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Original article

Correlation of FcγRIIIa polymorphisms and responses to rituximab in Thai population

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Background: Rituximab is a chimeric IgG_1 monoclonal antibody against CD20, approved for the treatment of B-cell non-Hodgkin's lymphoma (NHL). Antibody dependent cellular cytotoxicity (ADCC) has been suggested to be an important mechanism of rituximab via binding to the Fc gamma IIIa receptor (FcγRIIIa) on natural killer (NK) cells. FcγRIIIa has two expressed alleles that differ at amino acid position 158 in the extracellular domain, valine (V158) and phenylalanine (F158). These allelic variants have been demonstrated to differ in IgG_1 binding and ADCC. V/V homozygotes and V/F heterozygotes bind to IgG with higher affinity than F/F homozygotes.

Objectives: We identified the frequencies of FcγRIIIa polymorphism and investigate the correlation between FcγRIIIa polymorphism and rituximab responses, both *in vitro* and *in vivo* in Thai population.

Methods: The RFLP-Nested PCR and allele specific amplification was used to identify the FcγRIIIa polymorphism in the study. The correlation between FcγRIIIa polymorphism and rituximab responses, both *in vitro* and *in vivo*, was also studied.

Results: The distributions of FcγRIIIa-158 polymorphism in these subjects are V/V 40.26%, V/F 16.88%, and F/F 42.86%. Higher rituximab-induced Ramos cell cytotoxicity (mean 33.16%, 36.87%) was observed in the subjects with VV and VF genotypes, respectively. However, the lower cytotoxicity (mean 20.07%) was determined in subjects with FF genotype. As for the *in vivo* study, the NHL patients with V/V or V/F genotypes had a primary response as complete response; whereas, the NHL patients with F/F genotype had partial response.

Conclusion: FcγRIIIa polymorphism and the primary response in NHL patients tend to correlate. The higher number of patients is necessary for further study. These results provide useful information to understand beneficial response of rituximab as well as other IgG_1 therapeutic antibody in Thai patients.

Keywords: Anti CD20, FcγRIIIa polymorphisms, NK cell, non-Hodgkin's lymphoma, rituximab

Non-Hodgkin's lymphoma (NHL) is a heterogeneous group of B- and T-lymphocyte derived hematological malignancies. More than 80% of NHL is B-cell lymphoma [1-3]. In Thailand, NHL is the most common hematological cancer. Because the majority of B-cell NHL differentiated from B-lymphocytes, the surface molecules of B-cell lymphoma, such as CD20, could be expressed similar to B-lymphocytes. The first clinically approved monoclonal antibody-based immunotherapy of lymphoma involved the anti-CD20 chimeric monoclonal antibody, called rituximab [4, 5].

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Rituximab, a chimeric IgG1 monoclonal antibody (mAb), binds specifically to CD20 [12]. *In vitro*, rituximab can induce antibody-dependent cellular cytotoxicity (ADCC) through the FcR/Fc binding, and it binds human C1q and induces complement-mediated lysis (CDC), apoptosis, and direct growth arrest. There are some evidences that show the involvement of these mechanisms *in vivo* [6-12].

FcγRIIIa or CD16 is the Fc receptor that is dominantly expressed on human NK cells. The FcγRIIIa recognizes the IgG that bound to the surface of a target cell. Activation of FcγRIIIa by IgG causes the release of cytokines such as perforin and granzyme, which can promote cell death by triggering apoptosis. The FcγRIIIa is the low affinity receptor for the Fc region of IgG. Normally, the low affinity FcRs cause the specific binding and cell activation.

Therefore, FcγRIIIa is more crucial for anti-tumor responses and monoclonal antibody-based therapy than the high affinity receptors [12, 13].

