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as a new member of the cancer-related FOX family in cancer cells.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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*Original article*

# Second-line chemotherapy with irinotecan plus cisplatin after the failure of S-1 monotherapy for advanced gastric cancer

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**Abstract**

***Background.*** **For advanced gastric cancer (AGC), second-line chemotherapy after the failure of S-1 has not yet been established. The present study aimed to retrospectively evaluate the efficacy and safety of irinotecan plus cisplatin (IP) therapy after the failure of S-1 in patients with AGC.**

***Methods.*** **The subjects included 87 patients with AGC who received IP therapy as second-line chemotherapy. Irinotecan (70 mg/m$^2$) was administered by intravenous infusion followed by an intravenous infusion of cisplatin (80 mg/m$^2$) on day 1. On day 15, irinotecan (70 mg/m$^2$) alone was administered. The treatment was repeated every 4 weeks until disease progression, patient refusal, or severe adverse events.**

***Results.*** **The median patient age was 62 years (range, 39–75 years), and the median number of treatment cycles was 3 (range, 1–9). Out of the 87 patients, 70 were assessable for clinical response. There were 2 complete responses and 18 partial responses. The overall response rate was 28.6% (95% confidence interval [CI], 18.4%-40.6%) and the disease control ratio was 70.0%. The median time to progression and median survival time from the first day of IP therapy were 4.3 months and 9.4 months, respectively. The 1-year survival rate was 34.6%. Severe (grade 3/4) leukopenia, neutropenia, anemia, and thrombocytopenia were observed in 34%, 40%, 28%, and 8% of patients, respectively. Grade 3/4 nonhematologic toxicities included anorexia (17%), febrile neutropenia (10%), diarrhea (6%), fatigue (5%), nausea (2%), and elevated creatinine (1%).**

***Conclusions.*** **The combination of irinotecan plus cisplatin as second-line chemotherapy for AGC appears to be an effective and feasible treatment option after S-1 failure.**

**Key words** Irinotecan · Cisplatin · Gastric cancer · Second-line chemotherapy · S-1 failure

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## Introduction

For the first-line treatment of advanced gastric cancer (AGC), the Japan Clinical Oncology Group (JCOG) reported the results of a three-arm phase III study comparing 5-fluorouracil (5-FU), irinotecan hydrochloride (CPT-11) plus cisplatin (CDDP) combination chemotherapy (IP), and S-1 [1]. The results showed that IP therapy did not demonstrate statistically significant superiority to 5-FU (median survival time [MST], 12.3 months vs 10.8 months; $P = 0.055$), although it was potentially promising.

In contrast, S-1 showed significant noninferiority to 5-FU (MST, 11.4 months vs 10.8 months; $P < 0.001$). Furthermore, Koizumi et al. [2] reported that in the S-1 plus CDDP versus S-1 in RCT in the treatment for stomach cancer (SPIRITS) trial, S1 plus CDDP established superiority over S-1 monotherapy (MST, 13.0 months vs 11.0 months, respectively; $P = 0.037$). However, another phase III study, comparing S-1 and S-1 plus CPT-11 (GC0301/TOP-002 trial), could not demonstrate a significant survival benefit for S1 plus CPT-11 [3].

According to these results, S-1 plus CDDP is suitable for first-line chemotherapy for AGC, and CPT-11-based regimens failed as first-line chemotherapy. Additionally, Sakuramoto et al. [4] have reported that S-1 is effective as adjuvant chemotherapy in patients who have undergone curative gastrectomy for locally advanced gastric cancer (adjuvent chemotherapy trial of TS-1 for gastric cancer; ACTS-GC-trial). Thus, S-1 is currently used for gastric cancer in both first-line and adjuvant settings. As such, it is expected that the number of S-1-refractory cases will increase in the near future, and therefore, establishing second-line chemotherapy for S-1-refractory AGC is very important.

However, there are few data for second-line IP therapy for AGC refractory to S-1. Therefore, we decided to retrospectively evaluate the efficacy and

safety of IP therapy in 87 patients who received IP therapy only after failure of S-1 monotherapy.

## Patients, materials, and methods

### *Patient information*

The subjects in this retrospective study included 87 patients with primary AGC who received IP therapy as second-line chemotherapy for unresectable or recurrent tumors at the National Cancer Center Hospital (Tokyo, Japan) between March 2001 and January 2007. The following inclusion criteria were used: (1) histologically proven adenocarcinoma of the stomach; (2) age 75 years or younger; (3) performance status (Eastern Cooperative Oncology Group) 0 to 2; (4) refractory to or unable to tolerate prior chemotherapy with S-1 monotherapy (given in 6-week cycles; 4 weeks of S-1 administration and 2 weeks' rest); (5) adequate organ function; (6) lack of massive ascites; and (7) written informed consent.

### *Treatment schedule*

On day 1, CPT-11 (70 mg/m$^2$) was administered by intravenous infusion for 90 min, followed by intravenous infusion of CDDP (80 mg/m$^2$) for 120 min with adequate hydration. On day 15, CPT-11 (70 mg/m$^2$) alone was administered. The treatment was repeated every 4 weeks until disease progression, patient refusal to receive further treatment, or the occurrence of severe adverse event(s). Administration of CPT-11 on day 15 was delayed in the case of leukopenia or thrombocytopenia of grade 2 or more, diarrhea of grade 1 or more, or infection, until recovery from these adverse reactions. If the adverse reaction continued beyond day 22, CPT-11 was not given. If grade 4 leukopenia or thrombocytopenia or any grade 3/4 nonhematologic adverse reaction occurred, the doses of CPT-11 and CDDP were reduced to 60 mg/m$^2$ and 70 mg/m$^2$, respectively. If one of these severe adverse reactions occurred a second time, treatment was stopped. And if severe renal dysfunction (serum creatinine >2.0 mg/dl) developed, CDDP administration was halted, and CPT-11 monotherapy was continued until progression.

### *Clinical evaluation*

Clinical response in measurable lesions was evaluated every 8 weeks by computed tomography (CT) using the Response Evaluation Criteria in Solid Tumors. Toxicity was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. We defined overall survival (OS) as the number of days between the date of initial chemotherapy and the date of death or last follow-up visit. Time to progression was also measured from the beginning of treatment to the date of disease progression, which was evaluated by each physician. Survival analysis was performed using the Kaplan-Meier method, and differences between curves were analyzed using the log-rank test. The time to an event was calculated beginning with the start of treatment. All analyses were performed using the statistical software package StatView, version 5.0 (SAS Institute, Cary, NC, USA).

## Results

### *Clinicopathological features*

Patient clinicopathological characteristics are listed in Table 1. Between May 2000 and October 2006, 427 patients with AGC received first-line S-1 monotherapy. After failure, 298 patients subsequently received second-line chemotherapy. Of these, 96 patients received IP therapy, and we evaluated 87 patients who fulfilled the inclusion criteria (the excluded patients consisted of 7 patients aged >75 years and 2 who did not have adenocarcinoma). The primary reasons for discontinuation of S-1 therapy were progressive disease ($n$ = 80 [92%]); followed by adverse events ($n$ = 6 [7%]), including acneiform eruption ($n$ = 3), anorexia ($n$ = 2), edema ($n$ = 1), and diarrhea ($n$ = 1); and patient refusal ($n$ = 1 [1%]). The median number of prior S-1 courses administered was 3 (range, 1–16). The median follow-up was 5.0 years (range, 2.4–8.2 years). The median number of IP cycles administered after S-1 failure was 3 (range, 1–9 cycles; total, 300 cycles).

**Table 1.** Patient characteristics ($n$ = 87)

| Factor | No. of patients | Percentage |
|---|---|---|
| Age | | |
| Median (years) | 62 (39–75) | |
| Sex | | |
| Male | 65 | 75 |
| Female | 22 | 25 |
| ECOG performance status | | |
| 0/1/2 | 29/53/5 | |
| Histological type | | |
| Intestinal | 46 | 53 |
| Diffuse | 41 | 47 |
| Metastatic site | | |
| Lymph node | 53 | 61 |
| Liver | 44 | 51 |
| Peritoneum | 20 | 23 |
| Lung | 7 | 8 |
| Bone | 1 | 1 |
| Discontinuation of S-1 therapy | | |
| PD | 80 | 92 |
| Adverse event | 6 | 7 |
| Patient refusal | 1 | 1 |

ECOG, Eastern Cooperative Oncology Group; PD, progressive disease

**Table 2.** Overall response ($n$ = 70)

| CR | PR | SD | PD | RR | DCR |
|---|---|---|---|---|---|
| 2 | 18 | 29 | 21 | 28.6%[a] | 70.0%[b] |

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; RR, response rate; DCR, disease control ratio (CR+PR+SD/all)
[a]95% confidence interval (CI): 18.4–40.6
[b]95% CI: 57.9–80.4

