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Impact of the genetic variants of *GLCCI1* on clinical features of asthmatic patients

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A short running title: genetic variants of GLCCI1 of asthmatic patients

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Authorship statement:

Shinji Chiba collected and analyzed data, and wrote the paper.

Yutaka Nakamura designed research, performed research, and wrote the paper.

Tomoki Mizuno, Kazuyuki Abe, and Yosuke Horii performed research and collected data.

Hiromi Nagashima and Nobuhito Sasaki designed study and collected data.

Hiroyuki Kanno, Tatsuo Tanita, and Kohei Yamauchi analyzed data and wrote the paper.

Disclosure statement:

All authors state potential conflict of interest do not exist.

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Abstract

Background Several gene variants are associated with a response to an inhaled corticosteroids (ICSs) treatment in patients with bronchial asthma. A variant of the *glucocorticoid-induced transcript 1* (*GLCCI1*) genes has previously been associated with decreased lung function improvement upon treatment with inhaled corticosteroids in patients with bronchial asthma. Another report has also demonstrated that this genetic biomarker did not influence the change in flow volume in one second. However, no studies have considered the treatment content and the *GLCCI1* variants. We were able to determine the relationship between the pulmonary function and clinical features and the variant of the *GLCCI1* in Japanese asthmatic patients receiving long-term ICS treatment.

Materials and Methods In this study, 386 patients with bronchial asthma, who were receiving ICS and living in Japan, were recruited, genotyped, and underwent pulmonary function tests. To identify the GLCCI1 protein expression cells, endobronchial biopsy specimens were examined.

Results We found that the pulmonary function was not significantly different in the TT and GG homozygotes compared to the wild types. There are significant differences in the amount of risk when increasing the treatment steps for asthma between

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homozygotes and others. GLCCI1-positive cells were localized to the bronchial epithelial cells. The amount of GLCCI1 that cultured epithelial cells harboring *GLCCI1* variants produced was less than the *GLCCI1* wild type in the presence of a corticosteroid.

*Conclusion*s A worsening of pulmonary function might be prevented due to other recently used medications which may have a positive impact on it.

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Introduction

Bronchial asthma is a disorder of the conducting airways that leads to variable airflow obstructions in association with airway hyperresponsiveness and a local accumulation of inflammatory cells, particularly eosinophils, mast cells, and T lymphocytes (1). Inhaled corticosteroids (ICSs) are the primary medication used in the treatment of bronchial asthma based on the efficacy of a strong anti-allergic agent on the inflammatory cells and induced-mediators (2-3). Although most asthmatic patients show a beneficial response to ICS, the intraindividual variability in the level of treatment response is large (4). There is accumulating evidence that this variability is partly caused by intraindividual genetic differences (5-6). Recently, Tantisira et al. identified a genetic variation in *glucocorticoid-induced transcript 1 genes* (*GLCCI1*) and *T* genes on chromosome 7Q21.3 to be associated with a decreased lung function in those who have been treated with ICS (7-8). However, Hosking et al. demonstrated that they could not replicate the results of Tantisira et al. in post hoc analyses of seven clinical trials including 1924 adolescents and adults with asthma (9). In northern European populations of asthmatic children and young adults, *GLCCI1* genotype rs37972 has been demonstrated to not be associated with an ICS efficacy decrease in lung function and asthma symptom scores (10). Recently our prospective studies have demonstrated that *STAT4* TT of rs925847 and *IL13* AA of rs20541 were potential genomic biomarkers

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that predict a lower pulmonary function. Administration of high-dose ICS to asthmatic patients with genetic variants of *STAT*4 did not respond to it, whereas the genetic variants of *IL13* AA might avoid the advancing of airway remodeling (11-12). Thus, increased FEV1 or recovered airway remodeling were dependent on the genetic background and treatment content. In the current study, we aimed to assess whether variants in the *GLCCI1* rs37972 and rs37973 genotypes were associated with relationships between lung function and treatment in Japanese adults receiving ICS treatment, and to identify GLCCI1 positive cells in the bronchial airways.

