (ELISA) to investigate the potential utility of Reg IV measurements in the diagnosis of GC.

Results

Forced expression of Reg IV inhibits the mitochondrial apoptotic pathway

4

To investigate the biologic significance of Reg IV, the TMK-1 GC cell line was stably transfected with vector expressing Reg IV. TMK-1 cells were selected because they express low levels of Reg IV (Oue *et al.*, 2005). Clones were selected in G418 and examined for Reg IV expression by Western blot. Two clones, TMK-1-Reg IV-1 and TMK-1-Reg IV-2, expressed Reg IV at significantly higher levels than TMK-1 cells transfected with empty vector (Figure 1a). To determine the effect of Reg IV on 5-FU treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were performed. Cell growth of TMK-1 cells transfected with empty vector was inhibited by 5-FU in a dose-dependent manner (Figure 1b). This inhibition was partially ameliorated in Reg IV-overexpressing cells (Figure 1b), suggesting that cell proliferation was activated or apoptosis was inhibited in Reg IV-overexpressing cells. We investigated the effect of Reg IV on cell proliferation activity. Reg IV transfectants did not show significant differences in proliferation activity compared with cells transfected with empty vector (data not shown). We next examined the effect of forced Reg IV expression on the apoptotic susceptibility of these cells to 5-FU. As shown in Figure 1c, overexpression of Reg IV in both TMK-1-Reg IV-1 and TMK-1-Reg IV-2 cells significantly inhibited 5-FU-induced apoptosis in comparison with cells transfected with empty vector.   Apoptosis is controlled by two major pathways, the mitochondrial pathway (Green and Reed, 1998) and the membrane death receptor (DR) pathway (Ashkenazi and Dixit, 1999). In the mitochondrial pathway, release of cytochrome c by mitochondria into the cytosol is the rate-limiting step for the activation of caspases and endonucleases (Martinou *et al.*, 2000). Cytosolic cytochrome c activates procaspase-9 by binding to Apaf1 in the presence of dATP, leading to caspase-9 activation and subsequent activation of downstream effector caspases, including caspase-3, with triggering of apoptosis (Li *et al.*, 1997). Caspase-8 plays an important role in the DR-mediated apoptotic pathway, which is independent of cytochrome c release (Ashkenazi and Dixit, 1999). In our previous study, the mitochondrial apoptotic pathway was activated in 5-FU-induced apoptosis in TMK-1 cells (Tahara *et al.*, 2005). To determine the associated pathway inhibited by Reg IV overexpression, we examined expression of cytosolic cytochrome c protein in cytosolic extracts of 5-FU-treated and untreated cells by Western blotting. Incubation of cells with 5-FU induced cytochrome c expression in empty vector-transfected cells (Figure 1d). Cytochrome c release was inhibited in cells overexpressing Reg IV (Figure 1d). Next, we examined the activities

of caspase-3, -8 and -9. As shown in Figure 1e, treatment of cells with 5-FU significantly increased caspase-9 and -3 activities; but had no effect on caspase-8 activity. The activities of caspase-9 and -3 were significantly lower in Reg IV-overexpressing cells than in empty vector-transfected cells. The nuclear DNA repair enzyme poly(ADP-ribose)polymerase (PARP) is a target of caspase-3, and its cleavage can serve as a biochemical marker of apoptosis (Kaufmann *et al.*, 1993). We examined whether 5-FU-induced apoptosis is associated with PARP cleavage by Western blotting. Cleaved PARP was detected in 5-FU-treated empty vector-transfected cells; however, cleavage of PARP was reduced in Reg IV-overexpressing cells (Figure 1d). These results indicate that overexpression of Reg IV suppresses 5-FU-induced apoptosis by inhibiting the mitochondrial apoptotic pathway.

Reg IV activates phosphorylation of EGFR

Recombinant human Reg IV has been shown to induce rapid phosphorylation of EGFR at Tyr^{992} and Tyr^{1068} and Akt at Thr^{308} and Ser^{473}, resulting in increased AP-1 transcription factor activity (Bishnupuri *et al.*, 2006). In addition, HCT116 and HT29 colon cancer cell lines treated with recombinant Reg IV showed increased expression of Bcl-2 (Bishnupuri *et al.*, 2006). Bcl-2 is an antiapoptotic protein located on mitochondria and expressed at high levels in some tumor cells and tissues (Vander Heiden and Thompson, 1999). In the mitochondrial pathway, antiapoptotic Bcl-2 family proteins prevent mitochondrial membrane permeabilization and thereby inhibit changes in the mitochondrial membrane potential and cytochrome *c* release (Vander Heiden and Thompson, 1999). We examined phosphorylation of EGFR at Tyr^{992} and Tyr^{1068} in Reg IV-overexpressing cells. EGFR was phosphorylated at Tyr^{992} (Figure 1a) but not Tyr^{1068} in our Reg IV-overexpressing cells (data not shown). Expression of Bcl-2 was also examined by Western blotting, and induction of Bcl-2 in Reg IV-overexpressing cells was confirmed (Figure 1a). These results suggest that expression of Bcl-2 contributes to inhibition of the mitochondrial apoptotic pathway in Reg IV-overexpressing cells.

It was recently reported that AP-1 induces expression of dihydropyrimidine dehydrogenase (DPD) (Ukon *et al.*, 2005). DPD, an initial and rate-limiting enzyme in 5-FU catabolism, has significance for the pharmacokinetics and toxicity of 5-FU (Harris *et al.*, 1990). Overexpression of DPD in tumor cell lines is associated with resistance to 5-FU (Takebe *et al.*, 2001). Degradation of 5-FU by induction of DPD expression may

also inhibit 5-FU-induced apoptosis. We examined expression of DPD in Reg IV-overexpressing cells by Western blotting. Induction of DPD expression was observed in Reg IV-overexpressing cells (Figure 1a). We also examined expression of other enzymes involved in 5-FU metabolism. Expression of thymidylate synthase (TS) and orotate phosphoribosyl transferase (OPRT) was not changed significantly (Figure 1a). These results indicate that degradation of 5-FU by induction of DPD is also involved in inhibition of apoptosis by Reg IV.