The genetic polymorphisms have been discovered in various FcγR. For FcγRIIIa, the guanine (G) to thymidine (T) point mutation at nucleotide 559 results in the amino acid substitution at position 158 from valine (V) to phenylalanine (F) [14]. The FcγRIIIa-158 V allele shows higher affinity for IgG1 and IgG3 than FcγRIIIa-158F, and is able to bind IgG4. Meanwhile, FcγRIIIa-158F is not able to bind the IgG4 [15, 16]. Following the incubation of NK cells from FcγRIIIa-V/V homozygous donors with IgG, the influxes of calcium and the induction of apoptosis from FcγRIIIa-V/V homozygous donors is more than FcγRIIIa-F/F homozygous donors [17]. Recent studies have shown that FcγRIIIa polymorphism is associated with the therapeutic efficacy of rituximab in non-Hodgkin's lymphoma patients. The homozygous V/V allele patients provided a higher response to the treatment than the homozygous F/F allele patients [18-22]. Additionally, a higher response has been shown in rituximab treated follicular lymphoma patients who have the homozygous for FcγRIIIa-Val158 and FcγRIIb-His131 alleles [21]. On the other hand, some experiments have been shown that FcγRIIIa 158 V/F polymorphism is not associated with the response of R-CHOP (Rituximab combined with CHOP chemotherapy) in most Caucasian patients with follicular lymphoma treated with R-CHOP [23-25], which is different from the study in Korean patients with diffuse large B-cell lymphoma. The role of FcγRIIIa polymorphism in the efficacy of mAbs is still controversial. Furthermore, it might depend on the stage and type of disease or ethnicity of the patient [26].

Several studies have shown the variation of ethnicity in the distribution of the FcγRIIIa genotypes [26-30]. The distribution of homozygous V/V158, heterozygous V/F158, and homozygous F/F158 has been reported to be largely varied from 4% to 47%, 32.1% to 50%, and 5% to 63.2%, respectively. As for clinical benefit of rituximab in Thai population, this study aims to investigate the distribution of the FcγRIIIa genotypes in Thai population, and evaluate the correlation of the FcγRIIIa polymorphism and the response of rituximab both *in vitro* and in clinical outcome.

Methods

ADCC assay

Peripheral blood mononuclear cells (PBMCs) containing NK cells from Thai blood donors with informed consent were used as the effector cells (E). These cells were prepared by ficoll gradient centrifugation method. Ramos cells from ATCC that express CD20 molecules were used as target cells (T). These cells were stained with a 5 μM fluorescent dye CFSE for 5 minutes before assay. The effector: target (E:T) ratio, 10:1 was used in this assay.

In ADCC assay, 2×10^5 cells/ml CFSE-stained Ramos cells were pre-treated with 10 μM rituximab for 1 hour at 37°C, mixed with 2×10^6 cells/ml human PBMCs, and further incubated for 4 hours at 37°C. These co-culture cells were collected, stained with 5 ng/ml propidium iodide (PI) for 15 minutes, and analyzed dead Ramos cells by fluorescence flow cytometer.

This study was approved by Ethical Review Board of Faculty of Medicine, Chulalongkorn University.

FcgRIIIa polymorphism analysis

Genomic DNA was extracted from the whole blood of 60 healthy Thai male blood donors and 17 NHL patients treated with rituximab-containing anticancer drug regimen by using a blood DNA extraction kits (Vivantis®). The DNA was used for genotyping by RFLP-nested PCR with two sets of primers that gave a 1.2 Kb PCR product from the genomic DNA template and a 96 PCR product amplified from the 1.2 Kb PCR product, respectively.

The first amplification reaction was performed by using 2 μl of the genomic DNA, 0.2 mM dNTP, 1 U *Taq* polymerase, 1.5 mM $MgCl_2$ and 0.5 μM the first set FcγRIIIa primers (**Table 1**). The FcγIIIa PCR product was amplified by the following conditions: initial denaturation 95°C for 10 minutes, followed by 40 cycles of PCR amplification protocol (95°C for 1 minute, 56°C for 1 minute 30 seconds, and 72°C for 1 minute 30 seconds), and finally the final extension at 72°C for 8 minutes.

Then, 2 μl of PCR product from the first reaction was added into the master mix solution with the second set FcγRIIIa primers. The second FcγRIIIa PCR product was amplified by using the following conditions (**Table 1**); initial denaturation 95°C for 5 minutes, followed by 40 cycles of PCR amplification protocol (95°C for 1 minute, 67.5°C for 1 minute 30 seconds and 72°C for 1 minute 30 seconds). Finally, it was

extended at 72°C for 9 minutes 30 seconds. The nested PCR product was subjected for restriction fragment length polymorphism (RFLP) by being digested with 10 U *Nla*III in 20 µl the PCR product. The digestion was performed at 37°C for 3 hours.

