ができれば異常なゲノムが生成される可能性は少ない。修復機能が完全に対応することができないために、細胞の生存と引き換えに異常なゲノムが生成されて、それが放射線発がんの原因の一つになると考えられている。本研究では、放射線発がんの根本的な機構を理解するためにRad51パラログを中心とした相同組換えの分子機構を解明することを目的とする。

B．研究方法

ヒト細胞でジーン・ターゲティングが比較的容易であることが知られている大腸がん細胞株HCT116において、Rad51Bをノックアウトする。これらの変異細胞と元の野生株を用いて、DNA損傷に対する感受性、相同組換えの頻度を表す指標、蛍光免疫染色による放射線照射後のRad51の核内フォーカス形成、γ-tubulin染色による中心体の数と形態、FISHによる染色体の数的異常を解析する。また、異常がみられた表現型に対して、正常cDNAを変異細胞に導入することによって、それが補正されるかどうか確認する。

（倫理面への配慮）

ヒト由来の株化細胞を用いた細胞生物学的実験であり、直接ヒト由来の材料を用いることはないので特に倫理面への配慮は必要ない。

C．研究結果

HCT116でRad51Bを順次ノックアウトした結果、この細胞ではRad51Bが4コピー存在することが判明した。正常な遺伝子が1コピーのみ存在するトリプル・ノックアウト細胞は増殖も遅く、ジーン・ターゲティングの頻度もほぼ0であったために、この細胞を変異細胞の代表として用いた。

放射線、マイトマイシンC、シスプラチンに対する感受性は野生株と比べて変異細胞では2-3倍程度亢進していた。相同組換え機能の一つの指標ともなる姉妹染色分体の交換頻度は野生株の60から70％のレベルまで低下していた。Rad51はDNA損傷時に核内の損傷部位にリクルートされるが、変異細胞ではこの頻度が有意に低下していた。

中心体は正常では1つの細胞に1つあるいは2つのみ観察され、3つ以上は異常である。野生株における異常頻度は5％であるのに対して、Rad51B変異細胞では14％まで上昇していた。染色体7番のFISHによる染色体解析を行ったところ、野生株細胞では1つあるいは3つで示される異数体の頻度は5.4％であるのに対して、変異細胞では14％であった。また、染色体17番では野生株では3.8％であるのに対して、変異細胞では10.2％であった。これらの異常は正常cDNAを導入することによって野生株のレベルまで回復した。また変異細胞はHCT116において4コピー存在するRad51Bのうち3コピーを欠損して1コピーのみ有する細胞を用いたが、2コピーのみ有する細胞を用いても程度は軽いが同様の異常が観察された。

D．考察

Rad51B変異細胞で観察されたDNA損傷に対する感受性の亢進、相同組換えの低下、Rad51の核内フォーカス形成の低下はいずれもがRad51Bが相同組換えの前期においてRad51と協調してDNA修復の役割を果たすことを示唆する。これに加えて、中心体の数の増加と異数体の増加が観察されたが、これらの表現型はRad51Bの関しては初めての報告となる。すでに他の動物ではRad51Bの変異細胞の作製が報告されているが、後者の異常は報告されていなかった。このような染色体の異常が今回初めて明らかになった理由としては、この異常がヒト細胞に特異的である可能性、あるいはがん細胞に特異的である可能性が考えられる。特にがん細胞においては数多くの遺伝子の異常が存在するために、その細胞の遺伝学的バックグランドに応じて染色体

の異常が出現しやすい可能性も考えられる。

今回の研究によってRad51B遺伝子のコピー数が一つ変化するだけでも染色体の安定性には大きな影響がでることが明らかとなった。これまで相同組換え修復の研究は酵母とニワトリ細胞を用いた研究が先行していたが、これらの生物では相同組換えに関わる遺伝子で1つのコピー数の違いによる異常、すなわちhaploinsufficiencyが報告されていなかった。それに対してマウスのノックアウト細胞では同じRad51パラログであるRad51DやXRCC2で1コピーの重要性が報告されている。これらの結果より、マウスやヒトなどの高等動物では遺伝子の量的効果が重要になっていることも考えられる。