Expression and distribution of Reg IV and EGFR in GC tissues

Because forced Reg IV expression induces phosphorylation of EGFR at Tyr^{992} in TMK-1 cells, we examined whether expression of Reg IV activates phosphorylation of EGFR at Tyr^{992} in human GC tissue samples. Immunostaining of Reg IV and EGFR was observed in 61 (37.9%) and 40 (24.8%) of 161 GC cases, respectively. Immunostaining of Tyr^{992} phospho-EGFR was also performed in 40 EGFR-positive GC cases. Interestingly, some, but not all, EGFR-positive cells showed phosphorylation at Tyr^{992}. Immunohistochemical analysis revealed that Reg IV was expressed in almost all EGFR-positive GC cases. Of 40 EGFR-positive GC cases, 37 (92.5%) were positive for Reg IV, whereas of 121 EGFR-negative GC cases, only 24 (19.8%) ($P<0.0001$, Fisher's exact test) were positive for Reg IV. In 37 GC cases expressing both Reg IV and EGFR, Reg IV and EGFR were rarely expressed in the same GC cells; however, Reg IV-positive GC cells were found near EGFR-positive GC cells (Figure 2a–f). Some GC cells were positive for both Reg IV and EGFR. Triple-immunofluorescence staining revealed that GC cells positive for both Reg IV and EGFR did not show phosphorylation of EGFR at Tyr^{992}. In addition, GC cells positive for phosphorylation at Tyr^{992} were located near Reg IV-positive cells (Figure 2g–j). We then analysed the relation of Reg IV and EGFR expression to clinicopathologic characteristics. There was no clear association between Reg IV expression and clinical characteristics (Table 1). In contrast, expression of EGFR was associated with advanced T grade (depth of invasion, $P=0.0004$, Fisher's exact test) and N grade (degree of lymph node metastasis, $P=0.0218$, Fisher's exact test) (Table 1). Moreover, EGFR staining was observed more frequently in stage III/IV cases (27 of 84 cases, 32.1%) than in stage I/II cases (13 of 77 cases,

16.9%, P=0.0291, Fisher's exact test) (Table 1). No statistically significant prognostic effect of Reg IV was found in the 101 advanced GC patients (P=0.9857, log-rank test) (Figure 2k); however, expression of EGFR was associated with poor survival (P=0.0006, log-rank test) (Figure 2k). These results suggest that Reg IV-positive GC cells were different from EGFR-positive GC cells, but GC cases containing EGFR-positive GC cells also contained Reg IV-positive GC cells, resulting in phosphorylation of EGFR at Tyr[992] in human GC tissues.

Relation between Reg IV expression and response of GC to a combination chemotherapy of low-dose 5-FU and cisplatin

We next examined the relation between Reg IV expression and response of GC to combination chemotherapy of low-dose 5-FU and cisplatin in recurrent GC tissue specimens. Reg IV expression was investigated in primary tumor samples obtained by surgical resection before the initiation of chemotherapy. We did not investigate the Reg IV expression in metastatic lesions because of lack of biopsy materials from the metastatic sites. The overall results are summarized in Table 2. Among the 36 patients treated with the combination chemotherapy, all 14 patients with Reg IV expression showed no change (NC) or a progressive disease (PD) to the combination chemotherapy, whereas eight (36.4%) of 22 patients without Reg IV expression showed a partial response (PR) (P=0.0132, Fisher's exact test). There was no association between EGFR expression and response to combination therapy (P=0.1596, Fisher's exact test). In these 36 GC cases, Reg IV expression was observed in all EGFR-positive GC cases.

Serum Reg IV concentration in healthy subjects, non-cancer patients and GC patients

We next examined whether Reg IV could be detected by ELISA in sera from patients with GC. Western blot analysis did not detect Reg IV protein in culture media of the MKN-1 and TMK-1 GC cell lines, whereas high levels of Reg IV protein were found in culture media of Reg IV-transfected TMK-1 cells (TMK-1-Reg IV-1 and TMK-1-Reg IV-2) and the MKN-45 GC cell line (Figure 3a). We confirmed by anti--actin Western blot that contamination of cells in culture medium was minimal. We used ELISA to test culture media from these cell lines. Reg IV protein was detected in culture media from TMK-1-Reg IV-1, TMK-1-Reg IV-2 and MKN-45 cell lines (Figure 3b), and the levels of Reg IV protein detected by ELISA were similar to those obtained by Western blot

8

analysis (Figure 3a). Reg IV protein was not detected in culture media of MKN-1 and TMK-1 cell lines by ELISA (Figure 3b). Culture media of Reg IV-transfected TMK-1 and MKN-45 cells were preabsorbed with recombinant Reg IV protein before being tested by ELISA. The specificity of Reg IV recognition was confirmed by the marked decrease in the ELISA signals after preabsorption (Figure 3b). The levels of serum Reg IV in healthy individuals, patients with chronic-active gastritis (*Helicobacter pylori* positive) and patients with GC before surgery are shown in Figure 3c. The serum Reg IV concentration was similar between healthy individuals (*n*=101, means.e., 0.520.05 ng/ml) and patients with chronic-active gastritis (*n*=20, 0.360.09 ng/ml). However, the serum Reg IV concentration in presurgical GC patients (*n*=61, 1.960.17 ng/ml) was significantly elevated (healthy individuals vs all GC patients, *P*<0.0001, Mann–Whitney *U*-test), even at stage I (healthy individuals vs stage I GC patients, *P*<0.0001, Mann–Whitney *U*-test) (Figure 3c). In Cases 36 and 42, which showed high serum concentrations of Reg IV, strong and extensive Reg IV staining was observed in the primary GC samples. In contrast, in Case 18, in which the serum Reg IV concentration was very low, no Reg IV staining was observed in the primary GC sample (Figure 3d). The Reg IV concentration in serum samples from patients with GC showing Reg IV-positive immunostaining (*n*=12, 2.510.40 ng/ml) was statistically significantly higher than that with GC showing Reg IV-negative immunostaining (*n*=49, 1.820.18 ng/ml) (*P*=0.0251, Mann–Whitney *U*-test). When the cutoff level for Reg IV was set at 2.00 ng/ml, the sensitivity and specificity for detection of GC were 36.1% (22/61) and 99.0% (100/101), respectively.

