Technical and regulatory gaps
Critical / Non-critical

- **Determination of Criticality**
  - Two or 3 tier approach
  - Process
  - Justification
  - Regulatory submission/requirement
- **Variability in risk acceptance decisions (residual risk) and justification for QRM decision**
- **Regulatory expectation of manufacturing process description**
  - Post Approval regulatory requirement
- **Inclusion or not of non-critical parameters in reg. submission**
  - Regulatory requirement of changes of non critical parameters
  - Monitoring/management through QS of non critical parameters
  - Inclusion of non critical parameters in DS

Technical and regulatory gaps
Level of documentation in submission

- **Level of details in the regulatory submission and/or at manufacturing site**
  - Balance between data / information / knowledge
  - Prescriptive / descriptive / informative
  - Information in submission versus availability during inspection
  - Documentation and presentation of the prior knowledge in the regulatory submission
  - Level of details of QbD type information in submission (Design Space, Control Strategy, RTRT, Quality Risk Management, etc.)
- **Location of QbD type information in regulatory submission**
  - Design Space, Control Strategy, Quality Risk Management, etc.
  - Potential need to revise CTD
- **Regulatory flexibility**
  - To facilitate continual improvement
- **Communication between industry and regulators**
- **Communication between assessors and inspectors**

Technical and regulatory gaps
Process validation / Process verification

- **Harmonised definitions of process validation, verification and process qualification**
- **Need for clarity and (harmonized) regulatory expectation of continuous verification**
- **The relationship between control strategy and process validation / continuous verification**
- **Need of clarity of validation studies with design space**
  - Relationship between validation and Design Space
- **Process validation approaches and regulatory expectations for continuous manufacturing processes**

Summary

- **Control Strategy**
  - Evolution & Suitability
  - RTRT etc & Certificate of Analysis (CoA)
  - Batch release decision
- **Role of modelling in QbD**
  - Development
  - Presentation in submission
  - Relationships: Modelling - DS - RTRT
  - Verification, scale up, updating and maintenance
  - Site changes
- **Design Space**
  - Beyond ICH Q8(R2)
  - Verification, updating & maintenance
  - Scale up
  - Technical transfer
  - Clinical relevance
- **Critical / Non-critical**
  - Determination of Criticality
  - Variability in RA decisions and variability
  - Manuf. process description
  - Non-critical parameters
- **Level of documentation in submission**
  - Regulatory submission and/or at manuf. site
  - Location of QbD type information
  - Regulatory flexibility
  - Communication industry & regulators
  - Communication assessors & inspectors
- **Process validation / Process verification**
  - Harmonised definitions
  - Regulatory expectation of CPV
  - Relatisonship CS & PV / CPV
  - Validation studies with DS
  - Continuous manufacturing processes

Next Steps

- **Remaining Q&As**
  - 4 pending Q&As - will be further discussed in Fukuoka
  - Q&As to address the influence of Q8, Q9, Q10 on the existing ICH Q-guidelines - June 2011
- **Draft training workshop summary report**
  - February 2011
- **Continue collaboration with GCG on training outside ICH region** - on request e.g. HC, APEC
- **Promote basic training on Q8, Q9 & Q10**
  - To be managed by outside groups
- **Address remaining technical and regulatory gaps**

Address remaining
Technical and regulatory gaps

Q-IWG Working plan

- To be completed by End 2011
- Regional working groups to develop initial drafts
- Several ICH-Q-IWG telecons to discuss
- Final versions to be adopted at F2F on ICH meetings 2011

Proposal for Endorsement

- **Implementation documents ('Points to Consider') to address 6 remaining technical and regulatory gaps**
  - Completed by end of Q2 2011
    - Level of documentation in submission
    - Criticality
    - Control Strategy
  - Completed by end of 2011
    - Process validation
    - Design Space
    - Role of Modelling in QbD
  - Clearance procedure for the 'Points to consider' same as Q&A
- **Q-IWG to complete activities by End of 2011**

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分担研究報告書

臨床試験における海外の規制状況の調査研究

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研究要旨
欧米における販売承認の更新、条件付き販売承認、市販後臨床試験等の監視のための方策等に関する規制及びその運用状況を調査し、我が国での制度のあり方について検討した。欧州における販売承認の更新制度は着実に運用されてきているが、個別品目ベースでの適時の安全対策との関係を含めて、その意義を継続して評価していく必要がある。米国では、近年、市販後試験に対するFDAの監視権限の強化が図られ、関連規則やガイドラインが逐次整備されてきている。我が国においても、医薬品の適正使用の推進及び安全対策の強化の一環として、既承認医薬品の承認内容の見直しをより積極的、機動的に行っていくことは重要な作業であり、今後、米国の状況等も参考にしながら、関連規制の見直しが検討されるべきであろう。