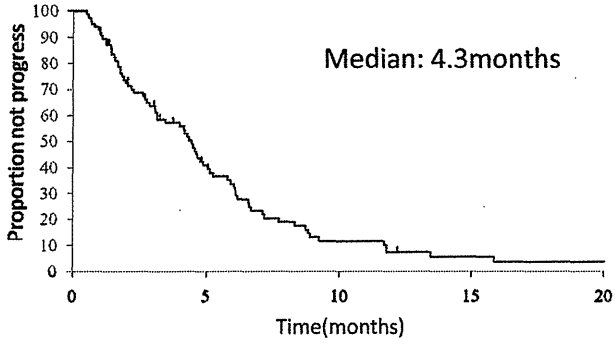


**Fig. 1.** Time to progression

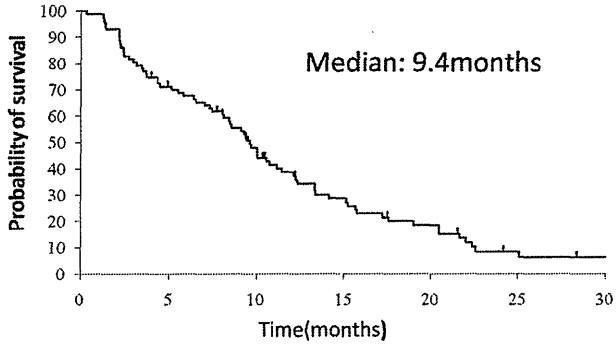


**Fig. 2.** Overall survival

*Response and survival*

A total of 70 patients had measurable lesions and were assessable for clinical response. There were 2 complete responses (CRs) and 18 partial responses (PRs). The overall response rate (ORR) was 28.6% (95% CI, 18.4–40.6%), and the disease control ratio was 70.0% (Table 2). The median time to progression (TTP) and MST from the first day of IP therapy were 4.3 months and 9.4 months, respectively (Figs. 1, 2). In addition, the MST from the first day of first-line S-1 was 14.3 months. The 1-year survival rate was 34.6%.

*Toxicities*

Toxicities experienced by patients treated with IP therapy are summarized in Table 3. Severe (grade 3/4) leukopenia, neutropenia, anemia, and thrombocytopenia were observed in 34%, 40%, 28%, and 8% of patients, respectively. Grade 3/4 nonhematologic toxicities included anorexia (17%), febrile neutropenia (10%), diarrhea (6%), fatigue (5%), nausea (2%), and elevated creatinine (1%).

**Table 3.** Adverse events ($n$ = 87)

| Grade | 0/1 | 2 | 3 | 4 | Grade ≥3 (%) |
|---|---|---|---|---|---|
| Leukopenia | 38 | 19 | 24 | 6 | 34 |
| Neutropenia | 44 | 8 | 11 | 24 | 40 |
| Anemia | 42 | 21 | 18 | 6 | 28 |
| Thrombocytopenia | 73 | 7 | 5 | 2 | 8 |
| Anorexia | 52 | 20 | 15 | 0 | 17 |
| Nausea | 75 | 10 | 2 | 0 | 2 |
| Diarrhea | 76 | 6 | 5 | 0 | 6 |
| Neutropenic fever | — | — | 8 | 1 | 10 |
| Fatigue | 60 | 23 | 4 | 0 | 5 |
| Creatinine | 76 | 10 | 1 | 0 | 1 |

There were four patients (5%) who died less than 30 days from the initiation of therapy; one death was due to febrile neutropenia and infection, while the three other deaths were assumed to have been due to rapidly progressive disease.

*Reasons for discontinuation and additional chemotherapy administered*

The primary reasons for discontinuation of IP therapy were progressive disease ($n$ = 73 [84%]), followed by adverse events ($n$ = 9 [10%]), including renal dysfunction ($n$ = 4), neutropenia ($n$ = 1), anorexia ($n$ = 1), liver dysfunction ($n$ = 1), acneiform eruption ($n$ = 1), anaphylactic shock ($n$ = 1), patient refusal ($n$ = 4 [5%]), and discontinuation because of CR ($n$ = 1 [1%]).

A total of 46 (53%) patients received additional chemotherapy. The most commonly used regimens were paclitaxel monotherapy ($n$ = 37 [80%]), docetaxel monotherapy ($n$ = 6 [13%]), and mitomycin C (MMC)-based therapy ($n$ = 3 [7%]).

## Discussion

The clinical value of second-line chemotherapy for AGC remains controversial. However, in Japan, CPT-11 is widely used both as a single agent and as combined therapy with CDDP or MMC [5, 6]. Futatsuki et al. [7] reported that CPT-11 monotherapy (100 mg/m$^2$, weekly or 150 mg/m$^2$, biweekly) achieved ORRs of 20% (9/45) in previously treated gastric cancer patients, and 18.9% (7/37) in patients who were only pretreated with 5-FU. CPT-11 monotherapy therefore appears to be somewhat effective for 5-FU-refractory gastric cancer. In a more recent randomized phase III study, albeit of small

sample size ($n$ = 40), Thuss-Patience et al. [8] reported that second-line CPT-11 monotherapy (250 to 350 mg/m$^2$, triweekly) significantly prolonged overall survival (OS) compared to best supportive care (BSC); the median survival in the CPT-11 arm was 123 days compared to 72.5 days for BSC; OS, hazard ratio [HR] = 2.85 (95% CI, 1.41–5.79); $P$ = 0.0027. These results indicate that second-line chemotherapy using CPT-11 can now be considered as a treatment option in GC.

There have been two phase II studies evaluating IP therapy. Boku et al. [9] reported an ORR of 26.7% (4/15), and Ajani et al. [10] reported an ORR of 31% (9/29) and an MST of 5 months for AGC refractory to 5-FU therapy. In addition, in a retrospective study, Ueda et al. [11] reported a 28% (8/28) ORR, a progression-free survival (PFS) of 3.4 months, and an MST of 9.4 months.

Our present study had a selection bias, with comparatively few patients (23%) having peritoneal metastases, because such cases tend to be treated with taxanes; however, our results (28.6% ORR, TTP of 4.3 months, MST of 9.4 months, and 34.6% 1-year survival rate) indicate that second-line IP therapy for AGC appears to provide almost the same efficacy as that seen in other second-line trials, even for patients who have experienced S-1 failure.

We also demonstrated greater feasibility for IP therapy by using it in a second-line rather than first-line setting. In the first-line setting, IP therapy did not show statistically significant superiority to 5-FU because of its toxicity; more than 30% of patients receiving IP therapy discontinued for toxicity-related reasons, as opposed to fewer than 10% stopping for toxicity due to 5-FU and S-1 [1]. In the present study, grade 3/4 leukopenia or neutropenia were relatively mild, and only 10% of patients stopped treatment because of toxicity-related reasons. The reasons for these results may be that first, the duration of IP therapy is shorter in the second-line setting than in the first-line setting, and, second, in this study, dose reduction and discontinued treatment were carried out exactly according to protocol.

Another recent well-known IP regimen is biweekly CPT-11+CDDP. Koizumi et al. [12] reported on their phase I/II study using it as first-line therapy, where CPT-11 (60 mg/m$^2$) and CDDP (30 mg/m$^2$) were administered on days 1 and 15. In 2008, Nakae et al. [13] reported a phase II study of biweekly IP after S-1 failure. The ORR was 28.6%, and the median OS was 389 days. The most common grade 3/4 toxicities were: neutropenia (22.9%), anemia (11.4%), anorexia (14.3%), fatigue (8.6%), and diarrhea (2.9%). The efficacy and toxicity of this biweekly regimen were almost the same as those seen in our study. The biweekly regimen is available for outpatients; however, there are no phase III data on this regimen.

Currently, either CPT-11 (monotherapy or combined with CDDP) or taxanes [14–16] would be selected as second-line chemotherapy for AGC. However, it is not yet clear which of these regimens is most effective. Therefore, there are some currently ongoing clinical trials on the second-line treatment of AGC (after S-1 or S-1+CDDP failure), including phase III studies comparing CPT-11 and paclitaxel, CPT-11 alone and CPT-11+CDDP, CPT-11 alone and CPT-11+S-1, and so on. The results are anxiously awaited.

In conclusion, the combination of CPT-11 and CDDP as second-line chemotherapy for AGC appears to be effective and feasible, and should therefore be considered as a promising treatment option for patients who have experienced S-1 failure. Because CDDP has been widely used as first-line treatment for AGC patients, this regimen is suitable for patients who failed S-1 monotherapy used as adjuvant chemotherapy.

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# Regular Article

## Genetic Polymorphisms of *FCGRT* Encoding FcRn in a Japanese Population and Their Functional Analysis

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**Summary:** Neonatal Fc receptor (FcRn) plays an important role in regulating IgG homeostasis in the body. Changes in FcRn expression levels or activity caused by genetic polymorphisms of *FCGRT*, which encodes FcRn, may lead to interindividual differences in pharmacokinetics of therapeutic antibodies. In this study, we sequenced the 5′-flanking region, all exons and their flanking regions of *FCGRT* from 126 Japanese subjects. Thirty-three genetic variations, including 17 novel ones, were found. Of these, two novel non-synonymous variations, 629G>A (R210Q) and 889T>A (S297T), were found as heterozygous variations. We next assessed the functional significance of the two novel non-synonymous variations by expressing wild-type and variant proteins in HeLa cells. Both variant proteins showed similar intracellular localization as well as antibody recycling efficiencies. These results suggested that at least no common functional polymorphic site with amino acid change was present in the *FCGRT* of our Japanese population.