CEA and CA19-9 levels were also measured in the same serum samples. The sensitivity and specificity of CEA for detection of GC were 11.5% (7/61) and 100.0% (101/101), respectively. The sensitivity and specificity of CA19-9 for detection of GC were 13.1% (8/61) and 100.0% (101/101), respectively. Spearman's rank correlation test revealed only a weak correlation between serum Reg IV and CEA (*r*=0.0173, *P*=0.3123) or CA19-9 (*r*=0.0107, *P*=0.4279) (Figure 3e and f). Of GC patients with normal serum CEA values, 31.5% were found to express Reg IV at 99.0% specificity, and 32.1% of GC patients with normal serum CA19-9 values were found to express Reg IV at 99.0% specificity. The sensitivities of serum Reg IV, CEA and CA19-9 with respect to tumor stage are shown in Table 3. In patients with stage I GC, the sensitivity of serum Reg IV

(36.1%) was significantly higher than that of CEA (5.6%, P=0.0028, Fisher' exact test) or CA19-9 (8.3%, P=0.0093, Fisher' exact test).

Discussion

It is generally accepted that apoptosis suppresses oncogenic transformation. The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Apoptosis represents a major source of this attrition (Hanahan and Weinberg, 2000). Thus, resistance to apoptosis is a hallmark of most and perhaps all types of cancer. In the present study, we showed that overexpression of Reg IV inhibits 5-FU-induced apoptosis. At least two mechanisms are involved in inhibition of apoptosis by Reg IV, induction of Bcl-2 and induction of DPD.

In 5-FU-treated TMK-1 cells, overexpression of Reg IV inhibited the mitochondrial apoptotic pathway that involves cytosolic cytochrome c release and subsequent activation of caspase-9 and -3. Increased Bcl-2 by forced Reg IV expression may act to inhibit the mitochondrial apoptotic pathway. Bcl-2 induction by Reg IV is blocked by AG1478, a tyrosine kinase inhibitor specific for EGFR (Bishnupuri *et al.*, 2006), indicating that phosphorylation of EGFR is required for Bcl-2 induction and that EGFR plays an important role in inhibition of apoptosis by Reg IV. In the present study, immunohistochemical analysis of GC tissues revealed that almost all EGFR-positive GC cases (92.5%) also expressed Reg IV and that EGFR was phosphorylated at Tyr[992] in all EGFR-positive GC cases. Although it is possible that other molecules, such as EGF, induce phosphorylation of EGFR at Tyr[992], the present results suggest that in a subset of EGFR-positive GC, Reg IV affects phosphorylation of EGFR at Tyr[992] and stimulates tumor cell growth by inhibiting apoptosis. It is important to note that in the immunohistochemical analysis, GC cells expressing both Reg IV and EGFR were rare. Triple-immunofluorescence staining revealed that GC cells positive for both Reg IV and EGFR did not show phosphorylation of EGFR at Tyr[992]. We cannot explain completely the discrepancy between GC cell line data and GC tissue data. Because GC cells positive for phosphorylation at Tyr[992] were located near Reg IV-positive cells, Reg IV

10

expression may induce expression and secretion of EGFR ligands, such as EGF, which could lead to EGFR phosphorylation of the adjacent cells. EGF secretion in Reg IV-overexpressing cells should be examined. Furthermore, clinical characteristics of Reg IV-positive GC cases were quite different from those of EGFR-positive GC cases in the present study. Expression of Reg IV was found in both early- and late-stage GC, whereas expression of EGFR was detected mainly in late-stage tumors. These results led us to speculate that continuous expression of Reg IV may be a selective pressure for the development of EGFR-positive GC cells and may confer selective growth advantage to EGFR-positive GC cells. In Reg IV-positive GC, even at an early stage, EGFR-positive GC cells may develop in accordance with tumor progression.

Forced Reg IV expression induced expression of DPD. DPD is a catabolic enzyme of 5-FU (Harris *et al.*, 1990), and several studies have indicated an inverse correlation between expression or activity of DPD and sensitivity to 5-FU-based chemotherapy in GC (Inada *et al.*, 2000; Ishikawa *et al.*, 2000). Degradation of 5-FU by DPD may play an important role in inhibition of 5-FU-induced apoptosis. In the present study, Reg IV expression in primary tumors was associated with response to 5-FU/cisplatin combination chemotherapy in recurrent GC cases. Although we did not evaluate activation of AP-1 in the present study, EGFR-dependent activation of AP-1 by Reg IV has been reported (Bishnupuri *et al.*, 2006). Because DPD is a downstream target gene of AP-1 (Ukon *et al.*, 2005), phosphorylation of EGFR may be required for induction of DPD by Reg IV. As mentioned above, induction of Bcl-2 also depends on phosphorylation of EGFR. Phosphorylation of EGFR may be a crucial event in inhibition of 5-FU-induced apoptosis by Reg IV. Interestingly, EGFR expression in primary tumors was not associated with response to combination chemotherapy in the present study. We confirmed that all EGFR-positive GC cases were positive for phosphorylation of EGFR at Tyr^{992}. We investigated both Reg IV and EGFR expression in primary tumors, but the response to chemotherapy was evaluated in metastatic lesions. Several lines of evidence suggest that primary and metastatic lesions bear different biologic properties. DPD gene expression levels were reported to be lower in primary cancers than in liver metastases in several studies (Kuramochi *et al.*, 2006). Because expression of EGFR is a late event in GC progression, metastatic lesions may express EGFR even though EGFR is not expressed by the primary tumor. Because we

11

investigated neither Reg IV nor EGFR expression in metastatic lesions, further studies are needed to clarify the predictive value of Reg IV and EGFR expression analysis.