Rad51Bの量的異常をきたす疾患としては、Rad51Bが関わる染色体転座を有する子宮筋腫がある。このような腫瘍においてはRad51Bのhaploinsufficiencyが存在すると考えられている。これらは良性腫瘍であるが、腫瘍の一部のみにこの転座が存在することが明らかにされているために、この異常は腫瘍化そのものよりは悪性化における染色体不安定性に関与しているものと考えられる。原爆被ばく者にがんのみならず子宮筋腫などの良性腫瘍の発生率が高いことも既に報告されている。これらの事実を総合的に考察すると、被ばく者に多い疾患は、がんに限らず放射線によるDNA二重鎖切断に対する修復が不完全であるから発生する可能性もある。今後、このような仮説に基づいて分子機構の研究を継続することが重要であろう。

E．結論

Rad51と相同性を有するRad51Bの機能をヒト細胞で検討した。その結果、他のRad51パラログと同様に相同組換え前期におけるRad51と協調した機能が確かめられた。それに加えて、Rad51B変異細胞では中心体の数の増加と染色体の異数体の増加も認められた。しかもこれらの異常は遺伝子1コピーの変化とも相関していた。以上よりRad51Bは相同組換え修復を制御することによって染色体の数的安定性を保っていることが明らかとなった。この結果は、放射線に起因するがんの発症の制御に、相同組換え修復の異常と異数体の出現が関与する可能性を示唆する。

F．健康危険情報

なし

G．研究発表

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H．知的財産権の出願・登録状況（予定を含む）

なし

III. 研究成果の刊行に関する一覧表

書籍

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Li Z, Hosoi Y, Cai K, Tanno Y, Matsumoto Y, Enomoto A, Morita A, Nakagawa K and Miyagawa K	Src tyrosine kinase inhibitor PP2 suppresses ERK1/2 activation and epidermal growth factor receptor transactivation by X-irradiation	Biochem Biophys Res Commun	341	363-368	2006

IV. 研究成果の刊行物・別刷

T-Cell Homeostasis and Inflammatory Response among A-Bomb Survivors

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ABSTRACT

More than 50 years after damage to their immune systems by A-bomb radiation, we still find significant alterations in T-cell immunity among survivors. To test the hypothesis that immune reconstitution of T-cell homeostasis following radiation damage might have been incomplete and/or deteriorated, we evaluated the ability of individual subjects to maintain naïve and memory T-cell pools. It was suggested that there might be a dose-dependent decrease in the number of T-cell receptor rearrangement excision circles in the CD4 T-cell fraction of the survivors. Although maintenance of memory T-cell pools of A-bomb survivors appeared to be close to normal in terms of size, T-cell repertoire deviation possibly associated with clonal expansion of T-cell populations was also suggested. It seems likely that A-bomb radiation exposure perturbed the mechanisms responsible for T-cell homeostasis, by impairing the ability to maintain naïve T-cell pools with a supply of new T cells from the thymus and also by inducing clonal expansion of a small fraction of T cells, which may lead to a long-term reduction in the diversity of T-cell repertoire in memory T-cell populations. In addition, we found that the plasma levels of the inflammatory cytokines IL-6, TNF-α, and IFN-γ appeared to increase with A-bomb radiation dose. It was concluded that perturbation of T-cell homeostasis associated with reduced immune function might have lead to long-lasting inflammation among A-bomb survivors.

INTRODUCTION

The immune systems of A-bomb survivors were dose-dependently damaged 60 years ago, mainly due to radiation-induced cell death. Although the systems of the survivors regenerated as the hematopoietic system recovered from the radiation damage, we can still observe significant immunological alterations among A-bomb survivors, including impairments in both T-cell proliferation ability to respond to mitogens *(1, 2)* and alloantigens *(3)* and the frequency of T cells bearing the IL-2 production capability *(4, 5)*, and a decrease in CD4 T-cell population *(6)*. Based on these observations, we hypothesized that immune reconstitution to restore T-cell immunological homeostasis following radiation damage might have been incomplete and/or deteriorated. Two distinct mechanisms are possibly involved in ensuring immune reconsitution after T-cell depletion by radiation *(7)*: The first mechanism depends upon renewed proliferation of surviving mature T cells that can repopulate the memory T-cell pool, whereas the second relies upon the differentiation of hematopoietic stem cells into the new T cells that comprise the naïve T-cell pool. In the present study, we first evaluated the sizes of naïve and memory T-cell populations among A-bomb survivors. We also examined the number of T-cell receptor rearrangement excision circles (TRECs), which are markers of recently produced T cells in the thymus, to investigate whether the impairment in the ability to maintain normal-sized CD4 T-cell pools among A-bomb survivors could have resulted from an insufficient supply of new CD4 T cells from the thymus.