**Keywords:** ***FCGRT*; neonatal Fc receptor (FcRn); genetic polymorphism; novel non-synonymous variation**

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## Introduction

Neonatal Fc receptor (FcRn) is an immunoglobulin G (IgG) receptor related to major histocompatibility (MHC) class I molecules.[1,2] Like MHC class I, FcRn consists of a heavy chain with extracellular α1, α2, and α3 domains followed by a transmembrane segment and a short cytoplasmic tail and non-covalently bound β2-microglobulin (β2m). FcRn binds the Fc region of monomeric IgG. The FcRn heavy chain is encoded by *FCGRT*, which is located in chromosome 19q13.3 and comprises 6 exons.

In humans, FcRn expression has been observed in a wide variety of tissues including placenta, liver, kidney and vascular endothelium.[1] FcRn has multiple roles in the body such as absorption or secretion of IgG across the intestinal mucosa, and IgG recycling from endothelial cells. With regard to antibody recycling, FcRn binds to the Fc domain of IgG at acidic pH in endosomes after endocytosis, and recycles it back to the extracellular space via the exocytic pathway, thereby protecting IgG from intracellular degradation in lysosomes.[2] This mechanism contributes to the long serum half-life of IgG, and thus, IgG recycling activity is an important function of FcRn and could contribute to the efficacy of antibody therapeutics. Indeed, we previously reported that affinities of antibody therapeutics to FcRn were closely correlated with the serum half-lives reported in clinical studies.[3] The relatively short serum half-life of Fc-fusion proteins such as etanercept, a fusion protein consisting of the extracellular ligand-binding portion of the human tumor necrosis factor receptor linked to the Fc portion of human IgG1, is thought to arise from low affinity to FcRn.[3]

Genetic polymorphisms of genes related to drug metabolism and transport are one of the crucial factors for low-molecular-weight drugs. Pharmacokinetics or pharmacodynamics of biologicals including antibody therapeutics may also be influenced by genetic polymorphisms of transport or target proteins. In this context, changes in FcRn expression levels or activity caused by genetic polymorphisms of *FCGRT* may lead to interindividual differences in pharmacokinetics of antibody therapeutics. However, reports on *FCGRT* genetic polymorphisms in Japanese populations are lacking.

Here we sequenced the 5′-flanking region, all exons and their flanking regions of *FCGRT* from 126 Japanese subjects. We then examined the functional properties of two detected non-synonymous variations using mammalian expression systems focusing on intracellular localization and antibody recycling activities.

## Materials and Methods

**Human genomic DNA samples:** One hundred twenty-six Japanese cancer patients participated in this study. The ethical review boards of the National Cancer Center, Aichi Cancer Center and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all subjects. Genomic DNA for DNA sequencing was extracted from blood leukocytes.

**PCR conditions for DNA sequencing:** The following sequences obtained from GenBank were used for primer design and reference sequences: NW_927240.1 (genome) and NM_004107.3 (mRNA). For sequencing, two sets of long-range PCR were performed to amplify all 6 exons from 50 ng of genomic DNA with two sets of primers (0.5 μM) designed in the promoter or intronic regions as listed in "1st PCR" of **Table 1**. We used LA-Taq with GC buffer I (0.05 U/μl, Takara Bio Inc., Shiga, Japan) to amplify from the 5′-flanking region to exon 3 and Z-Taq (0.025 U/μl, Takara Bio. Inc.) from exons 4 to 6, as described in **Table 1**. The 1st PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min for LA-Taq, and 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 190 sec for Z-Taq. Next, each region was separately amplified in the 2nd PCR using the 1st PCR product as the template. We used LA-Taq with GC buffer I or II (0.05 U/μl) for amplifying regions from the 5′-flanking region to exon 3 and Ex-Taq (0.02 U/μl, Takara Bio. Inc.) from exons 4 to 6 as described in **Table 1**. The 2nd PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min for all regions. The PCR products were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) and the sequencing primers listed in **Table 1** (Sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany) and the eluates were applied to an ABI Prism 3730xl DNA Analyzer (Applied Biosystems). All relatively low frequent variations ($n \leq 5$) were confirmed by repeated sequencing analyses of PCR products generated from original (not amplified) genomic DNA. The nucleotide positions based on the cDNA sequence were numbered from the adenine of the translational initiation site or the nearest exons.

**Hardy-Weinberg equilibrium and linkage disequilibrium (LD) analyses:** Hardy-Weinberg equilibrium and LD analyses were performed by SNPAlyze software ver. 7 (Dynacom Co., Yokohama, Japan). Hardy-Weinberg equilibrium was assessed by the $\chi 2$ test and pairwise LDs between variations were obtained for the frequently used coefficients $|D'|$ and rho square ($r^2$). $|D'|$ is used to assess the probability for past recombinations, and $r^2$ is used as a parameter for the linkage between a pair of variations.

**Table 1.** Primers used for sequencing *FCGRT*

| | Enzyme* | Amplified or sequenced region | Forward primer (5′ to 3′) | Reverse primer (5′ to 3′) | Amplified length (bp) |
|---|---|---|---|---|---|
| 1st PCR | LA-GI | 5′-flanking to Exon 3 | CTCAGGCTGGTCCTTGAACTCA | ATTAGCCAGTTATGGTGGTATG | 5,244 |
| | Z | Exons 4 to 6 | CAAGTGTGGTGGTGGGCACCTA | GGGAGTTCGAGACCAGCCTGAT | 3,788 |
| 2nd PCR | LA-GI | 5′-flanking | CTGAACCAGCTGAACGTCCACT | CTGAGCGTGGTGGTGGGCCTGT | 1,058 |
| | LA-GII | | ATAGAGGTGACAGTTGCACAGC | GGTCCAGACTGACAACAATGCC | 1,477 |
| | LA-GII | Exon 1 | GAGCAGCAGCCTCCCACAGGAT | ACACAAGAGGCGACAGGTGGTT | 1,017 |
| | LA-GI | Exons 2 to 3 | ATTGTTGTCAGTCTGGACCG | GCTGCAGTGGGAGGCTGATGGA | 1,332 |
| | Ex | Exons 4 to 5 | CCAAGGAGGTGACATCTTGAGG | CATCTCTGGGTTTCTGTCTCCA | 1,383 |
| | Ex | Exon 6 | CCGCCTTGCCGCTGCTGATCCA | GAGCTGAGATCACGCAATTGTA | 1,632 |
| Sequencing | | 5′-flanking | CTGAACCAGCTGAACGTCCACT | CAGGGTCTGGCTCTGTCACTCA | |
| | | | GTGCAGAATAGGCAAATCTATC | AACCACATCCTTCTGCTAGGAC | |
| | | | CGGGTTCAAGCAATTCTCCTGT | TTGAGGGTGTCTGCCGCTCAGG | |
| | | | GAGCAGCAGCCTCCCACAGGAT | CCTCCTCTCTCAGACCCAGGAA | |
| | | | CCTGGGTCTGAGGGAGGAGT | CCTCCTCGTACCTGAAGAACTT | |
| | | Exon 1 | GGACTCTCAGCCTATCAAGT | ACACAAGAGGCGACAGGTGGTT | |
| | | | CCGCGGTGTCCCGGGAGGAA | | |
| | | Exons 2 to 3 | GTATCTGTCCCACTGCAGTCTA | AACTGAGGCAGGTGGGCATGAC | |
| | | Exon 4 | TGAGTCTCTGTCACCTAGGAAG | AGTTAACAGCTCTTCAGACTCA | |
| | | Exon 5 | CCGCCTTGCCGCTGCTGATCCA | GTCTCTGTCCTCCCAGGTCTGT | |
| | | Exon 6 | TCAGAGAGAGGTGGAGACAGAA | GATGTATAAAACTGGCAGGTTC | |
| | | | CCTTGGATCTCCCTTCGTGGAG | TGGCTCACACTTGTAATCCCAC | |
| | | | GACGGAGTCTTGCTCTGTTGCT | | |

*LA-GI: LA-Taq with GC buffer I, LA-GII: LA-Taq with GC buffer II, Z: Z-Taq, Ex: Ex-Taq.