It is important to note that while TMK-1-Reg IV-2 clone expresses more Reg IV than TMK-1-Reg IV-1, the intensity of the DPD band for TMK-1-Reg IV-1 looks slightly more intense than the intensity of the DPD band for TMK-1-Reg IV-2. In the present study, Western blotting was performed 1 or more months after the TMK-1 cell line had been stably transfected with vector expressing Reg IV. It has been reported that EGFR phosphorylation induced by treatment with recombinant human Reg IV occurred rapidly, with maximum effect at 5 min (Bishnupuri *et al.*, 2006), suggesting that EGFR phosphorylation levels may reach plateau in Reg IV-transfected TMK-1 cells and DPD expression may not increase further with increase in Reg IV expression.

Serum Reg IV is a novel biomarker for GC. Despite the reliability of CEA and CA19-9 as markers for detection of GC, CEA and CA19-9 are unsuitable for detection of early GC. In fact, in the present study, CEA and CA19-9 were found in serum in 5.6 and 8.3%, respectively, of patients with stage I GC. Of 36 serum samples from patients with stage I GC, 13 (36.1%) showed high levels of Reg IV, indicating that Reg IV is a good serum marker for early detection of GC. In addition, because Reg IV levels in serum samples from patients with GC expressing Reg IV were significantly higher than those of Reg IV-negative GC, Reg IV concentration in sera may be a marker for prediction of the response to 5-FU-based chemotherapy. However, all GC cases with high serum concentrations of Reg IV did not necessarily show Reg IV immunostaining of the primary tumor. This discrepancy between immunostaining and ELISA results may be due to methodologic differences. Reg IV immunohistochemistry results were evaluated as the percentage of stained cancer cells; the intensity of immunostaining was not evaluated because we had no suitable internal control for the immunohistochemistry. More detailed quantitative methods for the measurement of Reg IV protein, such as intratumor Reg IV concentration, are needed to clarify the relation between levels of Reg IV protein in sera and levels in primary GC samples.

In conclusion, our present data show that Reg IV can confer resistance to 5-FU-induced apoptosis in GC cells, suggesting that overexpression of Reg IV may represent a novel mechanism of intrinsic drug resistance in human GC. We detected Reg IV in sera from

patients with GC. This indicates not only that serum Reg IV is a novel biomarker for GC but also that serum Reg IV may have some effect on normal organs in patients with GC. Identification of a cell-surface receptor for Reg IV may further improve our understanding of the basic biology of Reg IV.

Materials and methods

Cell line, expression vector, transfection and 5-FU treatment
A human GC-derived cell line, TMK-1, was established in our laboratory (Ochiai et al., 1985). TMK-1 cells were maintained in Roswell's Park Memorial Institute 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Whittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For constitutive expression of the REG4 gene, cDNA was PCR amplified and subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA, USA). The pcDNA-Reg IV expression vector was transfected into TMK-1 cells with FuGENE6 (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. Stable transfectants were selected after 2 weeks of culture with 100 g/ml G418 (Invitrogen). The effect of 5-FU (Acros Organics, Fairlawn, NJ, USA) on apoptosis was studied. 5-FU was dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted in cell culture medium for experiments. The final concentration of DMSO was maintained at 0.1%. For apoptosis assay, caspase activation assays and Western blot analysis, both floating and attached cells were collected after 5-FU treatment.

MTT, cell proliferation, apoptosis and caspase activation assays
For MTT assay, the cells were seeded at a density of 2000 cells per well in 96-well plates. The cells were then treated with 5-FU for 48 h. Cell growth was monitored by MTT assay (Alley et al., 1988). Cell proliferation activity was determined with a Cell Proliferation ELISA (Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's instructions. For apoptosis assay, cultured cells were treated for 48 h with 2 mm 5-FU, and apoptosis was evaluated with a Cell Death Detection ELISA[Plus] Kit (Roche Diagnostics), according to the manufacturer's instructions. For caspase activation assays, cultured cells were treated for 36 h with 2 mm 5-FU and the activities

of caspase-3, -8 and -9 were determined with caspase-3, -8 and -9 Colorimetric Activity Assay Kits, respectively (Chemicon, Temecula, CA, USA), according to the manufacturer's instructions. *P*-values for all the assays were calculated using Student's *t*-test.

Western blot analysis

Western blot analysis was performed as described previously (Yasui *et al.*, 1993). The filter was incubated for 1 h at room temperature with an anti-Reg IV antibody (rabbit polyclonal antibody raised in our laboratory; Oue *et al.*, 2005), anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-EGFR antibody (Cell Signaling Technology, Beverly, MA, USA), anti-phospho-EGFR (Tyr^{992}) antibody (Cell Signaling Technology), anti-phospho-EGFR (Tyr^{1068}) antibody (Cell Signaling Technology), anti-DPD antibody (Taiho Pharmaceutical, Tokyo, Japan), anti-TS antibody (Taiho Pharmaceutical), anti-OPRT antibody (Taiho Pharmaceutical), anti-PARP p85 fragment antibody (Promega, Madison, MD, USA) or anti--actin antibody (Sigma Chemical, St Louis, MO, USA). To quantify cytochrome *c* release into the cytosol, floating and attached cells were collected after incubation with 2 mm 5-FU for 36 h, and cytochrome *c* in cytosolic extracts was detected with an ApoAlert Cell Fractionation Kit (Takara Bio, Shiga, Japan).

Tissue samples

In all, 161 primary tumors and 61 serum samples were collected from patients diagnosed with GC. Patients were treated at the Hiroshima University Hospital or an affiliated hospital.

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 161 patients who had undergone surgical excision of GC. Of the 161 patients, 27 had early GC and 134 had advanced GC. Early GC is limited to the mucosa or the mucosa and submucosa regardless of nodal status. Advanced GC is a tumor that has invaded beyond the muscularis propria (Hohenberger and Gretschel, 2003). Information on patient survival was available for 101 of the 134 advanced GC cases.

