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SNPs rs4656317 and rs12071048 located within an enhancer in *FCGR3A* are in strong linkage disequilibrium with rs396991 and influence NK cell-mediated ADCC by transcriptional regulation

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ABSTRACT

CD16 receptors are mainly expresses on the surface of NK cells and mediate antibody-dependent cellular cytotoxicity (ADCC). The authors previously reported that NK cell-mediated ADCC is influenced by the single nucleotide polymorphism (SNP) rs396991 (T > G; F158V), and the structure and expression levels of CD16 differed among these genotypes. The authors examined haplotype frequency distributions among rs396991 and other SNPs, rs10917571 (G > T), rs4656317 (C > G), and rs12071048 (G > A), located in an enhancer of the *FCGR3A* gene. A total of 101 healthy Japanese were genotyped for the presence of these SNPs. The authors also measured ADCC activity, *FCGR3A* transcript levels, and surface CD16 expression on NK cells. We found that the regulatory SNPs (rSNPs) rs4656317 and rs12071048 were in strong linkage disequilibrium with rs396991. These two SNPs with major alleles had higher ADCC activity than those with minor alleles. In addition, *FCGR3A* transcript levels and surface CD16 expression levels were regulated by these SNPs. These findings suggest that NK cell-mediated ADCC could be influenced by transcriptional regulation of these rSNPs. These findings help to clarify our understanding of the linkage disequilibrium among functional SNPs in the *FCGR3A* gene, and provide a resource for investigating the roles of functional SNPs in NK cell-mediated ADCC.

1. Introduction

Natural killer (NK) cells constitute approximately 10% of the lymphocytes in human peripheral blood. They have important effector functions to directly lyse tumor cells and virus-infected cells without prior sensitization or MHC class restriction in the innate immune system [1–6].

The CD16 receptor is a low affinity Fc gamma receptor (FcγRIIIa), mainly expressed on the surface of NK cells. CD16 contains an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain and can bind to the constant (Fc) region of immunoglobulin when immobilized on the cell surface [7]. This receptor-ligand binding is followed by a CD16-mediated activation signal that results in antibody-dependent cellular cytotoxicity (ADCC). NK cell-mediated ADCC is used for the treatment of some tumors when using the tumor-targeted mAbs [8–13]. Therefore, ADCC is an important effector mechanism in the treatment of tumor cells.

The CD16 receptor is encoded by the *FCGR3A* gene. A nucleotide substitution of the *FCGR3A gene* (rs396991; T > G) results in either a valine (V) or a phenylalanine (F) expression at amino acid position 158 [14–18]. NK cell-mediated ADCC activity is speculated to depend on this single nucleotide polymorphism (SNP). Cartron et al. and Weng et al. reported that rs396991 was involved in the rituximab response in follicular lymphoma [19,20]. Rituximab is

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a chimeric anti-CD20 IgG1 mAb that is used to treat patients with B-cell lymphomas and various autoimmune diseases [21–23]. Similar results also found that rs396991 enhanced the effects of immunotherapies that use the tumor-targeted mAb for various tumors, like as cetuximab, trastuzumab, and infliximab [24–27].

The authors reported that NK cell-mediated ADCC was influenced by rs396991, and structure and expression levels of the CD16 receptor differed among these genotypes in healthy Japanese subjects [28]. However, a consensus has not been reached regarding the difference in CD16 expression levels among rs396991 genotypes yet. Hatjiharissi et al. also reported that this SNP influenced CD16 binding affinity to the Fc region of rituximab, CD16 transcript levels, and surface CD16 expression levels [29]. Conversely, Congy-Jolivet et al. suggested that CD16 expression levels were not associated with rs396991 genotypes [30]. As rs396991 is a coding SNP (cSNP), this SNP is not expected to directly affect transcriptional regulation. Therefore, the authors also assumed that rs396991 might link with other SNPs, such as rs10917571, rs4656317, and rs12071048, in regulatory regions (rSNPs) as a haplotype. In general, the extent of linkage disequilibrium often differs among populations. The authors also investigated the influence of these rSNPs on NK cell-mediated ADCC activity. The present study could contribute to a clearer understanding of the linkage disequilibrium of the *FCGR3A* gene and provide a resource for investigating the roles of functional SNPs in NK cell-mediated ADCC.

2. Materials and methods

2.1. Subjects

A total of 101 healthy Japanese volunteers (46 males and 62 females) between the ages of 18 and 33 were enrolled in the present study. SNP genotyping was performed for all volunteers. Other NK cell-associated analyses were conducted for 24 volunteers as part of 101 volunteers. The study protocol was approved by the Ethics Committee for Kagawa Prefectural University of Health Sciences, and written consent was obtained from all participating subjects according to the Declaration of Helsinki.