**Construction of FcRn expression plasmid:** Wild-type human FcRn cDNA was originally obtained from pME18SFL3 (AK075532) (Toyobo, Osaka, Japan). The coding region of FcRn cDNA subcloned into pcDNA3 was amplified by PCR, and then inserted into the EcoRI/SalI site of pEGFP-(C) plasmid. The resulting plasmid encodes hFcRn with C-terminally fused enhanced green fluorescent protein (EGFP) containing the eight amino acid-linker peptide VDSRGSRV between the two proteins. Mutations were introduced by an inverse PCR method. Primers consisted of 5′-AAG GCC CAA CCC AGC AGC CCT GGC TTT-3′ (forward) and 5′-CAG GCG CAT GGA GGG GGG CC CTT CCA-3′ (reverse) for R210Q, 5′-TCC ACC GTC CTC GTG GTG GGA ATC GTC-3′ (forward) and 5′-CTT GGC TGG AGA TTC CAG CTC CAC CCT-3′ (reverse) for S297T. The underlines indicate the mutated nucleotides. The variant plasmids were sequenced on both strands for the entire cDNA region to confirm the introduction of the mutation only at the target sites. Human $\beta$2 microglobulin ($\beta$2m) cDNA was obtained from pME18SFL3 (FCC106E07) (Toyobo). $\beta$2m cDNA was subcloned into pcDNA3.1/
Hygro. The $\beta$2m construct was used because FcRn becomes a heterodimer with $\beta$2m, which is necessary for the proper intracellular localization of FcRn.[4,5]

**Cell culture and plasmid transfection:** HeLa cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Nichirei, Tokyo, Japan). The plasmids encoding the wild-type or variant FcRn fused with EGFP along with the plasmid encoding $\beta$2m were transfected into HeLa cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Plasmids encoding wild-type or variant FcRn fused with EGFP were used for all experiments, including the intracellular localization and antibody recycling activity of FcRn.

**Western blot analysis:** Wild-type and variant FcRn-EGFP transfected into HeLa cells in 35-mm-diameter dishes were lysed with 500 $\mu$L of RIPA buffer [50 mM Tris HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40 and 0.25% sodium deoxycholate] supplemented with protease inhibitors (Nacalai Tesque, Kyoto, Japan). After incubation on ice for 30 min, the lysates were centrifuged at 15,000 rpm at 4°C for 20 min. An aliquot (3 $\mu$L) of the supernatant was diluted in SDS-sample buffer and applied to 10% SDS-polyacrylamide gel. After electrophoresis, separated proteins were transferred onto polyvinylidene fluoride membrane. Immunochemical detection of FcRn-EGFP proteins was performed using rabbit anti-human FcRn antibody raised against a peptide antigen (residues 135–148, LNGEEFMNFDLKQG). Visualization of the proteins was achieved with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA, USA) and the ECL Plus Western blotting detection reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Protein band densities measured by LAS-3000 (Fuji Film, Kanagawa, Japan) were quantified with Multi Gauge software (Fuji Film).

The relative expression levels are shown as means ± SD of three separate transfection experiments. To verify that the samples were evenly loaded, the blot was reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) antibody (R&D Systems, Minneapolis, MN, USA).

**Fluorescent labeling of antibodies:** As a model antibody, we used infliximab, a clinically used chimeric anti-human TNF$\alpha$ antibody which has the Fc domain of human IgG1. The binding of infliximab to human FcRn was shown by surface plasmon resonance analysis in our previous study.[3] Infliximab, kindly provided by Tanabe Pharmaceutical Co. Ltd. (Osaka Japan), was labeled with CypHer5 (GE Healthcare Bio-Sciences, Uppsala, Sweden) by incubating with CypHer5E mono NHS ester in PBS containing 0.5 M $Na_2CO_3$ (pH 8.3) for 1 hr at room temperature. After the reaction, unbound dye was removed by dialysis in PBS. The protein concentration and degree of labeling were determined by spectrophotometry. IgY (Jackson Immuno Research Laboratories, West Grove, PA, USA) was also labeled with CypHer5 and used in control experiments.

**Imaging with fluorescence microscopy:** HeLa cells transfected with wild-type or variant FcRn-EGFP cDNA and the $\beta$2m cDNA were cultured on 35-mm poly-L-lysine-coated glass-bottom dishes (0.08–0.12 mm thickness) (Matsunami, Osaka, Japan) for 2–4 days. The intracellular localization analyses of wild-type and variant FcRn-EGFP were carried out by confocal laser scanning fluorescence microscopy using a Carl Zeiss LSM510 system (Carl Zeiss, Jena, Germany). For co-localization experiments, wild-type or variant FcRn-EGFP-transfected HeLa cells were incubated with CypHer5-labeled infliximab diluted in cell culture medium containing 200 mM sodium phosphate buffer (pH 6.0) for 2–3 hr at 37°C. Note that throughout this study, the cell culture media used for incubation with the labeled antibody was acidified (pH 6.0) to obtain enhanced incorporation of antibodies into the cells, as reported previously.[6,7] The fluorescent signal was observed in neutral pH medium after washing the cells twice. The 488- and 633-nm laser lines were used to image FcRn-EGFP and CypHer5 labeled-infliximab, respectively.

**Biotin labeling of antibodies:** Infliximab and IgY were labeled with biotin using EZ-link sulfo-NHS-biotin (Pierce, Rockford, IL, USA). Antibodies and sulfo-NHS-biotin were mixed at the molar ratio of 1:20 and incubated for 60 min at room temperature. Biotinylated antibodies were purified using Zeba desalt spin column (Pierce). Protein concentration was determined by BCA protein assay (Pierce) using bovine serum albumin as a standard.

**Recycling assay:** HeLa cells were transfected with the wild-type or variant FcRn-EGFP construct along with the $\beta$2m construct. The day after transfection, cells were seeded on 96-well plates at $4 \times 10^4$ cells/well. After further culturing for one day, recycling assays were performed. Hanks' balanced salt solutions (HBSS) (pH 6.0 and 7.4) were prepared supplemented with 10 mM MES (pH 6.0) and 10 mM Hepes (pH 7.4). The cells were washed with HBSS (pH 7.4) and pre-incubated with HBSS (pH 7.4) for 30 min at 37°C. After washing with HBSS, 10 $\mu$g/ml of biotinylated infliximab diluted in HBSS (pH 6.0) containing 0.5% fish gelatin was added to each well. The cells were incubated at 37°C for 1 hr to allow the antibody to be incorporated into the cells. Cells were then washed five times with HBSS (pH 7.4). Then, HBSS (pH 7.4) supplemented with 2% ultra-low IgG FCS (Invitrogen) was added to each well and incubated at 37°C for the indicated periods of time. The supernatant was collected and subjected to ELISA for quantitating the recycled antibody. In order to determine the amount of biotinylated infliximab incorporated into the cells during the 1-hr incubation at 37°C, cells were lysed using RIPA buffer supplemented with protease inhibitors (Nacalai Tesque, Kyoto, Japan) after washing five times with HBSS, and the lysate was subjected to ELISA. Biotinylated IgY was also used as a negative control in some experiments.

**Enzyme linked immunosorbent assay (ELISA) for biotinylated antibody:** NeutrAvidin (Pierce, Rockford, IL) was bound on Maxisorp 96-well black plates (Thermo Fisher Scientific, Roskilde, Denmark) using IMMUNO-TEK ELISA construction system (ZeptoMetrix, Buffalo, NY, USA). Supernatants or lysates obtained from the recycling assay were applied on the wells and incubated for 16 hr at 4°C. The plates were washed three times with Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBST). Peroxidase-conjugated goat anti-human IgG (Pierce) diluted with TBST was added to the plate and incubated for 1 hr at room temperature. After washing three times with TBST, chemiluminescent reagent (SuperSignal ELISA Femto, Pierce) was added and incubated for 1 min at room temperature. The chemiluminescent signal was detected using an ARVO 1420 multilabel counter (Perkin Elmer, Waltham MA, USA). When the amount of biotinylated IgY was measured, peroxidase-conjugated rabbit anti-chicken IgY (Promega, Madison, WI, USA) was used. For generation of a standard curve, 0.1 to 10 ng/ml of biotinylated corresponding protein was used.

## Results

***FCGRT* variations found in a Japanese population:** Thirty-three genetic variations were found, including 17 novel ones, in 126 Japanese subjects (**Table 2**). Of these variations, 14 were located in the 5′-flanking region, 4 (2 synonymous and 2 non-synonymous) in the coding exons, 13 in the introns, 1 in the 3′-untranslated region (UTR), and 1 in the 3′-flanking region. All detected variations were in Hardy-Weinberg equilibrium