A．研究目的

承認審査、市販後調査を含め医薬品規制の国際調査を推進することにより、医薬品のグローバルな開発環境の整備及び安全確保体制を確立するための調査研究の一環として、欧米における販売承認の更新、条件付き販売承認、市販後臨床試験等の監視のための方策等に関する規制及びその運用状況を調査し、将来の我が国での制度のあり方について検討することを目的とする。

B．研究方法

初年度の研究で調査検討した欧州における販売承認の更新制度について、引き続き文献及びweb-site情報に基づき、その実際的な運用状況の調査を行った。また、米国における医薬品の市販後の有効性、安全性の確保のための制度のうち、特に市販後試験の実施及びそのフォローアップに関して調査を行い、これらを踏まえて、我が国における関連規制及びその運用のあり方について検討した。

（倫理面への配慮）外国の公的機関・組織を対象とした調査及び公開された情報を対象とした調査であり、倫理的な問題はない。

C．研究結果

1. 欧州における販売承認の更新制度

(1) 制度の概要

欧州における「販売承認の更新制度」の下では、原則として全ての医薬品の販売承認は初回承認から5年間有効となり、承認5年後に、承認保持者からの申請に基づき規制当局によるリスク・ベネフィットの再評価を経て販売承認が更新される。通常、販売承認は、ひとたび更新されるとその後の期限はなくなるが、市販後の安全監視の観点から必要な場合には、一度に限り再度5年の期限を付すことができるとされている。なお、「条件付き販売承認」（C.2. 参照）に該当する品目については、この更新が1年ごとに必要となる。なお、申請者は、更新申請に際して、1製品ごとに12,500ユーロの手数料を支払う。
（EC規則 No.726/2004 第14条1-3[1)]）

更新に関する審査の結果、①通常の使用状況において危険がある場合、②有効性がない場合、③品質が不良な場合、④申請書類が不正確である場合においては、販売承認の一時停止又は取消を行うとされている。（EC 指令 2001/83 第 116 条[2)]）

現行の更新制度は2005年11月から施行されたものである。それ以前は、製品が販売されている間は（永久に）5年ごとに販売承認を更新する制度であったが、原則1回の更新に改められた。旧制度は、承認保持者、規制当局ともに業務量の負担が大きく、ベネフィットとのバランスがとれていなかったために見直されたものであり、そもそも更新制度を無くす案もあったようであるが、各方面の意見も踏まえて、原則として承認後1回の更新とする内容となったとのことである。市販後の安全対策に関しては、承認更新のタイミングを待たなくとも、PSUR（Periodic Safety Update Reports：定期的安全性最新報告）等の情報を基に適時の対応が行われているという点も背景としてあるようである。

(2) 制度の運用状況

2010年に、CHMP（Committee for Medicinal Products for Human Use：人用医薬品委員会）において販売承認の更新が了承された品目数は47であり、うち10品目については5年後に再度の更新が必要と判断されている。（右表参照）

EMA（European Medicines Agency：欧州医薬品庁）は、更新手続きが終了すると、当該製品の EPAR（European Public Assessment Report：審査報告書）を改訂し、公表する（更新に関する CHMP の結論の簡単な記述が追加される）。また、販売承認の一時停止又は更新否決となった製品については、その理由を記した文書を公表する。

例えば、Rotarix（rotavirus vaccine）は販売承認の更新が行われたが、腸重積症のリスクを評価するための市販後臨床試験が進行中であり、安全性プロファイルを注意深く監視するため、5年後に再度の更新申請が必要と判断された。また、Keppra（levetiracetam：抗てんかん薬）についても承認の更新が行われたが、途中、小児適応の追加が行われており、小児における安全性データを収集するため、再度の更新申請が必要とされている。

2010年	更新が了承された品目数		
	合計	期限なし	5年後に再更新
1月	1	1	0
2月	6	4	2
3月	1	0	1
4月	3	3	0
5月	4	3	1
6月	10	8	2
7月	7	6	1
8月	—	—	—
9月	6	5	1
10月	2	1	1
11月	2	1	1
12月	5	5	0
合計	47	37	10