Of the 161 patients, 36 had recurrent GC and were treated with a combination of low-dose 5-FU and cisplatin (Kim *et al.*, 1999). All 36 patients with recurrent GC

14

provided a medical history and underwent physical examination, including evaluation of performance status, complete blood cell count, serum chemistry profile, chest X-ray and computed tomography (CT) and/or magnetic resonance imaging, at the time of enrollment. Tumor markers, including CEA and CA19-9, were checked monthly. The responses of metastatic lesions to treatment were assessed according to the World Health Organization criteria. Metastatic lesions were evaluated by CT, ultrasonography and other radiographic examinations. Complete response (CR) was defined as disappearance of all evidence of cancer for more than 4 weeks. PR was defined as at least 50% reduction in the sum of the products of the perpendicular diameters of all the lesions for more than 4 weeks without any evidence of new lesions or progression of existing lesions. NC was defined as less than 50% reduction or less than 25% increase in the sum of the products of the perpendicular diameters of all lesions without any evidence of new lesions. PD was defined as more than a 25% increase in more than one lesion or the appearance of new lesions.

Among the 161 GC cases used for immunohistochemical analysis, serum samples were available for ELISA from 61 cases (36 men and 25 women; age range, 35–88 years; mean, 67.5 years). Serum samples were collected before surgery and before initiation of therapy, and were stored at -80°C until analysis. Serum samples from 20 patients with chronic active gastritis with *H. pylori* infection (13 men and 7 women; age range, 57–85 years; mean, 68.8 years) were also collected. Control serum samples were obtained from 101 healthy individuals (75 men and 26 women; age range, 32–79 years; mean, 59.4 years).

Tumor staging was according to the TNM classification system (Sobin and Wittekind, 2002). 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

For immunostaining of EGFR and phospho-EGFR (Tyr^{992}), a Dako LSAB Kit (Dako, Carpinteria, CA, USA) was used as described previously (Oue *et al.*, 2005). Sections were incubated with the following antibodies: goat anti-Reg IV (diluted 1:50, R&D Systems, Abingdon, UK), mouse anti-EGFR (1:20, Novocastra, Newcastle, UK) and rabbit anti-phospho-EGFR (Tyr^{992}) antibody (1:20, Cell Signaling Technology). For immunostaining of Reg IV, peroxidase-conjugated anti-goat IgG was used as the secondary antibody. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. The percentage of stained cancer cells was evaluated for each antibody. A result was considered positive if at least 10% of cells were stained. When fewer than 10% of cancer cells were stained, the immunostaining was considered negative.

For triple-immunofluorescence staining, Alexa Fluor 405-conjugated anti-goat IgG (Molecular Probes, Eugene, OR, USA), Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) and Alexa Fluor 546-conjugated anti-rabbit IgG (Molecular Probes) were used as secondary antibodies.

ELISA

For measurement of the serum concentration of Reg IV, a sandwich ELISA was developed. First, polystyrene microtiter plates were coated with mouse monoclonal anti-Reg IV antibody (R&D Systems) by overnight incubation of 50 l/125 ng/well antibody diluted in Tris buffer (pH 7.4). The plates were then washed three times with washing buffer. After the plates were blocked with 1% milk in phosphate-buffered saline, 50 l of recombinant Reg IV standard or sample was added to each well and incubated overnight at 4°C. After three washes, 50 l of biotinylated goat polyclonal anti-Reg IV antibody (R&D Systems) in assay buffer (1% bovine serum albumin (BSA), Tris buffer (pH 7.4), 0.05% normal goat serum) was added to each well (75 ng antibody/well). The mixture was then incubated for 1 h with shaking at 37°C and washed three times with washing buffer. The plates were incubated with 50 l/well alkaline phosphatase-conjugated streptavidin (Dako) diluted 2000-fold in diluent containing 1% BSA and Tris buffer (pH 7.4) for 1 h at 37°C and washed three times. Color development was performed with the addition of pNPP chromogenic substrate

(Sigma) followed by incubation at 37°C for 1 h. Absorbance at 405 nm was measured with an ELISA plate reader. As a reference standard, known concentrations of human recombinant Reg IV (Oue *et al.*, 2005) from 0 to 30 ng/ml were tested in triplicate.

Measurement of CEA and CA19-9

CEA and CA19-9 were measured with a commercially available automated immunoassay method (Modular Analytics, Roche Diagnostics). The upper limits of normal for this method are 5.0 ng/ml for CEA and 37 U/ml for CA19-9.

Statistical methods

Associations between clinicopathologic parameters and Reg IV or EGFR expression were analysed by Fisher's exact test. Kaplan–Meier survival curves were constructed for Reg IV- or EGFR-positive and Reg IV- or EGFR-negative patients. Survival rates were compared between Reg IV- or EGFR-positive and Reg IV- or EGFR-negative groups. Differences between survival curves were tested for statistical significance by log-rank test (Mantel, 1966). Differences in the serum concentration of Reg IV between two groups were tested by non-parametric Mann–Whitney *U*-test. Correlation between the serum concentration of Reg IV and that of CEA or CA19-9 was assessed by Spearman's rank correlation. A *P*-value of less than 0.05 was considered statistically significant.

References

Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL *et al*. (1988). Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* 48: 589–601.

Ashkenazi A, Dixit VM. (1999). Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol* 11: 255–260.

Aung PP, Oue N, Mitani Y, Nakayama H, Yoshida K, Noguchi T *et al*. (2006). 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.

Bishnupuri KS, Luo Q, Murmu N, Houchen CW, Anant S, Dieckgraefe BK. (2006). Reg IV activates the epidermal growth factor receptor/Akt/AP-1 signaling pathway in colon adenocarcinomas. *Gastroenterology* 130: 137–149.

Buckhaults P, Rago C, St Croix B, Romans KE, Saha S, Zhang L *et al*. (2001). Secreted and cell surface genes expressed in benign and malignant colorectal tumors. *Cancer Res* 61: 6996–7001.

Green DR, Reed JC. (1998). Mitochondria and apoptosis. *Science* 281: 1309–1312.

Hanahan D, Weinberg RA. (2000). The hallmarks of cancer. *Cell* 100: 57–70.

Harris BE, Song R, Soong SJ, Diasio RB. (1990). Relationship between dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels with evidence for circadian variation of enzyme activity and plasma drug levels in cancer patients receiving 5-fluorouracil by protracted continuous infusion. *Cancer Res* 50: 197–201.

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.

Hohenberger P, Gretschel S. (2003). Gastric cancer. *Lancet* 362: 305–315.