**Table 2.** Summary of *FCGRT* variations detected in this study

| SNP ID | | Location | Position | | Nucleotide change | Amino acid change or known VNTR | Frequency | |
|---|---|---|---|---|---|---|---|---|
| This Study | dbSNP (NCBI) or reference | | NW_927240.1 | From the translational initiation site or from the end of the nearest exon | | | | 95% Confidence interval |
| MPJ6_FRT001[a] | | 5′-flanking | 1557122 | −2230 | agaacctgaactA > Ccctgaccagcag | | 0.004 | 0.000–0.012 |
| MPJ6_FRT002[a] | | | 1557195 | −2157 | gggtgtcttgcaC > Actgtcatcccag | | 0.008 | 0.000–0.019 |
| MPJ6_FRT003 | rs78889190 | | 1557207 | −2145 | cctgtcatcccaG > Ctgctttgggagg | | 0.020 | 0.003–0.037 |
| MPJ6_FRT004[a] | | | 1557221 | −2131 | gctttgggaggcC > Taaggtgggaggc | | 0.004 | 0.000–0.012 |
| MPJ6_FRT005[a] | | | 1557498_1557505 | −1854_−1847 | ggaaggaaggaaGGAAGGAA/-ggaggcaaggaa | | 0.024 | 0.005–0.043 |
| MPJ6_FRT006 | rs60964075 | | 1557502_1557505 | −1850_−1847 | ggaaggaaggaaGGAA/-ggaggcaaggaa | | 0.103 | 0.066–0.141 |
| MPJ6_FRT007 | rs60964075 | | 1557505_1557506 | −1847_−1846 | ggaaggaaggaaggaa-/GGAAggaggcaaggaa | | 0.099 | 0.062–0.136 |
| MPJ6_FRT008[a] | | | 1557505_1557506 | −1847_−1846 | ggaaggaaggaaggaa-/GGAAGGAAggaggcaaggaa | | 0.020 | 0.003–0.037 |
| MPJ6_FRT009[a] | | | 1557506 | −1846 | ggaaggaaggaaG > Agaggcaaggaag | | 0.004 | 0.000–0.012 |
| MPJ6_FRT010[a] | | | 1557540_1557547 | −1812_−1805 | aaggaaggaaggAAGGAAGG/-aggcaaggaagg | | 0.004 | 0.000–0.012 |
| MPJ6_FRT011 | rs2335534 | | 1557671 | −1681 | tctgggagcagcG > Agctgtttaacgg | | 0.028 | 0.007–0.048 |
| MPJ6_FRT012[a] | | | 1558366 | −986 | gatacagaggggT > Gaggaggaggatc | | 0.004 | 0.000–0.012 |
| MPJ6_FRT013 | ref. 8 | | 1558963_1558999 | −389_−353 | cgagggtagagcGGTTGGGGGCCCGGACTCCTGG GTCCGAGGGTAGAGC/-ggttgggggccc | VNTR3 > VNTR2 | 0.032 | 0.010–0.053 |
| MPJ6_FRT014[a] | | | 1559173 | −179 | actgagatccagT > Gtcaggggtgaaa | | 0.028 | 0.007–0.048 |
| MPJ6_FRT015 | rs59774409 | Intron 1 | 1559442 | IVS1 + 18 | ggccgctccgggC > Tcagggccctgct | | 0.028 | 0.007–0.048 |
| MPJ6_FRT016[a] | | | 1559453 | IVS1 + 29 | gccagggccctgC > Ttgcaggcgggcg | | 0.147 | 0.103–0.191 |
| MPJ6_FRT017 | rs11551281 | Exon 2 | 1559885 | 126[b] | ctcgcctgccccG > Tgggactcctgcc | Pro42Pro | 0.044 | 0.018–0.069 |
| MPJ6_FRT018 | rs2878342 | Exon 3 | 1560418 | 582[b] | ggagaggggccgC > Tggaaacctggag | Arg194Arg | 0.028 | 0.007–0.048 |
| MPJ6_FRT019[a] | | Exon 4 | 1570485 | 629[b] | gcctgaaggcccG > Aacccagcagccc | Arg210Gln | 0.004 | 0.000–0.012 |
| MPJ6_FRT020 | rs3810194 | Intron 4 | 1570734 | IVS4 + 7 | agctgggtgaggT > Ccccgccaggtgg | | 0.048 | 0.021–0.074 |
| MPJ6_FRT021 | rs1132990 | | 1570857 | IVS4 + 130 | gccttgaacctcA > Gcgcctgtcagtg | | 0.048 | 0.021–0.074 |
| MPJ6_FRT022[a] | | | 1570915 | IVS4 + 188 | ccaactgccttcC > Tgtctcctgctgc | | 0.020 | 0.003–0.037 |
| MPJ6_FRT023 | rs10525267 | | 1571020_1571025 | IVS4 + 293_ + 298 | tgctgctgctgcTGCTGC/-gggtcttcctgg | | 0.083 | 0.049–0.117 |
| MPJ6_FRT024[a] | | | 1571170 | IVS4–238 | ctggcacagcccC > Tgccttgccgctg | | 0.020 | 0.003–0.037 |
| MPJ6_FRT025 | rs73582442 | | 1571235 | IVS4–173 | gctggttcttacG > Atccaacctgggg | | 0.048 | 0.021–0.074 |
| MPJ6_FRT026 | rs73582446 | | 1571314 | IVS4–94 | gctggaatctccG > Aaggctgggaggg | | 0.048 | 0.021–0.074 |
| MPJ6_FRT027[a] | | Exon 5 | 1571425 | 889[b] | ccagccaagtccT > Accgtgctcgtgg | Ser297Thr | 0.020 | 0.003–0.037 |
| MPJ6_FRT028 | rs55662447 | Intron 5 | 1571614_1571615 | IVS5 + 90_ + 91 | agagacccagagAG/-gggggacagaga | | 0.028 | 0.007–0.048 |
| MPJ6_FRT029[a] | | | 1571615 | IVS5 + 91 | gagacccagagaG > Tgggggacagaga | | 0.004 | 0.000–0.012 |
| MPJ6_FRT030 | rs77741672 | | 1571691 | IVS5 + 167 | gagaggggacgG > Cagacagagaccc | | 0.151 | 0.107–0.195 |
| MPJ6_FRT031[a] | | | 1571915 | IVS5–46 | gtcagacccagaG > Acgcctcagagat | | 0.020 | 0.003–0.037 |
| MPJ6_FRT032 | rs14769 | 3′-UTR | 1572276 | 1304 (*206)[c] | taacacgagtttG > Aggcccgaatcag | | 0.044 | 0.018–0.069 |
| MPJ6_FRT033[a] | | 3′-flanking | 1572364 | 1312 + 80 (*214 + 80)[d] | tgggcctcggatC > Ttctcctacaggt | | 0.004 | 0.000–0.012 |

[a]Novel variations detected in this study.
[b]Positions in cDNA (NM_004107.3).
[c]Numbered from the termination codon TGA.
[d]Positions were shown as 1312 (*214) (final base of exon 6) + bases from the end of exon 6.

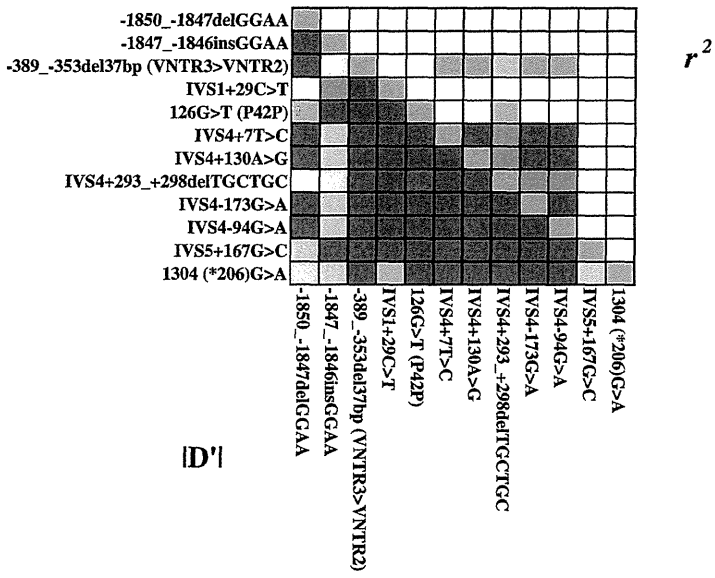



**Fig. 1.** Linkage disequilibrium (LD) analysis of *FCGRT*
Pairwise LD is expressed as $r^2$ (upper right) and |D′| (lower left) values (from 0 to 1) by 10-graded blue colors. A denser color represents closer linkage.

($p \geq 0.05$). Two novel non-synonymous variations, 629G>A (R210Q) and 889T>A (S297T), were found as heterozygotes. The allele frequencies were 0.004 for R210Q and 0.020 for S297T. The functional significance of these non-synonymous variations was explored *in vitro* in the following sections. The other coding variations were previously reported synonymous variations. A variable number of tandem repeats (VNTR) was detected in the 5′-flanking region as was found in Caucasian subjects,[8] and the frequencies of VNTR3 (with 3 repeats) and VNTR2 were 0.968 and 0.032, respectively. A short tandem repeat of GGAA was also detected in the 5′-flanking region with a repeat number of 8 (frequency: 0.024), 9 (0.103), 10 (0.754), 11 (0.099) and 12 (0.020). With the 12 detected variations with $\geq 0.03$ frequencies, linkage disequilibrium (LD) was analyzed using |D′| and $r^2$ values (**Fig. 1**). Because of relatively weak linkage between the variations in $r^2$ values, haplotype analysis was not performed.

**Intracellular localization of FcRn variants:** Two novel non-synonymous variations, R210Q and S297T, were functionally tested using a mammalian expression system. First, relative expression levels of wild-type and variant FcRn proteins were evaluated by Western blotting. As shown in **Figure 2**, similar levels of the proteins were detected in the three FcRn constructs, and we did not find any statistically significant differences ($p > 0.05$) between the wild-type and the two variants assessed by Dunnett's multiple comparison test when normalized by the expression levels of glyceraldehyde-3-phosphate dehydrogenase as a control. When the wild-type levels were set as 100%, R210Q and S297T levels were 95.08 ± 12.38% and 93.94 ± 13.24%, respectively.