2. 欧州における条件付き販売承認制度

著しく衰弱性の疾患又は生命を脅かす疾患に使用される薬剤、公衆衛生への脅威に対応すべく緊急な状況で使われる薬剤又はオーファンドラッグについて、通常よりも不完全な承認申請データ（特に臨床試験データ）で承認をせざるを得ない場合に、特定の条件を付して販売承認を行う制度である。この場合の条件とは、進行中の臨床試験又は新たに実施する臨床試験によってリスク・ベネフィットのバランスを再確認すること、市販後安全性監視データを収集することなどである。2005

年に導入されたルールであり、新規承認品目のみに適用される（すなわち効能追加の承認等は対象外）。

条件付き承認制度の下での販売承認は、1年ごとに更新しなければならない。ひとたび条件に従ったデータが揃えば、条件付き承認は解除され、通常の承認に移行する（EC規則No.726/2004 第14条7[1]、EC規則No.507/2006[3]、ガイドライン[4]）。

2011年3月時点で、本制度下で承認され販売されている品目は、以下の7品目である。

- Arzerra（ofatumumab：フルダラビン及びアレムツマブに無効の慢性リンパ球性白血病）
- Cayston（aztreonam：嚢胞性線維症患者における緑膿菌による慢性肺感染の抑制療法）
- Diacomit（stiripentol：てんかん強直間代発作）
- Humenza（split influenza virus, inactivated：インフルエンザワクチン）
- Tyverb（lapatinib：HER2陽性の進行又は転移性乳がん）
- Vectibix（panitumumab：EGFR陽性の転移性大腸がん）
- Votrient（pazopanib：進行性腎細胞がん）

3. 米国における市販後試験の実施及びそのフォローアップ

(1) 関連する制度の概要と変遷

1997年のFDA近代化法（FDA Modernization Act）において、医薬品の市販後試験の進行を監視する権限がFDAに付与された（FDC Act 504B項）。ここで言う「市販後試験」とは、市販後に行われる各種の臨床試験及び非臨床試験のうち、FDAが実施を指示したもの又は企業がその実施についてFDAと合意したものを指す。（すなわち、市販後に企業が自主的に実施する試験は含まれない。）

この制度の下では、企業は、年次報告において、試験の実施状況をFDAに報告することが義務付けられる。米国における当該品目の承認日が基準となり、企業は、毎年の基準日から60日以内にFDAに報告書を提出する。報告書には、以下に示す情報を含めることとされている（21 CFR 314.81、ガイダンス[5]）。

－申請者名
－製品名、NDA番号
－米国での承認日
－市販後試験の約束日
－市販後試験の約束の内容
（目的、試験タイプ、被験者集団、適応・用量など）
－市販後試験のスケジュール
－市販後試験の現在の状況
（保留中／実施中／遅延／中止／提出済）
－試験状況の説明
（被験者の集積率など）

一方FDAは、企業から報告された情報を、年に1回Federal Register（連邦公報）で公表しなければならない。

従前は、市販後に臨床試験等が実施されるのは、FDAと承認保持者（企業）との自主的な合意に基づく場合か、あるいは以下に示すような特殊な状況にある場合に限られていた。（これらはPMC (Post-MarketingCommitment) と呼ばれる。）

- 迅速承認（Accelerated Approval）の対象とされた品目で、市販後に臨床的利点の証明が必要とされたもの（21 CFR 314.510）
- 小児研究平準化法（Pediatric Research Equity Act）に基づいて小児臨床試験が必要とされた品目（21 CFR 314.55(b)）
- 動物試験による有効性データをもって承認された品目で、市販後に臨床での有効

性、安全性の証明が必要とされたもの（21 CFR 314.610(b)(1)）

その後、2007年のFDA改正法（FDA Amendments Act）による市販後安全対策強化の一環として、新薬（医療用に限る）の承認時又は承認後に製薬企業に対して市販後臨床試験等の実施を求める新たな権限がFDAに付与された。（PMR (Post-Marketing Requirement) と呼ばれる。）そして、このPMRについても、PMCと同様の形で、その状況に関するFDAへの報告が求められる。

なお、正当な理由なくタイムスケジュールに従わなかった場合や進捗状況の定期的な報告を怠った場合は、承認取消し、民事上の罰金の罰則規程が設けられている。(実際には、罰則適用にまで至った例は知られていない。)

(2) 市販後試験の実施及びそのフォローアップ状況

2009年9月末時点において未完了のPMRs及びPMCsの状況がFDAにより公表されている[6]。PMRについては、NDA及びBLA合わせて、500件程度のうち約90%が予定通り進行し、PMCについては、1,300件程度のうち約85%が予定通り進行している。（下表参照）