Inada T, Ogata Y, Kubota T, Tomikawa M, Yamamoto S, Andoh J *et al*. (2000). 5-Fluorouracil sensitivity and dihydropyrimidine dehydrogenase activity in advanced gastric cancer. *Anticancer Res* 20: 2457–2462.

Ishikawa Y, Kubota T, Otani Y, Watanabe M, Teramoto T, Kumai K *et al*. (2000). Dihydropyrimidine dehydrogenase and messenger RNA levels in gastric cancer: possible predictor for sensitivity to 5-fluorouracil. *Jpn J Cancer Res* 91: 105–112.

Kari C, Chan TO, Rocha de Quadros M, Rodeck U. (2003). Targeting the epidermal growth factor receptor in cancer: apoptosis takes center stage. *Cancer Res* 63: 1–5.

Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. (1993). Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res* 53: 3976–3985.

Kim R, Murakami S, Ohi Y, Inoue H, Yoshida K, Toge T. (1999). A phase II trial of low dose administration of 5-fluorouracil and cisplatin in patients with advanced and recurrent gastric cancer. *Int J Oncol* 15: 921–926.

Kochi M, Fujii M, Kanamori N, Kaiga T, Kawakami T, Aizaki K *et al*. (2000). Evaluation of serum CEA and CA19–9 levels as prognostic factors in patients with gastric cancer. *Gastric Cancer* 3: 177–186.

Kuramochi H, Hayashi K, Uchida K, Miyakura S, Shimizu D, Vallbohmer D *et al*. (2006). 5-Fluorouracil-related gene expression levels in primary colorectal cancer and corresponding liver metastasis. *Int J Cancer* 119: 522–526.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES *et al*. (1997). Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479–489.

Longley DB, Harkin DP, Johnston PG. (2003). 5-Fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 3: 330–338. |

Mantel N. (1966). Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep* 50: 163–170.

Martinou JC, Desagher S, Antonsson B. (2000). Cytochrome *c* release from mitochondria: all or nothing. *Nat Cell Biol* 2: E41–43.

Ochiai A, Yasui W, Tahara E. (1985). Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. *Jpn J Cancer Res* 76: 1064–1071.

Oue N, Hamai Y, Mitani Y, Matsumura S, Oshimo Y, Aung PP *et al*. (2004). 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.

Oue N, Mitani Y, Aung PP, Sakakura C, Takeshima Y, Kaneko M *et al*. (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.

Sobin LH, Wittekind CH (eds). (2002). *TNM Classification of Malignant Tumors* 6th edn. Wiley-Liss Inc: New York, pp 65–68.

Tahara Jr E, Tahara H, Kanno M, Naka K, Takeda Y, Matsuzaki T *et al*. (2005). G1P3, an interferon inducible gene 6–16, is expressed in gastric cancers and inhibits mitochondrial-mediated apoptosis in gastric cancer cell line TMK-1 cell. *Cancer Immunol Immunother* 54: 729–740.

Takebe N, Zhao SC, Ural AU, Johnson MR, Banerjee D, Diasio RB *et al*. (2001). Retroviral transduction of human dihydropyrimidine dehydrogenase cDNA confers resistance to 5-fluorouracil in murine hematopoietic progenitor cells and human CD34+-enriched peripheral blood progenitor cells. *Cancer Gene Ther* 8: 966–973.

Ukon K, Tanimoto K, Shimokuni T, Noguchi T, Hiyama K, Tsujimoto H *et al*. (2005). Activator protein accelerates dihydropyrimidine dehydrogenase gene transcription in cancer cells. *Cancer Res* 65: 1055–1062.

Vander Heiden MG, Thompson CB. (1999). Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? *Nat Cell Biol* 1: E209–216.

Violette S, Festor E, Pandrea-Vasile I, Mitchell V, Adida C, Dussaulx E *et al*. (2003). Reg IV, a new member of the regenerating gene family, is overexpressed in colorectal carcinomas. *Int J Cancer* 103: 185–193.

Yasui W, Ayhan A, Kitadai Y, Nishimura K, Yokozaki H, Ito H *et al*. (1993). Increased expression of p34cdc2 and its kinase activity in human gastric and colonic carcinomas. *Int J Cancer* 53: 36–41.

Yasui W, Oue N, Ito R, Kuraoka K, Nakayama H. (2004). Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implications. *Cancer Sci* 95: 385–392.