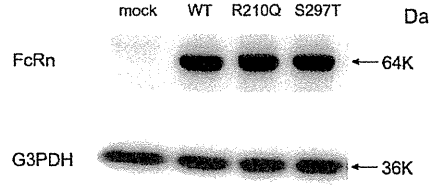



**Fig. 2.** Western blotting of wild-type and variant FcRns
Cell lysates obtained from the HeLa cells transfected with wild-type or either of the two variant FcRn-EGFP plasmids were subjected to electrophoresis, followed by transfer to the membrane. Detection of FcRn-EGFP was performed as described in Materials and Methods. One representative data of three independent transfections is shown. The FcRn band (64 KDa) consists of 37 KDa of FcRn and 27 KDa of EGFP. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) levels were used for normalization of the lysate proteins applied to electrophoretic gels.

In order to examine the differences of intracellular localization between wild-type FcRn and its variants, each EGFP fusion construct together with a human $\beta$2m construct was transfected into HeLa cells, and fluorescent images were observed by confocal microscopy. There have been several studies reporting the intracellular localization or trafficking of FcRn using fluorescent protein-tagged FcRn.[9–12] N- and C-terminally tagged FcRn showed similar localization.[13] Since FcRn is a type I membrane protein, N-terminal amino acid residues including R210 and S297 were located in the extracellular

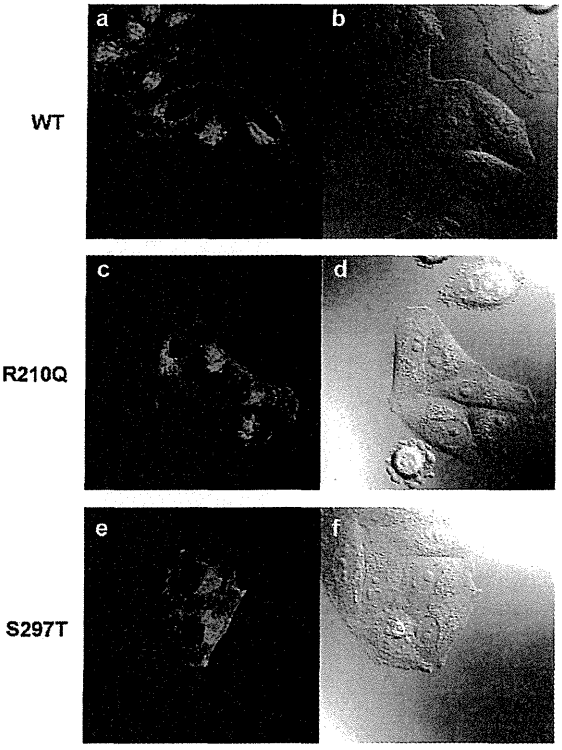



**Fig. 3.** Intracellular localization of wild-type (WT) and variant FcRns in HeLa cells
HeLa cells were transfected with wild-type (a) or variant (c; R210Q, e; S297T) FcRn-EGFP. The intracellular localization of FcRn-EGFP was observed by confocal laser scanning fluorescence microscopy. Differential interference contrast images of the field are also shown (b, d, f).

or intraluminal region. Therefore, we chose a C-terminal EGFP tag located in the cytoplasmic region of FcRn in order to minimize the effect of the fluorescent tag on the structural environment around the mutation sites.

As shown in **Figure 3a**, the fluorescent signal of wild-type FcRn-EGFP was located primarily in intracellular vesicular components, especially in the perinuclear region. Similar localization was observed for R210Q and S297T variants (**Figs. 3c** and **3e**), suggesting that these amino acid mutations do not affect the intracellular localization of FcRn.

**Intracellular co-localization of FcRn variants and incorporated antibody:** We then examined the co-localization of the incorporated CypHer5-labeled infliximab and FcRn-EGFP. The binding of CypHer5-labeled infliximab to FcRn was confirmed beforehand (data not shown).

As shown in **Figure 4**, co-localization of FcRn-EGFP and CypHer5-labeled infliximab in intracellular vesicular compartments was observed in HeLa cells expressing wild-type or variant FcRn. Since the fluorescence intensity of CypHer5 increases in acidic pH,[14] the observed fluorescent signal can indicate that CypHer5-labeled infliximab is localized in intracellular acidic compartments such as endosomes. Since the fluorescent images were obtained by confocal microscopy from cells which were washed with neutral pH media, the fluorescence is thought to be derived from incorporated antibodies and not from cell surface-bound antibodies. Therefore, these results showed that both types of FcRn variant, as well as wild-type FcRn, were in acidic endosomes in which incorporated antibodies localized.

**Antibody recycling activity of FcRn variants:** In order to elucidate the antibody recycling activity of wild-type and variant FcRn, we established the ELISA for biotinylated antibody (infliximab in this study), and measured the amount of recycled antibody from wild-type or variant FcRn-transfected cells. The binding of biotinylated infliximab to FcRn was confirmed by surface plasmon resonance (SPR) analysis (data not shown).

As shown in **Figure 5b**, recycled biotinylated infliximab was detected when the biotinylated infliximab had been loaded to the HeLa cells transfected with wild-type FcRn. The recycling was not detected in mock-transfected cells (**Fig. 5a**), showing that recycling was dependent on expression of FcRn. When the cells were incubated at 4°C for incorporation or recycling, the antibody was not detected in the supernatant. Therefore, recycling was mediated by intracellular trafficking of antibody and not by nonspecific mechanisms. As shown in **Figures 5c** and **5d**, similar levels of antibody recycling were also observed in HeLa cells transfected with either variant FcRn, suggesting similar IgG binding and intracellular trafficking properties of variant FcRns to those of wild-type FcRn. **Figure 6** shows the time course of antibody recycling from cells transfected with wild-type or variant FcRn. The amount of incorporated antibody was measured using the cell lysate at 0 min, and it is noteworthy that no statistical differences assessed by Dunnett's multiple comparison test were observed in the amount of incorporated antibodies between wild-type and either variant FcRn at time 0 (data not shown). The amount of recycled antibody at each time point was expressed as a percentage of the initially incorporated antibody. There was no significant difference between wild-type and the variant FcRns in the amount of recycled antibody, suggesting that these amino acid substitutions do not affect the antibody recycling activity of FcRn.

## Discussion

In general, antibody therapeutics have longer half-lives than those of chemical drugs, and the $T_{1/2}$ of IgGs, except for IgG3, in humans is around 21 days. IgG1, IgG2 and IgG4, which are currently used isoforms for antibody therapeutics, have high affinities for FcRn.[15] Escaping from intracellular degradation by binding to FcRn has shown to contribute to this long half-life of the IgGs.

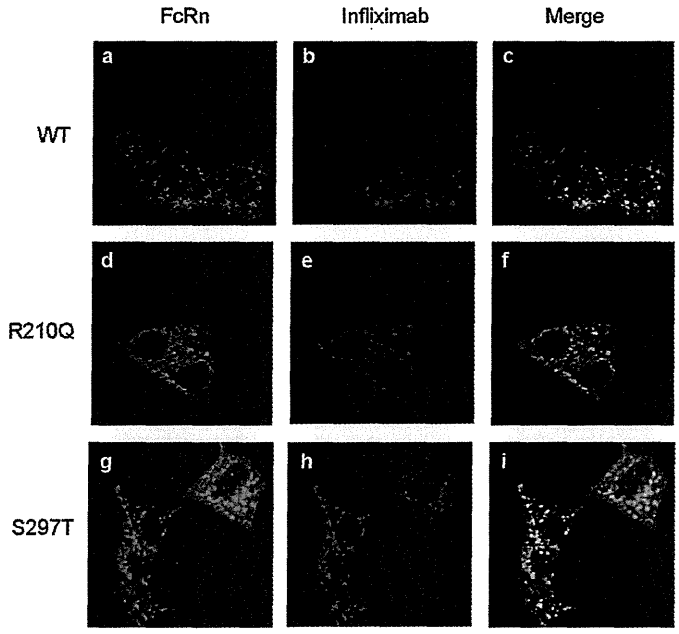


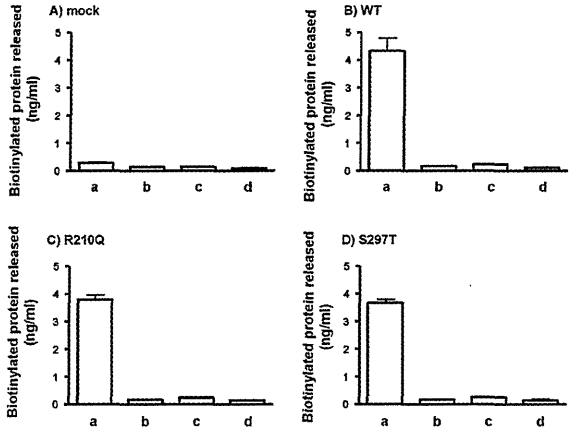

**Fig. 4.** Co-localization of CypHer5-labeled infliximab and FcRn in HeLa cells expressing wild-type (WT) or a variant FcRn
HeLa cells transfected with wild-type (a, b, c), or variant (d, e, f; R210Q, g, h, i; S297T) FcRn-EGFP were incubated with CypHer5-labeled infliximab in cell culture media containing sodium phosphate buffer (pH. 6.0) for 2–3 hr. After washing the cells twice with neutral pH medium, the fluorescent signal was observed. Panels (a, d, g) and (b, e, h) show the intracellular localization of FcRn-EGFP and the incorporated CypHer5-labeled infliximab, respectively. In panels (c, f, i) the fluorescent signal of FcRn-EGFP was merged with that of CypHer5-labeled infliximab.