A major question remains: Are the immunological changes detected in A-bomb survivors associated with disease development? The key to addressing this question is persistent inflammation that may be involved in the perturbation of T-cell homeostasis. It is noteworthy that advancing age accompanied by alterations in the immune system — particularly age-dependent decreases of T-cell count and function — can lead to persistent infections and chronic inflammation *(8)*. In the present study, we therefore examined inflammatory cytokine levels among A-bomb survivors.

MATERIALS AND METHODS

Study population

Blood samples were obtained from individuals of an A-bomb survivor cohort in which 1,280 survivors, distributed almost equally by age, gender, and radiation dose, had been selected from Hiroshima participants in the Adult Health Study (AHS) at the Radiation Effects Research Foundation (RERF) in 1992 *(2)*. Blood samples were obtained with the informed consent of the survivors. We obtained approval from the Human Investigation Committee at RERF before the work was started.

Flow cytometry

Analytical flow cytometry was conducted using a FACScan machine (BD Biosciences, San Jose, CA, USA). CD45RO and CD62L expressions were analyzed using a combination of FITC-labeled anti-CD45RO antibody (CALTAG Laboratories, Burlingame CA, USA), PE-labeled anti-CD62L and PerCP-labeled anti-CD4 or PerCP-labeled anti-CD8 antibodies (BD-PharMingen, San Diego, CA, USA). $CD45RO^{-}/CD62L^{+}$ naive,

$CD45RO^+$ and $CD45RO^-/CD62L^-$ memory cell fractions in CD4 and CD8 T-cell populations were determined using the Cell Quest software (BD Biosciences). Note that we used only CD8-bright expression to identify CD8 T cells in order to exclude NK cells which are dully CD8 positive.

Measurement of TREC numbers

TRECs in 1 x 10^5 cells from each CD4 or CD8 T-cell fraction were enumerated by the real-time PCR method previously reported by Yasunaga, et al *(9)* with some modifications. To measure cell equivalents in the real-time PCR, *RAG-1* sequence in each sample was similarly quantified. All experiments were performed and analyzed using ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems, Foster City, CA). The number of TRECs in each sample was calculated using the following formula:

Number of TREC copies per 10,000 cells

= 10,000 / 2 (cycles required for the significant amplification of TREC) - (cycles required for the significant amplification of *RAG-1*) - 1

Measurement of cytokine levels in the plasma

Plasma samples were obtained from heparinized blood and stored at –80 °C until use. Levels of TNF-α, IFN-γ, IL-6 and IL-10 in the plasma were measured in duplicate using a highly sensitive enzyme-linked immunosorbent assay kit (Quantikine HS, R&D systems, Minneapolis, MN).

RESULTS

Naïve and memory T-cell populations among A-bomb survivors

In the present study, we used double labeling with CD45RO and CD62L to ensure reliable identification of naïve and memory cell subsets in both CD4 and CD8 T-cell populations among 533 Hiroshima A-bomb survivors (Table 1). In the CD4 T-cell population, the percentage of naïve cells significantly decreased with age ($P < 0.01$) or increased radiation dose ($P < 0.05$), and a decrease in the percentage of naïve CD8 T cells was also statistically significant with age ($P < 0.01$) or dose ($P < 0.05$). And for CD8, but not CD4, T-cell population, the percentages of memory T cells in PBL were found to significantly increase with age for A-bomb survivors ($P < 0.01$). Furthermore, the percentages of memory T cells were found to significantly increase with increasing radiation dose in the CD8 T-cell population ($P < 0.05$), but not in the CD4 T-cell population. These results indicate that previous A-bomb exposure has induced long-lasting deficits in both naïve CD4 and CD8 T-cell populations along with an increased proportion of memory CD8 T-cell population.

Table 1. Alterations in the size of peripheral T-cell pools among 553 A-bomb survivors

T-cell subsets	Factors (unit)	
	Age (10 years)	Radiation (Gy)
CD4 total	Decrease (5.0%)*	Decrease (2.0%)
Naïve	Decrease (7.5%)	Decrease (4.5%)
Memory	Not significant	Not significant
CD8 total	Not significant	Not significant
Naïve	Decrease (42.3%)	Decrease (7.7%)
Memory	Increase (7.3%)	Increase (5.6%)

*Associations of percentage of each lymphocyte subpopulation with age at the time of examination, gender, and the radiation dose were analyzed based on a multiple-linear-regression model.