		NDA（化成品）	BLA（生物製剤）
PMRs	予定通り進行	372/405 (91.5%)	88/96 (92%)
	予定変更	33/405 (8.5%)	8/96 (9%)
PMCs	予定通り進行	867/978 (89%)	244/325 (75%)
	予定変更	111/978 (11%)	81/325 (25%)

（2009年9月30日データ）

予定通り進行しているPMR及びPMCは、さらに、保留中（試験は開始されてないが遅延の基準には該当しないもの）、実施中（スケジュール通りに試験が進行中であるもの)、提出済（試験は終了又は中止し試験報告書がFDAに提出されたもの）に分類される。予定変更のPMR及びPMCは、遅延（元々のスケジュールから遅れているもの）、中止（試験が完了前に中止されたが試験報告書がFDAに提出されていないもの)に分類される。PMCについて、予定通り進行しているものの中の内訳をみると、（NDA及びBLA合わせて）保留中のものが約48%、実施中のものが約20%、提出済みのものが約31%となっている。（下表参照）

PMCsの状況		NDA（化成品）	BLA（生物製剤）
予定通り進行	保留中	449/867 (46%)	82/244 (25%)
	実施中	147/867 (15%)	84/244 (26%)
	提出済	271/867 (28%)	78/244 (24%)
	小計	867 (89%)	244 (75%)
予定変更	遅延	100/111 (10%)	79/81 (24%)
	中止	11/111 (1%)	2/81 (1%)
	小計	111 (11%)	81 (25%)
合計		978	325

（2009年9月30日データ）

2008年10月から2009年9月までに完了したPMR及びPMCは合わせて423件あり、うち約75%において当初の要求が満たされたと判断されている。

米国におけるPMRs及びPMCsに関する情報はデータベース化され、FDAのweb-site

において、企業名、製品名、NDA/BLA 番号、試験の状況、試験実施の根拠規定などをキーワードとして検索が可能である[7]。

(3) 抗悪性腫瘍薬の市販後臨床試験

1995 年以後、FDA は、迅速承認制度の下で 49 の抗悪性腫瘍薬（効能追加を含む）について迅速承認を行った。このうち 27 件については、市販後臨床試験において臨床上の有用性が確認され、通常承認に移行した。一方、5 件については、有用性の証明がうまくできなかったなどの理由で承認が取り下げられる又はその過程にある。

例えば Mylotarg（gemtuzumab ozogamicin：急性骨髄性白血病）は、2000 年 5 月に迅速承認された後に市販後臨床試験が実施されたが、延命効果が示されず、死亡例も比較的多いとの結果が得られたことから、販売企業は当該製品を自主撤退させた。また、Avastin（bevacizumab）は、2008 年 2 月に迅速承認制度による転移性乳がんの効能追加の承認を受けたが、追加で行われた臨床試験の結果、PFS（無増悪生存期間）のわずかな延長が認められたものの種々の重篤な副作用が認められ、FDA はリスクがベネフィットを上回るものと判断し、当該効能の取扱いについて検討中である。

FDA は、迅速承認制度の下で承認された抗悪性腫瘍薬の市販後臨床試験の状況をフォローするとともに、市販後臨床試験全般についてその計画・実施を改善し、迅速承認制度をより効果的に運用するための方策について検討する目的で、2011 年 2 月に抗悪性腫瘍薬諮問委員会（Oncologic Drugs Advisory Committee）を開催した（同種の会合は過去 2003 年 3 月、2005 年 11 月にも開催）。会合では、Erbitux (cetuximab)、Bexxar (tositumomab)、Clolar (clofarabine)、Arranon (nelarabine)、Vectibix (panitumumab)、Gleevec (imatinib) の市販後臨床試験の進捗状況について報告され、確認・議論が行われている。

D．考察

欧州では、2005 年 11 月から施行された新たな販売承認の更新制度に基づく作業が軌道に乗ってきた段階にある。この制度は、対象品目の取扱いの差はあるものの、日本の再審査制度に類似した制度といえる（欧州は全医薬品が対象であり製品ベース、日本は新薬のみが対象であるが効能追加等も別途実施）。なお、米国には、日本の再審査制度や欧州の販売承認の更新制度のように、全ての新薬を対象として、承認から一定期間後に承認内容の見直しを行うなどという制度は存在しない。