Large interindividual variations in pharmacokinetic parameters have been reported for at least several antibody therapeutics. For example, trough concentrations in repetitive dosing of antibodies were reported to show 5.6-fold interindividual differences in 22 palivizumab-treated patients,[16] 18.2-fold differences in 16 cetuximab-treated patients,[17] and over 70-fold differences in 86 infliximab-treated patients.[18] In addition, large percent coefficients of variation were reported for $T_{1/2}$, such as 72.0% for gemtuzumab ozogamicin[19] and 76.4% for basiliximab,[20] after second dose of their treatments. We presumed that changes in FcRn expression levels and function caused by genetic variations of *FCGRT* may lead to these interindividual differences in pharmacokinetics of antibody therapeutics.

In order to identify genetic polymorphisms of *FCGRT*, we sequenced genomic DNA from 126 Japanese subjects. A total of 33 genetic variations, including 17 novel ones, were detected. A VNTR was detected in the 5′ -flanking region, as was the case in Caucasian subjects reported previously.[8] Although a recent study showed that no significant impact was observed in the rates of maternal-fetal IgG transfer,[21] VNTR3 is known to be associated with 1.66-fold higher transcriptional activity than VNTR2 *in vitro*. In addition, monocytes with VNTR3/3 showed increased binding of IgG compared to those with 2/3.[8] Thus, this variation may contribute to the interindividual differences in pharmacokinetics of antibody therapeutics. The allele frequency of VNTR2 in Japanese (0.032) was lower than that in Caucasians (0.075).[8]

In this study, two novel nonsynonymous variations were found and their functional significance was assessed *in vitro* using a mammalian expression system. However, the two FcRn variants did not show any changes in intracellular localization or recycling, suggesting that the two nonsynonymous substitutions found in a Japanese population probably do not contribute to the interindividual variations in the pharmacokinetics of antibody therapeutics. Since FcRn function is important for maintenance of IgG levels as well as maternal-fetal IgG transfer, functionally-affecting genetic variations might be few to retain its functional capability.

Amino acid residues of human FcRn that interact with IgG were reported to be E138, E139, D153 and W154, in the $\alpha$2 domain.[1] (Amino acid numbers shown in this paper include the signal peptide.). The electrostatic binding of these anionic amino acid residues in FcRn with H310 and H435 in IgG, which has an isoelectric point of pH 7.6, defines the strict pH-dependent binding of IgG to FcRn.[22] The variant amino acid residues identified in this study, R210Q and S297T, are both located in the $\alpha$3 domain of FcRn. According to the predicted higher order structure,[1] R210 and S297 are located very close to the




| Column | a | b | c | d |
|---|---|---|---|---|
| Reagents | Infliximab | | | IgY |
| Loading Temp. (°C) | 37 | 4 | 37 | 37 |
| Recycling Temp. (°C) | 37 | 37 | 4 | 37 |

**Fig. 5.** Recycling of biotinylated antibodies from wild-type (WT) or variant FcRn-transfected HeLa cells
HeLa cells transfected with wild-type or a variant FcRn were incubated for 1 hr with biotinylated infliximab. After washing, the cells were further incubated for 2 hr. The amount of recycled protein in the supernatant was determined by ELISA. Experimental conditions are shown in the table. For the samples shown as columns a-c, biotinylated infliximab was loaded, whereas biotinylated IgY was used for d. The temperature for antibody loading was 37°C (a, c, d) or 4°C (b). The temperature for recycling antibodies from antibody-loaded cells was 37°C (a, b, d) or 4°C (c).

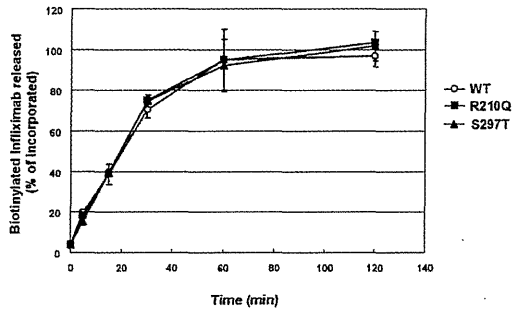



**Fig. 6.** Quantitative analyses of recycling of biotinylated infliximab; Time course of release of the biotinylated infliximab incorporated into the HeLa cells transfected with wild-type (WT) or variant FcRn
HeLa cells transfected with wild-type or a variant FcRn were incubated for 1 hr with biotinylated infliximab. After washing, cells were further incubated for the indicated periods of time. The amount of recycled protein was determined by ELISA. The amount of recycled antibody at each time point was expressed as a percentage of the initially incorporated antibody at time 0.

transmembrane region that is distant from the IgG binding site. Considering the results obtained here, where no difference in antibody recycling activity between wild-type and each variant FcRn was detected *in vitro*, the amino acid substitutions identified in a Japanese population may not have significant impact on structural and functional properties of FcRn. Although FcRn is known to bind with albumin as well as IgG, the albumin binding site of FcRn has been identified as H189, which also is located in the α2 domain.[23] The polymorphic sites are also far from the albumin binding site. However, the effect of amino acid substitutions R210Q and S297T on the albumin recycling activity via FcRn should be determined in a future study.

In the present study, we used HeLa cells to examine the localization and recycling activity of FcRn variants. Since endogenous expression of FcRn protein in HeLa cells has not been detected,[24] we considered HeLa cells suitable for examining the antibody recycling activity of variant FcRn since the background responses are negligible. In fact, as shown in **Figure 5**, antibody recycling was detected only in FcRn-transfected cells. Therefore, we concluded that HeLa cells can be used as a suitable model for evaluating the function of variant FcRn proteins.

Our results suggested that at least no common functional polymorphic site with amino acid change was present in *FCGRT* in our Japanese population. Since FcRn function is important for maintenance of IgG levels, there may be few functionally-affecting genetic variations. Further analysis is necessary for the functional significance of transcriptional regulatory regions.

**Acknowledgement:** We thank Ms. Chie Sudo for secretarial assistance.

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ORIGINAL ARTICLE

# Systemic chemotherapy for peritoneal disseminated gastric cancer with inadequate oral intake: a retrospective study

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**Abstract**
*Background* Oral fluoropyrimidines are widely used as standard treatment for gastric cancer, but peritoneal disseminated gastric cancer patients are often ineligible for chemotherapy using oral anticancer agents because of inadequate oral intake. The purpose of this study was to evaluate the treatment outcome and identify the prognostic factors in gastric cancer patients with inadequate oral intake resulting from peritoneal dissemination.
*Methods* Seventy-nine patients with peritoneal disseminated gastric cancer receiving systemic chemotherapy as the first-line treatment option at our hospital between April 1999 and December 2006, and who were administered intravenous drip infusion because of inadequate oral intake, were retrospectively analyzed.
*Results* All patients received 5-fluorouracil (5-FU)-based chemotherapy. Of the 79 treated patients, 71 had ascites as peritoneal dissemination and the remaining 8 had only gastrointestinal stenosis without ascites. Eleven (15%) patients showed an improvement in ascites. Proportion of oral intake improvement was 33%. Median time to progression and overall survival time was 1.7 months [95% confidence interval (CI), 0.9–2.4 months] and 3.3 months (95% CI, 2.1–4.5 months), respectively. Four independent poor prognostic factors were identified in multivariate analysis: serum albumin $< 3.0$ g/dl [hazard ratio (HR) 1.69, $P = 0.03$], performance status $\geq 3$ (HR 1.78, $P = 0.05$), massive ascites (HR 1.79, $P = 0.04$), and serum C-reactive protein $\geq 2.0$ mg/dl (HR 2.03, $P < 0.01$).
*Conclusion* The efficacy of 5-FU-based chemotherapy for peritoneal disseminated gastric cancer patients with inadequate oral intake was unsatisfactory.

**Keywords** Gastric cancer · Peritoneal metastasis · Inadequate oral intake · Chemotherapy

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## Introduction

Although the incidence and mortality rate of gastric cancer has decreased dramatically over the past several decades, gastric cancer remains one of the most common malignancies in the world, especially in Asia [1]. Gastric cancer can spread through various routes such as by local extension of direct serosal invasion, involvement of lymphatics, and distant metastasis through vascular diffusion. Peritoneal dissemination occurs mainly as a result of direct serosal invasion, omentum and peritoneal seeding, and/or lymphatic spread. Peritoneal dissemination is a common reason why gastric cancer cannot be resected [2]. Moreover, peritoneal recurrence after curative resection is identified as a major type (29–44%) of recurrence [3, 4]. Peritoneal dissemination may cause serious clinical complications, such as intestinal obstruction, massive ascites, obstructive jaundice, and hydronephrosis. These complications are associated with abdominal pain, abdominal fullness, vomiting, and malnutrition, leading to an extremely poor quality of life for the patient.