TREC analyses among A-bomb survivors

The number of TREC copies in CD4 T-cell fractions from 445 survivors and that in CD8 T-cell fractions from 426 survivors were examined: The number of TREC copies significantly ($P < 0.01$) decreased with age in both the CD4 and CD8 T-cell fractions. Multiple regression analysis was conducted for the number of TREC copies in the CD4 or CD8 T-cell fraction among survivors who were less than 20 at the time of the bombing (ATB), since the individual TREC number in this group appeared to be close to the normal distribution (especially in the CD4 T-cell fraction). As shown in Fig. 1, there appeared to be a dose-dependent decrease in the number of TRECs in the CD4 T-cell fraction of the survivors ($P < 0.1$), and the number of TRECs in the CD8 T-cell fraction of the survivors also appeared to decrease somewhat with increased radiation dose, but this dose trend was not statistically significant ($P > 0.1$). There was a strong correlation ($r = 0.7$) between the numbers of TREC copies in the CD4 and CD8 T-cell fractions for the same survivors who were age ATB <20.

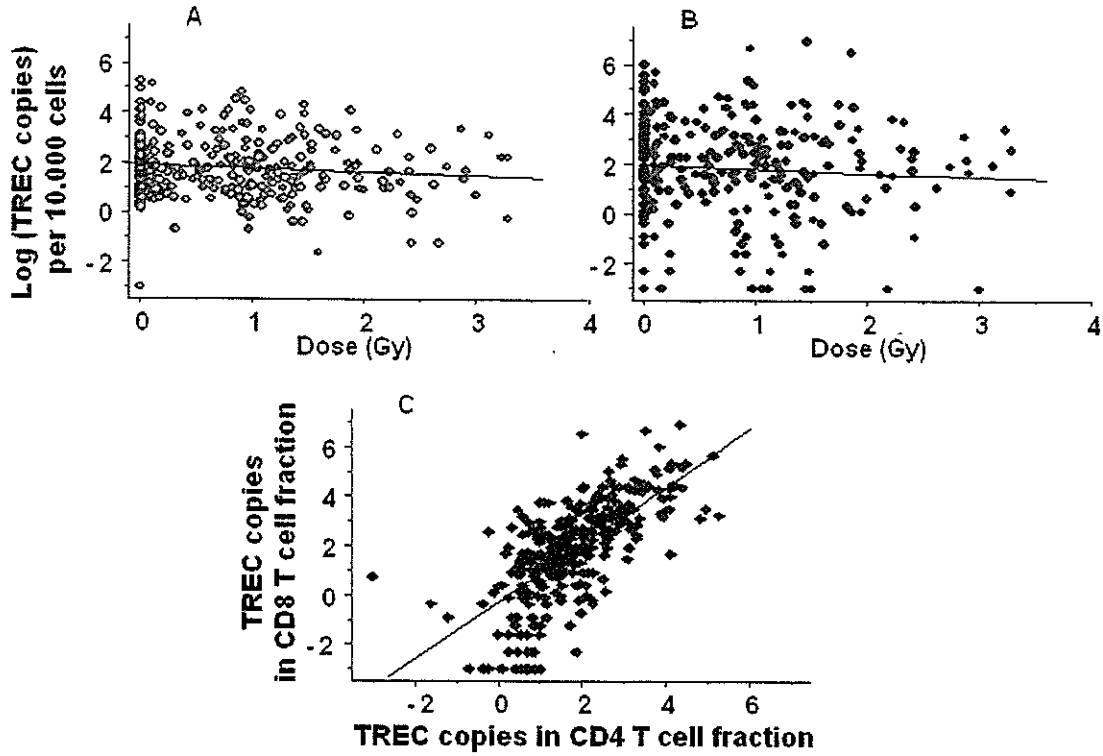

Figure 1. The number of T-cell receptor rearrangement excision circles (TRECs) in CD4 T-cell fractions from 313 individuals (panel A) and that in CD8 T-cell fractions from 300 individuals (panel B) among survivors who were age less than 20 at the time of the bombing (ATB). For the CD4 ($P < 0.1$) but not CD8 ($P > 0.1$) T-cell fractions, the radiation dose trend was suggestive. Pannel C: There was a strong correlation ($r = 0.7$) between the number of TREC copies in the CD4 and CD8 T-cell fractions from the same survivors who were age ATB <20.