2.2. Genomic DNA and total RNA isolation

Genomic DNA was isolated from peripheral blood anticoagulated with K2-EDTA, using a QIAamp DNA Blood Mini Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's protocol. For total RNA isolation, PBMCs were isolated from heparinized peripheral blood using Ficoll-Paque PLUS (GE Healthcare UK Ltd., Buckinghamshire, England). NK cells were separated from PBMCs using the EasyStep™ Human NK Cell Enrichment Kit (STEMCELL Technologies, Vancouver, BC, Canada) resulting in more than 95% purity. Total RNA was isolated from NK cells using an miRNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. The quantity and quality of genomic DNA and total RNA were evaluated using a NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. SNP genotyping

We genotyped SNPs located within an enhancer, i.e., rs10917571 (G > T), rs4656317 (C > G), and rs12071048 (G > A) in the *FCGR3A* gene (Fig. 1). SNP genotyping was achieved by the direct sequencing of genomic DNA [28]. *FCGR3A* gene sequence-specific primers for PCR and direct sequencing are shown in Table 1. The PCR reaction consisted of 100 ng of genomic DNA as template, 1 × PCR buffer (1.5 mM $MgCl_2$), 200 μM dNTPs, 1 μM each primer, 1 U AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific),

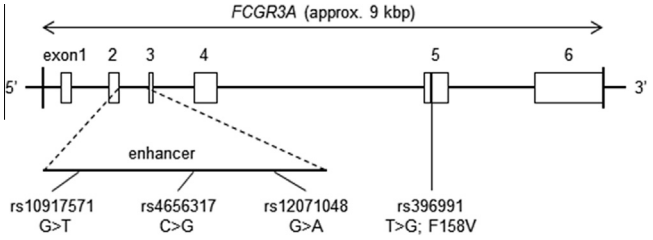

Fig. 1. A schematic representation of the target SNPs in the *FCGR3A* gene. Target SNPs in this study are rs10917571 (G > T), rs4656317 (C > G), and rs12071048 (G > A) in the *FCGR3A* gene. Positions of these SNPs are shown within an enhancer. rs396991 (T > G; F158V) was genotyped in our previous study.

and molecular grade water to make a final volume of 20 μL. Cycling conditions of the PCR were performed as follows: an initial denaturation at 95 °C for 5 min, followed by further denaturation with 40 cycles at 95 °C for 30 s, primer annealing at 53 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 7 min. Sequencing of specific PCR products was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and specific primers according to the manufacturer's instructions. After cleanup of reaction mixtures and denaturation to single-stranded DNA, purified samples were analyzed on an ABI PRISM® 310 Genetic Analyzer (Thermo Fisher Scientific).

2.4. Cell lines and cell culture

A Raji cell line (JCRB Cell Bank, Osaka, Japan) is derived from a patient with Burkitt's lymphoma and expresses the CD20 molecule on the cell surface. Raji cells were cultured in complete RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific), 2 mM L-glutamine, 100 U/mL penicillin (Thermo Fisher Scientific), and 100 μg/mL streptomycin (Thermo Fisher Scientific) at 37 °C in the humidified atmosphere of a 5% CO_2 incubator. One day prior to ADCC activity experiments, the Raji cells were sub-passaged to ensure they were growing in the log phase.

2.5. ADCC activity

A measurement of ADCC activity was performed by CFSE/7-AAD flow cytometric assay, as previously described [28,31,32]. Raji cells were labeled with 5 μM 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (BioLegend, San Diego, CA, USA) at 37 °C for 15 min. After labeling, cells were washed with complete medium and resuspended at 1×10^6 cells/mL. CFSE-labeled Raji cells were incubated with 100 μg/mL rituximab (Chugai Pharmaceutical, Tokyo, Japan) at 37 °C for 1 h, and then washed twice. Isolated NK cells were incubated with 1×10^5 CFSE-labeled Raji target cells at different effectors to target (E:T) cell ratios from 20:1, 10:1, 5:1, and 2.5 in 96-well V-bottom microplates. After coculture for 4 h at 37 °C with 5% CO_2, the cell mixture was washed twice in PBS and stained with 500 ng/mL 7-amino actinomycin D (7-AAD, BioLegend) in Cell Staining Buffer (BioLegend) at 4 °C for 15 min in darkness. In order to achieve complete cytolysis, 1% saponin was added to a maximum of lysis sample. Cells were washed twice with PBS and analyzed on a Cell Lab Quanta SC flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with a 488-nm laser air-cooled configuration. CFSE cell staining is measured in the FL-1 cannel and 7-AAD cell staining is measured in the FL-3 cannel. Dead Raji target cells were indicated as $CFSE^+7\text{-}AAD^+$ cells. ADCC activity was shown as % Specific Lysis calculated by the following formula: % Specific