Yasui W, Sumiyoshi H, Hata J, Kameda T, Ochiai A, Ito H *et al*. (1988). Expression of epidermal growth factor receptor in human gastric and colonic carcinomas. *Cancer Res* 48: 137–141.
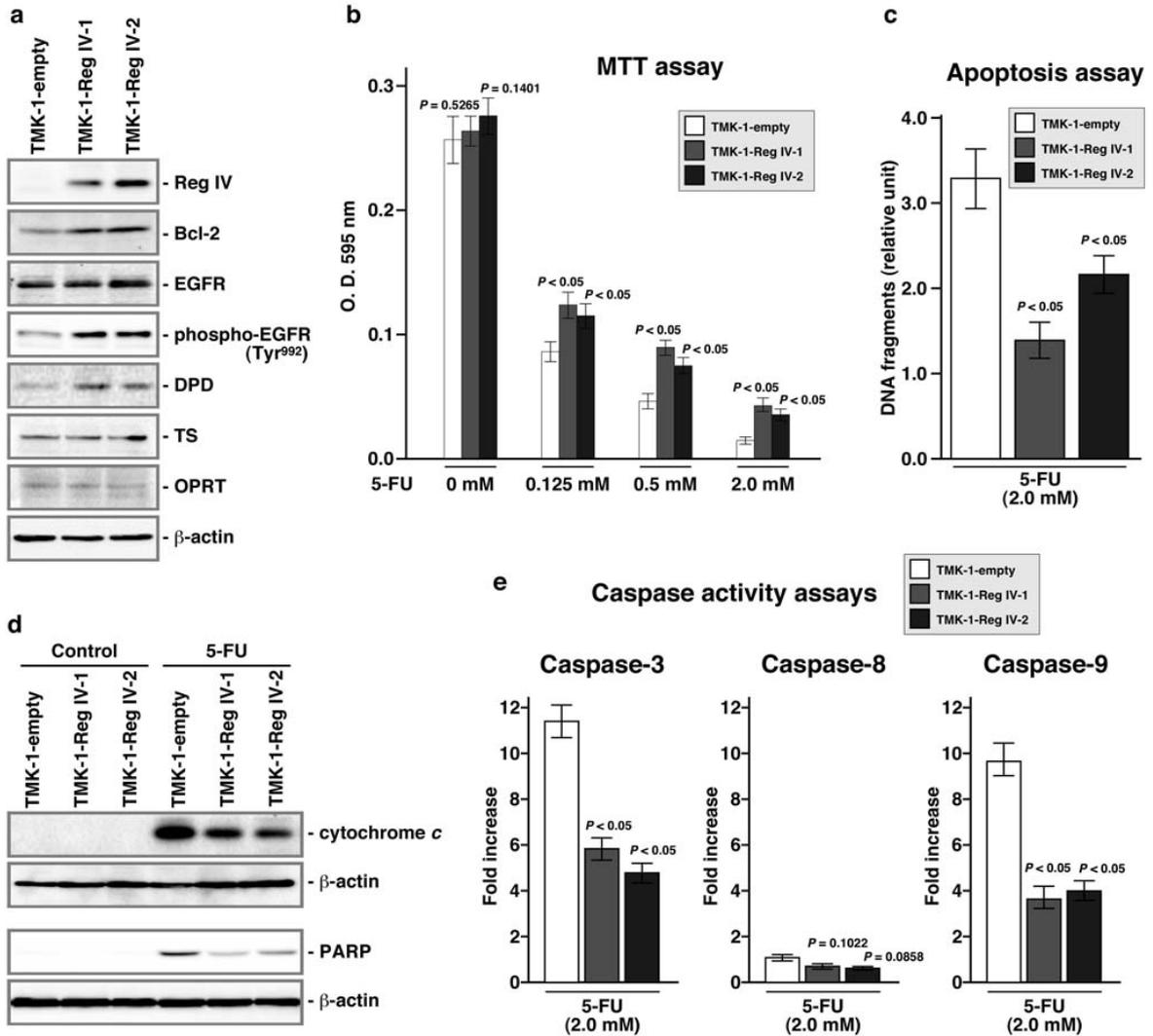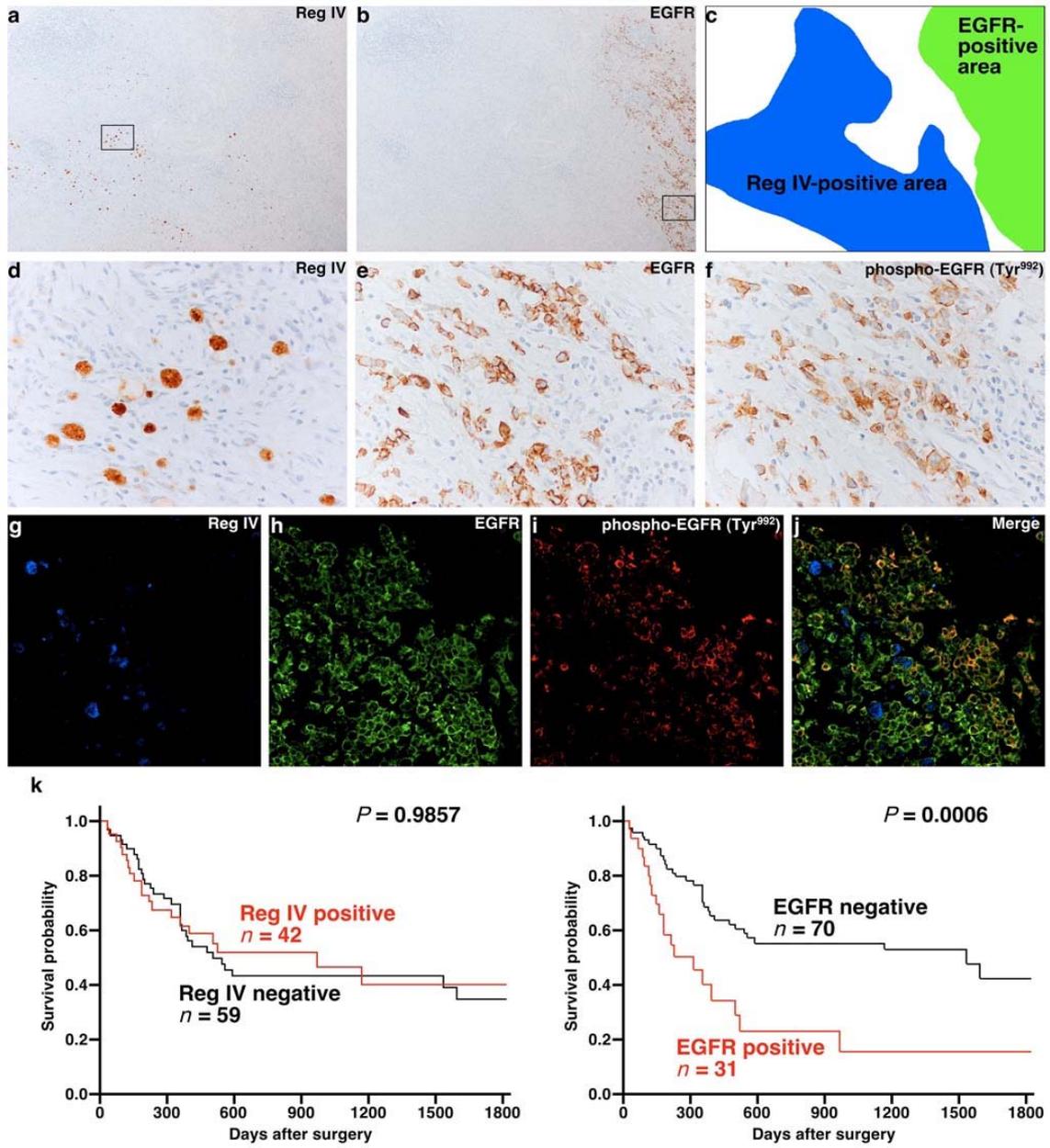
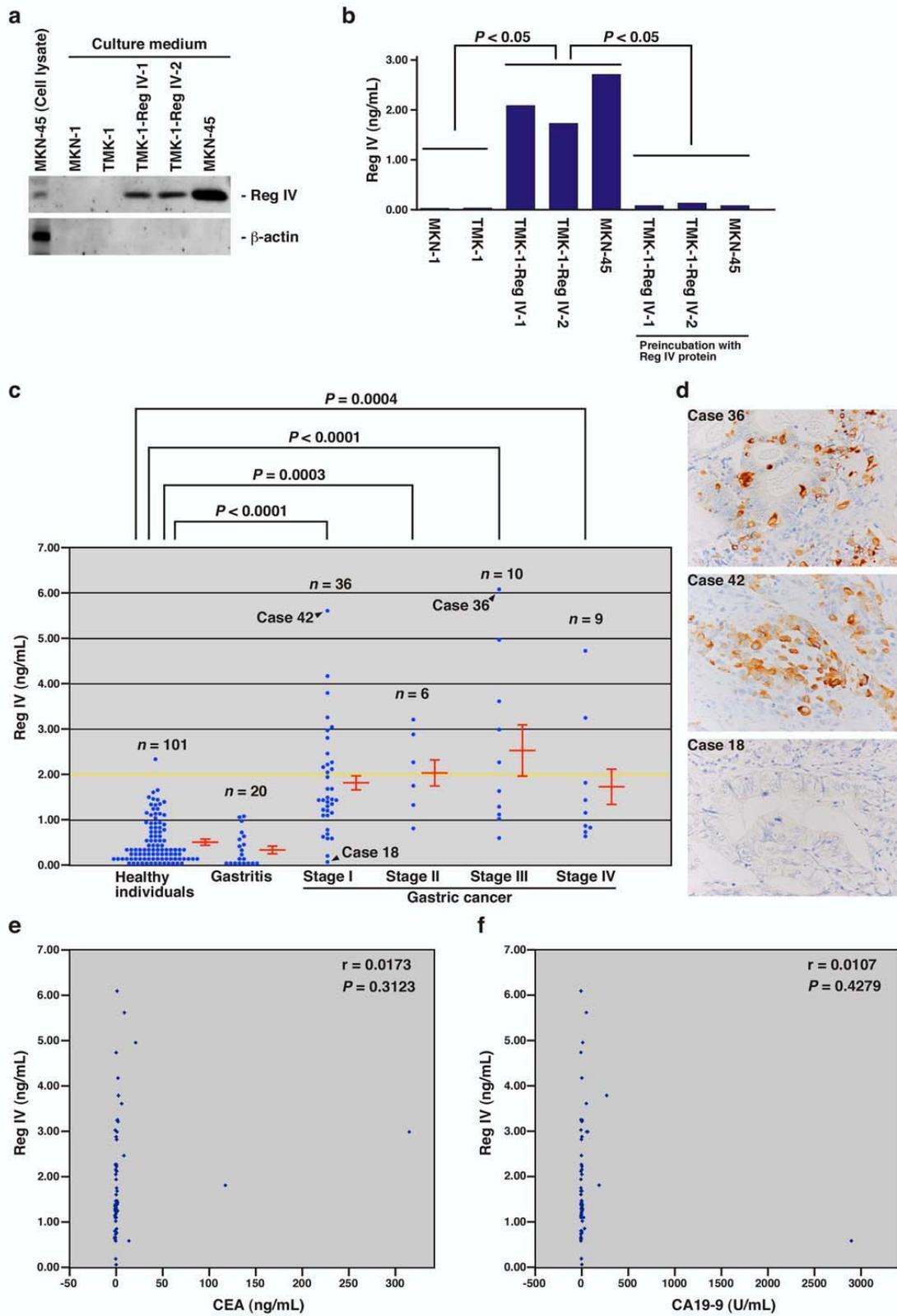Acknowledgements