Inflammatory cytokine levels among A-bomb survivors

The plasma levels of the inflammatory cytokines IL-6, IFN-γ, and TNF-α, and the anti-inflammatory cytokine IL-10 were examined among 442 A-bomb survivors (Table 2). In contrast to the age-dependent decreases in the proportion of naïve T-cell populations and the number of TRECs, plasma levels of IL-6, TNF-α, and IL-10 significantly increased with age among A-bomb survivors ($P < 0.01$). We also observed statistically significant dose-dependent increases in plasma levels of IL-6 ($P < 0.01$), TNF-α ($P < 0.01$), IFN-γ ($P < 0.01$), and IL-10 ($P < 0.05$).

Table 2. Alterations in the plasma cytokine levels among 442A-bomb survivors

Cytokines	Factors (unit)	
	Age (10 years)	Radiation (Gy)
IL-6	Increase (24%)*	Increase (13%)
IL-10	Increase (8%)	Increase (6%)
IFN-γ	Not significant	Increase (12%)
TNF-α	Increase (15%)	Increase (7%)

*Associations of each cytokine level with age at the time of examination, gender, and the radiation dose were analyzed based on a multiple-linear-regression model.

DISCUSSION

T-cell homeostasis is regulated and maintained by the balance between renewal and survival vs. death among naïve and memory T cells *(10)*. Naïve T-cell pools of A-bomb survivors are not appropriately maintained, probably because of lower proportions of naïve CD4 and CD8 T cells compared with those of unexposed controls of the same age. This may indicate that the naïve T cell pools insufficiently recovered after radiation-induced damage of the T cell system and did not reach normal size level. In this study, we also observed a dose-dependent decrease in the number of TRECs in CD4 T-cell fractions among A-bomb survivors. The results show a possibility that A-bomb radiation exposure induced long-term impairment in thymic CD4 T-cell production. To strengthen this hypothesis, we plan to investigate a larger study population.

In contrast to the naïve T-cell pools, the sizes of memory T cell pools of A-bomb survivors appeared to be almost normal (CD4), or somewhat larger (CD8) than those of controls. However, the extent of T-cell receptor

repertoire deviation in memory CD4 T cells appeared to significantly increase with increased radiation dose *(11)*. Further evidence for the perturbation of memory T-cell populations of A-bomb survivors was provided by studies unique to the Radiation Effects Research Foundation, which involved identification and characterization of clonally expanded T-cell populations using chromosome aberrations as genetic markers *(12)*. It is therefore likely that A-bomb radiation exposure perturbed the mechanisms responsible for T-cell homeostasis by impairing the ability to maintain naïve T-cell pools with a supply of new T cells from the thymus, and by inducing clonal expansion of a small fraction of T cells that may have lead to a long-term reduction in the diversity of T-cell repertoire in memory T-cell populations.

In this study, we found that the plasma levels of the inflammatory cytokines IL-6, TNF-α, and IFN-γ appeared to increase with increased A-bomb radiation dose. We also found that the plasma level of IL-6 was elevated significantly in survivors who had a lower percentage of peripheral blood CD4 T cells *(13)*, and that the prevalence of myocardial infarction was significantly higher in individuals who had reduced CD4 T-cell percentages *(14)* or elevated IL-6 levels *(13)*. These results suggest that pre-clinical inflammatory status linked to T-cell impairments may at least partly be involved in the development of the diseases, such as cardiovascular disease, which have been observed frequently in A-bomb survivor populations *(15, 16)*. In conclusion, we hypothesize that A-bomb radiation perturbed T-cell homeostasis and induced long-lasting inflammation, and that such immunological alterations might have lead in some way to disease development among A-bomb survivors. Clearly, prospective studies that will follow up the survivors who were examined for immunological and inflammatory endpoints will be required to directly test these hypotheses.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

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

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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 (mean±s.e., 0.52±0.05 ng/ml) and patients with chronic-active gastritis (0.36±0.09 ng/ml). However, the serum Reg IV concentration in presurgical GC patients was significantly elevated (1.96±0.17 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. *Oncogene* advance online publication, 22 January 2007; doi:10.1038/sj.onc.1210215