To confirm the genotyping, the allele specific amplification method was used. Two ml of the genomic DNA was mixed with 0.2 mM dNTP, 1 U Accuprime® *Taq* polymerase, and 0.5 µM the set of V or F allele specific primers in a 0.2 ml PCR tube. The FcγRIIIa PCR product was amplified by using the following conditions: initial denaturation 94°C for 2 minutes, followed by 40 cycles of PCR amplification protocol (94°C for 30 seconds, 65°C for 30 seconds and 68°C for 1 minute), and finally the final extension at 72°C for 8 minutes. The FcγRIIIa genotype of the PCR product was identified by 3% agarose gel electrophoresis.

Clinical outcome evaluation

Primary clinical outcomes of NHL patients treated with rituximab-containing drugs were assessed for correlating with their genotypes. The data of clinical outcomes recorded by experienced clinicians were collected from the patients' chart. Each response to the treatment of every patient was evaluated based on standard criteria, the International Workshop Criteria for Non-Hodgkin's Lymphoma [31], which divide the response into three levels as complete response (CR), partial response (PR), and progression disease (PD).

Statistical analysis

Data were individually presented. As for the *in vitro* ADCC study, the difference of the ADCC activity in each genotyping group was compared by using the nonparametric Kruskal-Wallis test. The correlation between the genotypes and the clinical responses of NHL-patient treated with rituximab-containing regimen was assessed by using two-tailed Fisher's exact test. The statistically significant value was considered at $p < 0.05$.

Results

The distribution of FcgRIIIa polymorphism in Thai population

The nested PCR products generated by two sets of primers were 1.5 Kb PCR product from the first primer set, and 94 bp PCR product from the second set of the primers. A restriction enzyme *Nla*III was used to cut the second PCR product at the 158 polymorphic site of the FcγRIIIa of V allele into 61 and 33 bp products. This enzyme theoretically generates one band (94 bp) for F/F genotype; two bands (61 and 33 bps) for V/V genotype; and, three bands (94, 61, and 33 bps) for V/F genotype. These genotypes were confirmed by PCR using V or F allele specific primers. Genotypes of three of the sixty genomic DNA samples from normal subjects were further confirmed by DNA sequencing.

The FcγRIIIa genotypes of 60 healthy subjects were 23 V/V, 10 V/F, and 27 F/F, respectively. The genotypes of 17 NHL were eight V/V, three V/F, and six F/F as can be seen in **Table 2**.

Table 1. Set of primers used in the study

First PCR primer	Forward primer	5'-ATA TTT ACA GAA TGG CAC AGG-3'
	Reverse primer	5'-GAC TTG GTA CCC AGG TTG AA-3'
Second PCR primer	Forward primer	5'- ATC AGA TTC GAT CCT ACT TCT GCA GGG GGC AT-3'
	Reverse primer	5'- ACG TGC TGA GCT TGA GTG ATG GTG ATG TTC AC-3'
Confirm PCR primer	V specific primer	5'-CTG AAG ACA CAT TTT TAC TCC CAAA-3'
	F specific primer	5'-CTG AAG ACA CAT TTT TAC TCC CAAC-3'
	Reverse primer	5'-TCC AAA AGC CAC ACT CAA AGA C-3'

Table 2. The distribution of FcγRIIIa polymorphism in Thai population

Genotype	VV	VF	FF	Total
Normal Volunteers	23	10	27	60
NHL patients	8	3	6	17
Total	31 (40.26%)	13 (16.88%)	33 (42.86%)	77

Correlation of the FcgRIIIa genotype and rituximab-mediated ADCC in vitro

The correlation between FcγRIIIa genotype and *in vitro* ADCC activity of NK cells from 60 healthy subjects was also evaluated in this study. ADCC of NK cells was induced by using rituximab-bound Ramos cells (CD20 positive cells) as the target cells. Cytotoxicity on Ramos cells was identified by staining the cells with fluorescent CFSE to separate them from PBMCs and by staining them with PI to detect cell death. Death of Ramos cells was determined as CFSE+/PI+ cells by fluorescence flow cytometer. A representative result of rituximab-induced death of Ramos cell by ADCC is presented in **Figure 1**. The mean values of the percentage of rituximab induced Ramos cell cytotoxicity were 31.16% in V/V genotype subjects, 36.87% in heterozygous V/F genotype subjects, and 20.07% in F/F genotype subjects (**Figure 2**). Both V/V and V/F genotypes had significantly higher ADCC activity than F/F genotype at p <0.001.