欧州において、2010 年は約 50 品目について販売承認の更新が行われており、昨年度の調査研究において EMA 担当者から聴取した件数の見込みと一致する。うち 2 割程度の品目について 5 年後に再度の更新が必要と判断されたものの、更新自体が否決されたものはない。基本的な安全対策については、5 年後の承認更新時を待たずとも PSUR や個別の副作用報告等の情報に基づいて適宜行われるという実態を反映したものと理解する。全ての医薬品を対象として、市販後一定期間を経過した時点で、リスク・ベネフィットのバランスの評価を改めて行うという機会は、個別品目ベースでの適時の安全対策を補完するものという位置づけになるであろう。なお、個別品目に関して、販売承認の更新を含めた市販後の種々の対応が、その簡単な背景等も含めて EPAR に記載され、web-site を介して公表されるというプロセスは、透明性確保の観点から重要なものであり、参考となる。

米国においては、1997 年の FDA 近代化法に基づき市販後試験に対する FDA の監視権限の強化が図られて以後、関連規則やガイドラインが逐次整備され、市販後に実施される臨床試験等の状況が体系的にフォローアップ

されるシステムが構築されている。具体的には、企業からFDAへの年次報告の内容に関する詳細なガイダンスの公表、報告された情報のFDAにおけるデータベース化、Federal Register（連邦公報）及びFDAのweb-siteを介した情報公表プロセスの確立とその実施である。

近年、我が国においても、既承認医薬品について市販後に臨床試験等が実施されるケースが増えてきている。これらの試験は、承認に付された条件（いわゆる承認条件）を履行するために行われる試験（薬事法第79条）、再評価指定を受けた医薬品について再評価申請資料の作成のために行われる試験（同第14条の6）など、その実施根拠が比較的明確なものと、承認審査の過程において規制当局から指示され、当局と申請企業との合意に基づいて実施される試験に大別できる。（後者についても、結果として、再審査申請資料の作成のための調査・試験として位置づけされることにはなる。）

そのような市販後試験の進捗状況やその後の予定等について、新薬に関しては安全性定期報告書に記載され、規制当局に提出されることになっている。しかし、当該報告書は安全性に関する情報の報告に主眼が置かれ、有効性の再確認を主たる目的として実施されるような市販後試験の状況を的確にフォローアップするための手段としては十分ではない。また、新薬以外の既承認医薬品について臨床試験等が行われる場合、その進捗状況等を体系的に把握する手段は設けられていない。

今後、医療に関連する技術や情報の進歩に応じて、既承認医薬品の承認内容の見直しをより積極的、機動的に行っていくことは、医薬品の適正使用の推進及び安全対策の強化の一環としても重要な作業となる。このためには、その評価判断の材料となる市販後試験の適切な実施と管理が不可欠であり、そうした観点からの我が国の関連規制の見直しが検討されるべきと考える。その際には、米国での状況も参考にしながら、我が国の現行の薬事規制及び医療システムを踏まえて、以下の事項について検討されることが望まれる。併せて、制度の適正な運用のためには、規制当局内における相応のリソースの確保についても検討する必要があろう。

- 市販後試験に係る情報の規制当局への報告に関する法的根拠の整備
- 報告内容及び手続きに関するガイダンス等の整備
- 市販後試験に関するデータベースの構築及びその維持管理
- 市販後試験の状況及び結果に関する情報の公表を含めたプロセスの透明性の確保

E．結論

欧米における販売承認の更新、条件付き販売承認、市販後臨床試験等の監視のための方策等に関する規制及びその運用状況を調査した。欧州における販売承認の更新制度は着実に運用されているが、個別品目ベースでの適時の安全対策との関係を含めて、その意義を継続して評価していく必要がある。米国では、1997年のFDA近代化法に基づき市販後試験に対するFDAの監視権限の強化が図られて以後、関連規則やガイドラインが逐次整備されてきている。今後、医療に関連する技術や情報の進歩に応じて、既承認医薬品の承認内容の見直しをより積極的、機動的に行っていくことは、医薬品の適正使用の推進及び安全対策の強化の一環としても重要な作業となる。このためには、その評価判断の材料となる市販後試験の適切な実施と管理が不可欠であり、米国の状況等も参考にしながら、そうした観点からの我が国の関連規制の見直しが検討されるべきであろう。

F．健康危険情報　なし

G．研究発表　なし

H．知的財産権の出願・登録状況　なし

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Regular Article

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

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

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 β2 microglobulin (β2m) cDNA was obtained from pME18SFL3 (FCC106E07) (Toyobo). β2m cDNA was subcloned into pcDNA3.1/
Hygro. The β2m construct was used because FcRn becomes a heterodimer with β2m, 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 β2m 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 μL 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 μL) 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 TNFα 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 β2m 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 β2m construct. The day after transfection, cells were seeded on 96-well plates at 4×10^4 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 μg/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.