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

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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

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}

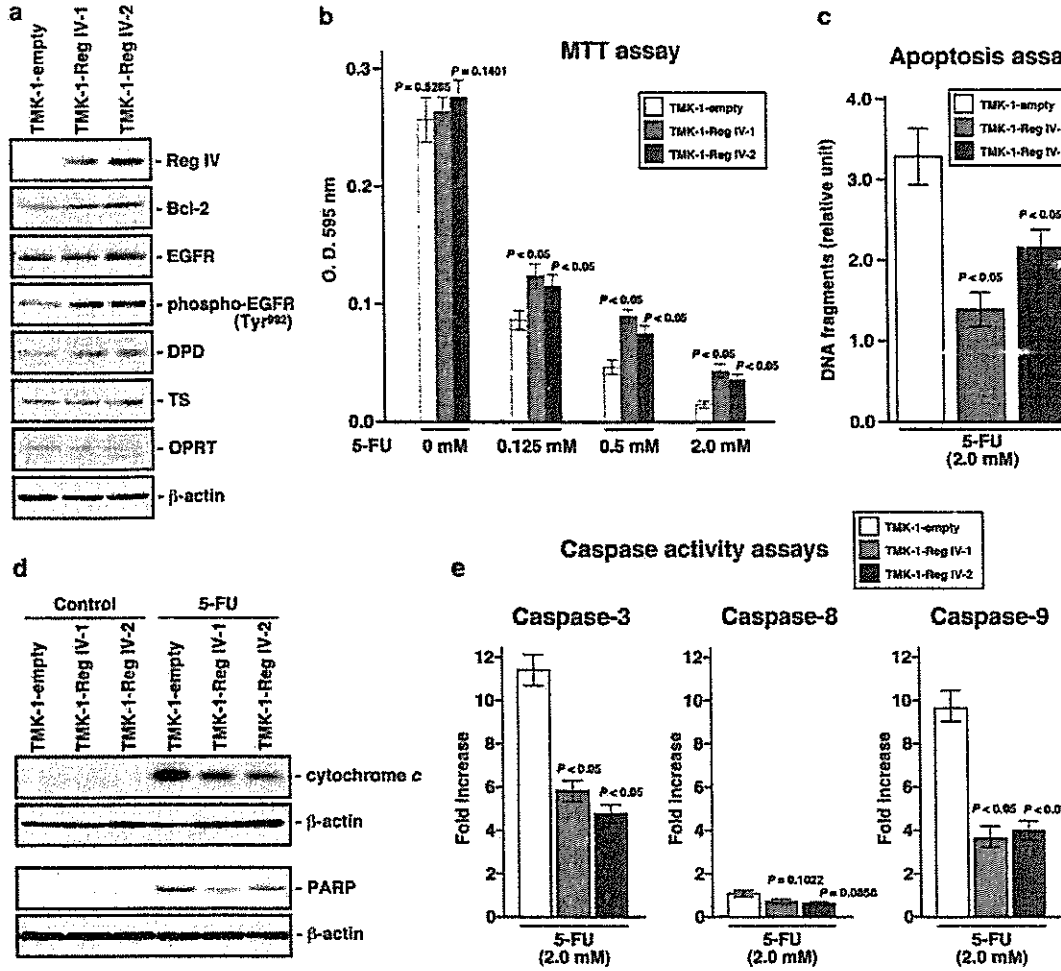

Figure 1 Forced expression of Reg IV inhibits 5-FU-induced apoptosis. (a) Western blot analysis of the TMK-1 GC cell line stably transfected with vector expressing Reg IV. Two G418 resistant clones, TMK-1-Reg IV-1 and TMK-1-Reg IV-2, overexpressing Reg IV protein were isolated. (b) Effect of forced Reg IV expression on cell growth of TMK-1 cells. Cell growth was assessed by MTT assay at 48 h after 5-FU treatment. Bars and error bars represent mean and s.d., respectively, from three different experiments. *P*-values for all the assays were calculated using Student's *t*-test. (c) Forced Reg IV expression inhibits 5-FU-induced apoptosis. Cells were incubated with 2 mM 5-FU for 48 h, and both floating and attached cells were collected. Apoptosis was determined with a Cell Death Detection ELISA^{Plus} Kit. Bars and error bars represent mean and s.d., respectively, from three different experiments. *P*-values for all the assays were calculated using Student's *t*-test. (d) Forced Reg IV expression inhibits cytochrome *c* release and PARP cleavage induced by 5-FU. Cells were incubated with 2 mM 5-FU for 36 h, and both floating and attached cells were collected for Western blot analysis. (e) Forced Reg IV expression inhibits caspase activation by 5-FU. Cells were treated with 5-FU (2 mM) for 36 h, and floating and attached cells were collected. 5-FU induced activation of caspase-9 and -3, and to a lesser extent, caspase-8. Reg IV-overexpressing cells (TMK-1-Reg IV-1 and TMK-1-Reg IV-2) showed significantly lower activation of caspase-9 and -3 in response to 5-FU than empty vector-transfected cells. Bars and error bars represent mean and s.d., respectively, from three different experiments. *P*-values for all the assays were calculated using Student's *t*-test.

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-β-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 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 ($n=101$, mean$\pm$s.e., 0.52 ± 0.05 ng/ml) and patients with