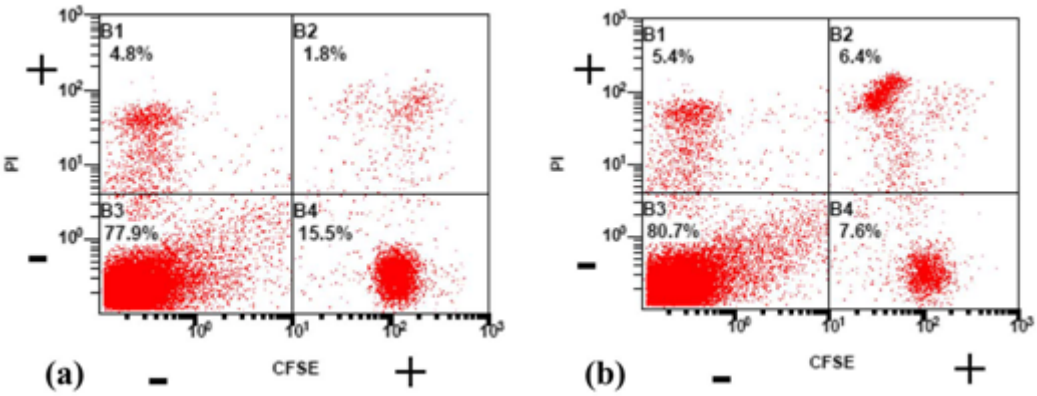

Figure 1. The representative results of rituximab-mediated Ramos cell death by ADCC at 10:1 PBMCs:CFSE$^+$ Ramos cells ratio using fluorescence flow cytometer: (a) without rituximab; and, (b) with rituximab. The amount of Ramos cell death is identified in B2 quadrant. The percentage of Ramos cell death was calculated from the percentage of cells in quadrant B2 and B4.

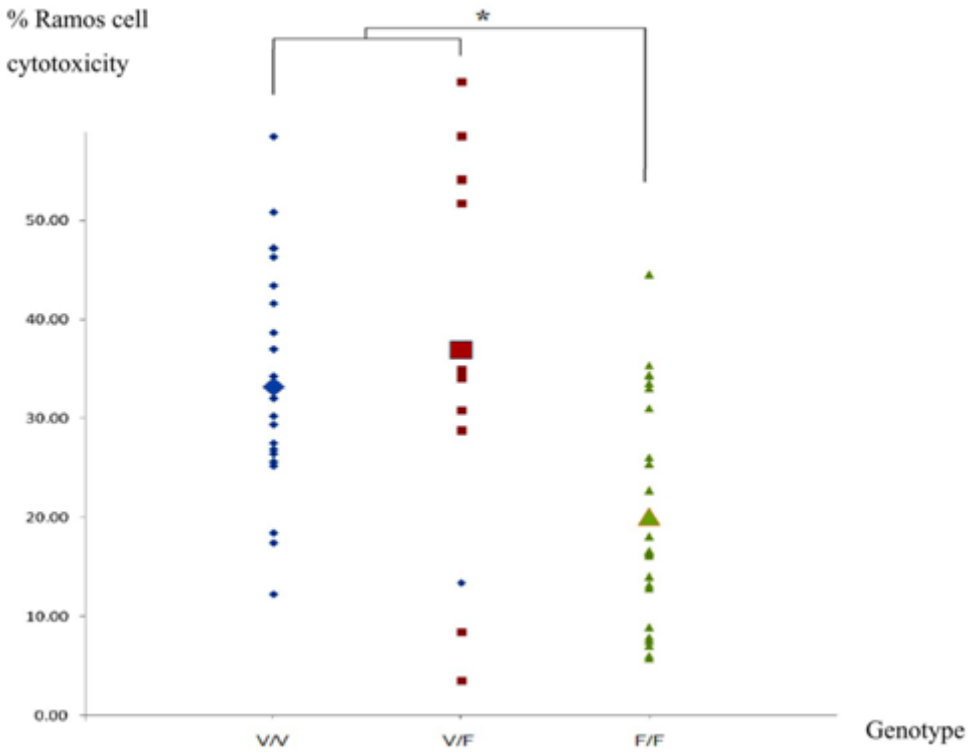

Figure 2. The correlation between FcγRIIIa genotypes and rituximab-mediated ADCC *in vitro* by using PBMCs from 60 healthy subjects as target cells. *statistically significant at p <0.001

Correlation of the FcgRIIIa polymorphism and the clinical outcome of rituximab-treated NHL patients

This study also investigated the correlation of FcγRIIIa genotypes and clinical outcomes of 17 NHL patients treated with rituximab-containing anticancer drug regimens. Their genotypes were eight V/V, three V/F, and six F/F. The treatment responses of these patients were evaluated by hematologists at King Chulalongkorn Memorial hospital.