Fig. 1

Fig. 2

Fig. 3

Table 1 Association of Reg IV and EGFR expression with clinicopathologic features of gastric cancer

	Reg IV expression			EGFR expression		
	Positive	Negative	P value[a]	Positive	Negative	P value[a]
T grade						
T1	8 (29.6%)	19	0.3895	0 (0.0%)	27	0.0004
T2/3/4	53 (39.6%)	81		40 (29.9%)	94	
N grade						
N0	20 (34.5%)	38	0.6120	8 (13.8%)	50	0.0218
N1/2/3	41 (39.8%)	62		32 (31.1%)	71	
Stage						
I/II	27 (35.1%)	50	0.5180	13 (16.9%)	64	0.0291
III/IV	34 (50.5%)	50		27 (32.1%)	57	

[a]Fisher's exact test.

Table 2 Association of Reg IV and EGFR expression with response to combination chemotherapy of low-dose 5-FU and cisplatin

	Reg IV expression			EGFR expression		
	Positive	Negative	P value[a]	Positive	Negative	P value[a]
CR and PR[b]	0 (0.0%)	8	0.0132	0 (0.0%)	8	0.1596
NC and PD[c]	14 (50.0%)	14		9 (32.1%)	19	

[a]Fisher's exact test. [b]Complete response and partial response. [c]No change and progressive disease.

Table 3 Diagnostic sensitivity of serum Reg IV, CEA, and CA19-9 with respect to tumor stage

	Reg IV	CEA	P value[a]	CA19-9	P value[b]
Stage I (n = 36)	36.1%	5.6%	0.0028	8.3%	0.0093
Stage II (n = 6)	50.0%	0.0%	0.1818	0.0%	0.1818
Stage III (n = 10)	50.0%	40.0%	1.0000	30.0%	0.6499
Stage IV (n = 9)	22.2%	11.1%	1.0000	22.2%	1.0000
Specificity	99.0%	100.0%		100.0%	

[a]Fisher's exact test. Reg IV vs. CEA. [b]Fisher's exact test. Reg IV vs. CA19-9.