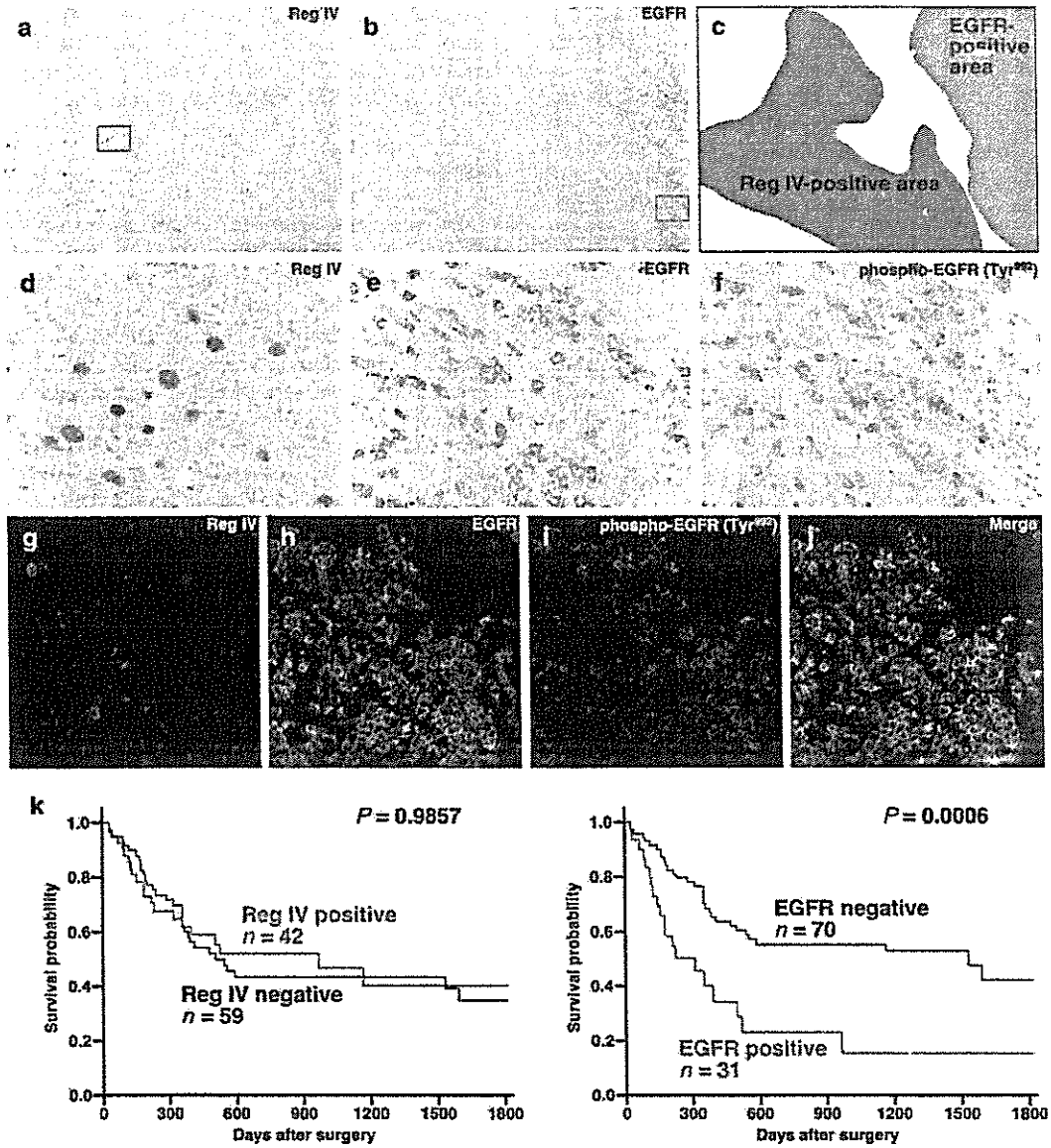

Figure 2 Expression and distribution of Reg IV and EGFR in GC tissues. Immunostaining of Reg IV (a, brown color) and EGFR (b, brown color) in both Reg IV- and EGFR-positive GC case (original magnification, × 40). (c) Schematic representation of Reg IV-positive GC cells (blue) and EGFR-positive GC cells (green). In both Reg IV- and EGFR-positive GC case, Reg IV-positive GC cells were different from EGFR-positive GC cells although Reg IV-positive GC cells were found near EGFR-positive GC cells. (d and e) High magnification images of the fields indicated by boxes in panels a and b (original magnification, × 400). A subset of EGFR-positive GC cells was positive for Tyr^{992} phospho-EGFR (f) (original magnification, × 400). (g–j) Triple-immunofluorescence staining of Reg IV (g), EGFR (h) and Tyr^{992} phospho-EGFR (i). Cells with EGFR with phosphorylation at Tyr^{992} were located near Reg IV-positive GC cells (j) (original magnification, × 400). (k) Prognostic value of Reg IV and EGFR staining.

chronic-active gastritis ($n=20$, 0.36 ± 0.09 ng/ml). However, the serum Reg IV concentration in presurgical GC patients ($n=61$, 1.96 ± 0.17 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 ($n=12$, 2.51 ± 0.40 ng/ml) was statistically significantly higher than that with GC showing Reg IV-negative immunostaining ($n=49$, 1.82 ± 0.18 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).

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	

Abbreviations: EGFR, epidermal growth factor receptor; Reg IV, regenerating gene IV. [a]Fisher's 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

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	0 (0.0%)	8	0.0132	0 (0.0%)	8	0.1596
NC and PD	14 (50.0%)	14		9 (32.1%)	19	