Recently, several phase III trials demonstrated that orally administered fluoropyrimidines, S-1 (containing

tegafur, 5-chloro-2,4-dihydroxypyridine, and potassium oxonate) or capecitabine, were not inferior to infusional 5-fluorouracil (5-FU) in advanced gastric cancer, so further clinical trials will demand greater feasibility of oral intake [5–7]. However, patients with severe peritoneal dissemination are excluded from drug development in accordance with inadequate oral intake. It is necessary to establish a treatment strategy for peritoneal disseminated gastric cancer patients with inadequate oral intake.

We retrospectively investigated the treatment outcome and prognostic factors in peritoneal disseminated gastric cancer patients with inadequate oral intake to determine the appropriate treatment strategy.

## Patients and methods

### Patients

Patients who received first-line chemotherapy treatment for gastric cancer at the National Cancer Center Hospital in Tokyo between April 1999 and December 2006 were retrospectively selected for this study according to the following criteria: (1) histological confirmation of adenocarcinoma as gastric primary lesion; (2) Stage IV disease or postoperative recurrence; (3) histological and/or radiologic confirmation of peritoneal dissemination; (4) no prior chemotherapy or radiotherapy; and (5) inadequate oral intake. We defined inadequate oral intake as requiring an intravenous drip infusion that had indeed been done. Patients who were administered an intravenous drip infusion for the purpose of renal protection or as a drug administration route such as for morphine were excluded.

Pretreatment clinical variables were evaluated: age (younger than 65 years of age or 65 years and older), gender (male or female), Eastern Cooperative Oncology Group (ECOG) performance status (PS) (0–2 or ≥3), serum albumin (<3.0 or ≥3.0 g/dl), serum C-reactive protein (CRP, <2.0 or ≥2.0 mg/dl), tumor histological type (diffuse or others), primary lesion status (present or absent), disease status (Stage IV or recurrence), ascites (massive or non-massive), number of metastatic sites (1 or ≥2), and the 5-FU administration method (bolus or continuous). Ascites was defined as four levels: none, mild, moderate, or massive. None was defined as undetected by computed tomography (CT) scan; mild ascites was localized in only one area such as the pelvic cavity or surface of the liver; moderate ascites did not correspond to either mild or massive ascites; and massive ascites extended continuously from the pelvic cavity to the upper abdominal cavity.

This retrospective study was approved by the National Cancer Center Institutional Review Board and conducted in accordance with ethical principles stated in Japanese ethics guidelines for epidemiological studies.

### Assessment of response

Responses were evaluated using the Response Evaluation Criteria in Solid Tumors. Ascites response was evaluated as follows: disappearance was defined as ascites unidentifiable by CT scan, decrease was defined as ascites decrease of more than one level, no change was defined as ascites remaining at the pretreatment level, and increase was defined as ascites increase of more than one level or ascites becoming clinically apparent. Oral intake improvement was defined as sufficient ingestion for 7 days or more without an intravenous drip infusion.

### Statistical methods

In univariate analysis, cumulative survival proportions were calculated using the Kaplan–Meier method, and any differences were evaluated using the log-rank test. Only those variables that achieved statistical significance in univariate analysis were subsequently evaluated in multivariate analysis using Cox's proportional hazard model. Progression-free survival (PFS) and overall survival (OS) were calculated using the Kaplan–Meier method. PFS was calculated from the date of the first treatment to the date of disease progression, death, or final follow-up. OS was calculated from the date of the first treatment to the date of death or final follow-up. All statistical analyses were performed using Dr. SPSS II software (SPSS Japan, Tokyo, Japan). All $P$ values presented in this report are of the two-tailed type. Differences with a $P$ value $\leq 0.05$ were considered significant.

## Results

### Patients and characteristics

From April 1999 to December 2006, a total of 1,747 consecutive patients with gastric cancer underwent systemic chemotherapy at the National Cancer Center Hospital in Tokyo. Of these, 340 patients with peritoneal metastasis underwent systemic chemotherapy as first-line treatment. Of these 340 patients, 82 patients had received an intravenous drip infusion before chemotherapy. However, 3 patients were excluded because of the usage of infusion as an opioid administration route. The remaining 79 patients were thus identified as participants in this study. The patient characteristics are summarized in Table 1. All patients had baseline Eastern Cooperative Oncology Group (ECOG) performance status (PS) greater than 1.

**Table 1** Patient characteristics

| Characteristics | *N* | % |
|---|---|---|
| Gender | | |
| Male | 43 | 54 |
| Female | 36 | 46 |
| Age (years) | | |
| Median | 58 | |
| Range | 20–77 | |
| ECOG performance status | | |
| 0 | 0 | 0 |
| 1 | 27 | 34 |
| 2 | 33 | 42 |
| 3 | 19 | 24 |
| Disease status | | |
| Unresectable | 59 | 75 |
| Recurrent | 20 | 25 |
| Primary tumor | | |
| Present | 46 | 58 |
| Absent | 33 | 42 |
| Histological type | | |
| Diffuse type | 71 | 90 |
| Intestinal type | 4 | 5 |
| Other not specified | 4 | 5 |
| Number of metastatic sites | | |
| 1 | 49 | 62 |
| ≥2 | 30 | 38 |
| Ascites | | |
| None | 8 | 10 |
| Mild | 34 | 43 |
| Moderate | 16 | 20 |
| Massive | 21 | 27 |
| Treatment regimen | | |
| Standard 5-FU ci | 10 | 13 |
| Low-dose 5-FU ci | 12 | 15 |
| MTX/5-FU | 56 | 71 |
| 5-FU/L-LV | 1 | 1 |

## Chemotherapy

First-line chemotherapy was based on 5-FU in all patients. 5-FU-based regimens of bolus administration were methotrexate (MTX)/5-FU and 5-FU/L-leucovorin (L-LV; or *l*-LV) therapy. The MTX/5-FU therapy consisted of weekly MTX [100 mg/m$^2$ administered intravenously (i.v.) as bolus] followed by 5-FU (600 mg/m$^2$ i.v. bolus) at 3-h intervals (Fig. 1a, b). The 5-FU/L-LV therapy consisted of weekly L-LV (200 mg/m$^2$ 2-h i.v. infusion) plus 5-FU (500 mg/m$^2$ i.v. bolus). The continuous 5-FU regimen included two different schedules: low-dose continuous infusion (ci) of a daily i.v. infusion of 5-FU (300 mg/m$^2$ 24-h ci) and standard 5-FU ci (800 mg/m$^2$ 24-h ci on days 1–5, q4w) (Fig. 1c, d). Median number (range) of each chemotherapy was 4 times (1–41 times) in MTX/5-FU, 6 times in 5-FU/L-LV, and 2 times (1–4 times) in standard 5-FU ci; median administration of low-dose 5-FU ci was 24 days (4–299 days).

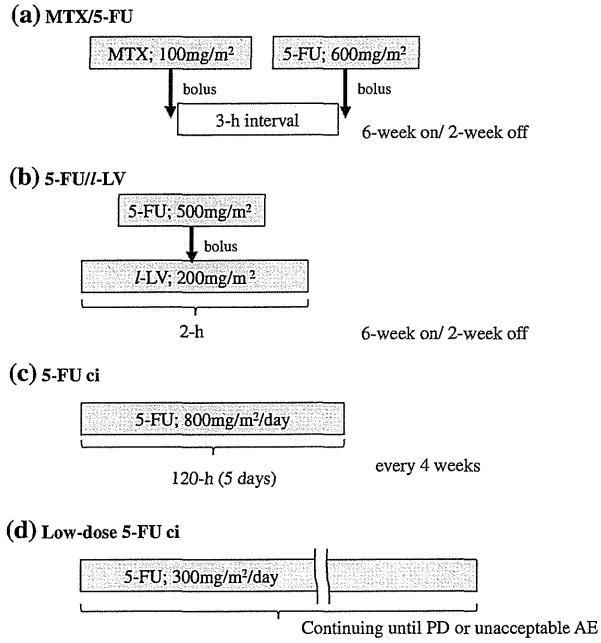


**Fig. 1** 5-Fluorouracil (*5-FU*)-based regimens used in the current study. *MTX*, methotrexate; *L-LV* (*l-LV*), L-leucovorin; *PD*, progressive disease; *AE*, adverse event; *ci*, continuous infusion

## Efficacy

Seventy-one (90%) of the 79 patients had evaluable ascites at initial diagnosis. The remaining 8 (10%) patients had gastrointestinal stenosis without ascites. Objective improvement in ascites was observed in 11 patients [15%, 95% confidence interval (CI) 8–26%]: 2 (3%) patients achieved disappearance of ascites and 9 (13%) patients had a decrease of ascites. Twenty-eight patients showed no change of ascites and 14 patients had an increase of ascites. The remaining 26 patients were not assessable because of the unavailability of posttreatment radiologic images, except for evident clinical disease progression that is unnecessary for radiologic evaluation (11 patients), transfer to other hospitals (7 patients), refusal (7 patients), and early death (1 patient). Oral intake improvement was observed in 26 patients (33%, 95% CI 23–44%). Two patients were excluded from analysis because they underwent endoscopic stent placement or ileostomy during chemotherapy. The most frequent reason for treatment discontinuation was disease progression (77%), followed by hospital transfer