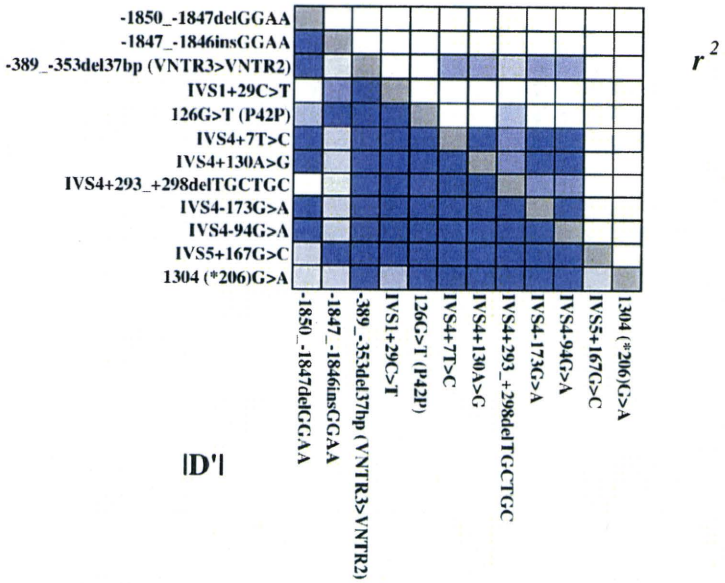

Fig. 1. Linkage disequilibrium (LD) analysis of *FCGRT*
Pairwise LD is expressed as r^2 (upper right) and |D′| (lower left) values (from 0 to 1) by 10-graded blue colors. A denser color represents closer linkage.

($p \geq 0{,}05$). 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 ≥ 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.

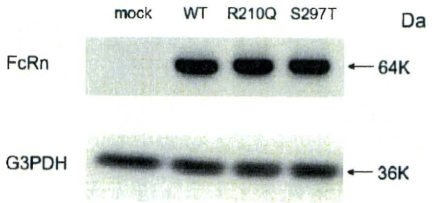

Fig. 2. Western blotting of wild-type and variant FcRns
Cell lysates obtained from the HeLa cells transfected with wild-type or either of the two variant FcRn-EGFP plasmids were subjected to electrophoresis, followed by transfer to the membrane. Detection of FcRn-EGFP was performed as described in Materials 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 β2m 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

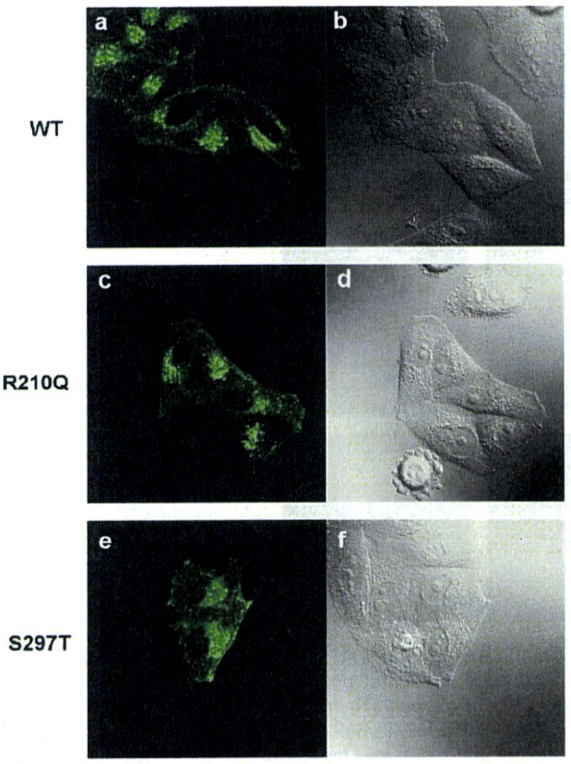

Fig. 3. Intracellular localization of wild-type (WT) and variant FcRns in HeLa cells
HeLa cells were transfected with wild-type (a) or variant (c; R210Q, e; S297T) FcRn-EGFP. The intracellular localization of FcRn-EGFP was observed by confocal laser scanning fluorescence microscopy. Differential interference 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.