Abbreviations: CR, complete response; EGFR, epidermal growth factor receptor; NC, no change; PD, progressive disease; PR, partial response; Reg IV, regenerating gene IV. [a]Fisher's exact test.

Figure 3 ELISA of serum samples from patients with GC. (a) Western blot analysis of Reg IV. Culture media of Reg IV-transfected TMK-1 cells (TMK-1-Reg IV-1 and TMK-1-Reg IV-2) and MKN-45 GC cell lines contain Reg IV. Extracts of MKN-45 cells served as a positive control. Western blotting with anti-β-actin antibody confirmed that there was minimal contamination of culture medium with cells. (b) Detection of Reg IV in culture media by ELISA. Reg IV was detected in culture media of Reg IV-transfected TMK-1 and MKN-45 cells but not MKN-1 and TMK-1 cells. Culture media of Reg IV-transfected TMK-1 and MKN-45 cells were preincubated with recombinant Reg IV and then tested by ELISA. A significant reduction in the signal intensity of the ELISA was observed. *P*-values were calculated using Student's *t*-test. (c) Detection of Reg IV protein in serum samples by ELISA. A high concentration (2.00 ng/ml) of Reg IV was detected in 22 serum samples from patients with GC. Yellow bars indicate the cutoff levels defined in this study. Red bars indicate the means$\pm$s.e. Differences in the serum concentration of Reg IV between two groups were tested by non-parametric Mann–Whitney *U*-test. (d) Immunostaining of Reg IV in primary GC samples. Strong and extensive Reg IV staining was observed in Cases 36 and 42, which also showed high concentrations of Reg IV in serum samples (c). In Case 18, no staining of Reg IV was observed, and the serum concentration of Reg IV was low (c). (e) Relation between serum concentrations of Reg IV and CEA. Correlation was examined using Spearman's rank correlation. (f) Relation between serum concentrations of Reg IV and CA19-9. Correlation was examined using Spearman's rank correlation.