As in the **Table 3**, all eight patients with V/V genotype had complete response. Two of three patients with V/F genotype had complete response. The other one could not be assessed due to rituximab complications. The antibody was withdrawn from this patient. Only one of six patients with F/F genotype had complete response. Three of them had partial response and the other two patients could not be assessed because of rituximab complications.

Discussion

This study reveals the genotyping distribution of FcγRIIIa in Thai population. The frequencies of FcγRIIIa-158 V/V, V/F and F/F genotype were 40.25%, 16.88%, and 42.85%, respectively. Genetic polymorphisms are influenced by race and ethnicity. Comparison of FcγRIIIa genotype with Caucasian population, Thai subjects have higher frequency of FcγRIIIa homozygous V/V genotype than the Caucasian (40.26% vs. 11%) but similar frequency of F/F homozygous (42.86% vs. 50%) [27, 28]. Thai population also differs in genotype distribution from population in other Asian countries. The frequencies of FcγRIIIa-158 V/V, V/F and F/F genotype in Korean are 47%, 48%, and 5%, respectively [26]. These frequencies in Japanese are 4%, 44%, and 52%, respectively [29].

It has been shown that homozygous FcγRIIIa 158V/V on NK cells was bound to IgG with higher affinity than FcγRIIIa 158F/F [32, 33]. Recent studies have suggested that healthy individuals expressing V/V and V/F genotypes increase expression of FcγRIIIa on NK cell surface, binding affinity with rituximab, and ADCC activity of rituximab [15]. This study also demonstrates good correlation between FcγRIIIa polymorphism and the response to rituximab *in vitro*. As for determining the major mechanism of action of rituximab ADCC, human B-lymphoma Ramos cells were used as the target cells recognized by rituximab and NK cells in human PBMCs were, used as the effector cells. The results demonstrated that the effector cells in PBMCs from subjects with V/V and V/F genotype induced higher rituximab-mediated Ramos cell cytotoxicity than effector cells from F/F allele individuals. The effector cells from V/V, V/F, and F/F individuals induced Ramos cell death 33.16%, 36.87%, and 20.07%, respectively. These results support the correlation of FcγRIIIa polymorphism and the response to rituximab *in vitro*. The FcγRIIIa 158V/V and V/F NK cells, which FcγRIIIa is stronger bound to IgG, induced higher ADCC than FcγRIIIa 158F/F NK cells, which FcγRIIIa is more weakly bound to IgG.

Several studies revealed the influence of FcγRIIIa polymorphisms on the response to rituximab containing anticancer regimen in different types of NHL [21-27]. Depend on types of lymphoma, NHL patients with FcγRIIIa 158 V/V genotype responded to rituximab monotherapy and/or rituximab-containing regimen better than F/F genotype. However, some studies in Caucasian population demonstrated no correlation between FcγRIIIa polymorphisms and the clinical response to rituximab [23-25]. This study demonstrates the correlation between the FcγRIIIa polymorphism and the clinical outcomes of NHL patients that were either DLBCL or FL patients treated with rituximab-containing anticancer regimens. The patients with FcγRIIIa-158 V/V and V/F genotypes responded to rituximab containing regimens better than FcγRIIIa-158 F/F genotype. However, the correlation between FcγRIIIa polymorphism and survival rate or long term clinical response to rituximab needs to be further evaluated.

Table 3. Clinical outcomes of 17 NHL patients treated with anticancer drug regimens containing rituximab

Primary clinical assessment	Genotype		
	VV	VF	FF
Complete Response (CR, CRu)	8	2	1
Partial Response (PR) or Progression Disease (PD)	-	-	3
No assessment	-	1	2
Total	8	3	6

In conclusion, the results from this study support several previous studies that ADCC is one of the mechanisms of rituximab actions on B lymphoma cells. There is correlation between FcγRIIIa polymorphism and the response of rituximab both *in vitro* and in Thai population. In Thailand, genetic polymorphism of FcγRIIIa may influence the clinical use of rituximab as well as other IgG_1 therapeutic antibodies that have ADCC as one of their mechanism of actions.

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