Materials and Methods

Study subjects

All study subjects were recruited from the Iwate Medical University Hospital. Patients aged ≥ 18 years were eligible if they had a diagnosis of asthma as defined by the American Thoracic Society criteria for ≥ 5 years and were using ICS at a stable low-mid dose for ≥ 1 year before screening. Well-controlled asthmatic patients who had no other medical disorders, who had smoked less than 10-pack-years, and who had not been exposed to environmental hazards were considered for the study. This study was approved by the Iwate Medical University Hospital Ethics Committee (H20-119) and

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registered with Clinical Trials (JMA-IIA00045 remodeling-ICS). Prospective patients were notified of our desire to include them in our study, and were asked whether they would be willing to participate. Upon acceptance, the subjects provided written informed consent according to the ethical protocols of our institution. Subjects were assessed for age, sex, asthma duration, smoking history, eosinophil counts, serum IgE concentration, and spirometry. DNA was isolated from lymphocytes using standard procedures. Subjects were genotyped for rs37972 and rs37973 using a 7500 Fast Real-Time PCR System (Life Technologies Japan, Tokyo, Japan) to evaluate the relationship between genotype and pulmonary function and asthma treatment steps. Treatment steps based on GINA's *Pocket Guide for Asthma Management and Prevention –A Pocket Guide for Physicians and Nurses Update 2015*, were used as follows: step 2 - use of low dose controller medication plus as-needed reliever medication; step 3 - use of one or two controllers plus as-needed reliever medication; step 4 - use of two or more controllers plus as-needed reliever medication and step 5 - use of higher level care and/or add-on treatment. This was determined when a sustained treatment step was achieved and maintained for at least 6 months and the treatment could not be successfully reduced further. Spirometry was performed and airway methacholine responsiveness was measured using an Astograph (Jupiter 21, CHEST,

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Tokyo, Japan) according to the method described by Takishima et al. (13).

Fibreoptic bronchoscopy and specimen handling

Asthmatic patients underwent fibreoptic bronchoscopic examinations during ICS treatment and then paused the use of ICS for two weeks. Asthmatic bronchial tissues were obtained from: i) five asthmatic patients harboring CC (wt) of rs37972 and AA (*wt*) of rs37973; ii) five asthmatic patients harboring TT (homozygous) of rs37972 and AA (wt) of rs37973; and iii) five asthmatic patients harboring CC (wt) of rs37972 and GG (homozygous) of rs37973. Asthmatic bronchial biopsies were performed by inserting a flexible fibreoptic bronchoscope (BF-Q290; Olympus Optical Co Ltd, Tokyo, Japan) under local anaesthesia as previously described in detail (14). ~~Bronchial epithelial cells were obtained using a standard sterile single sheathed nylon cytology brush (Olympus). Cells were harvested into 5 mL sterile PBS after each brushing.~~ Five biopsies were taken using forceps (Olympus) in the segmental bronchi of the right lobe and in the bifurcations of the right bronchi. Biopsied specimens were fixed in 4% paraformaldehyde, immersed in sucrose, embedded in OCT compound, and then 5-μm-thick sections were stained with haematoxylin and eosin (HE).

Immunolocalization of GLCCI1 in the bronchial tissues was evaluated by using an

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antibody against GLCCI1 (Atlas Antibodies AB, Stockholm, Sweden). All of the samples were then incubated with an Alexa Fluor 488-conjugated secondary antibody (Life Technologies) and imaged using a confocal laser-scanning microscope (C1si; Nikon, Tokyo, Japan).

Immunoblotting analysis of GLCCI1

To confirm whether the homozygous for *GLCCI1* patients expressed decreased protein levels in the bronchial epithelial cells, which had been indicated in the immunohistochemistry, we performed a immunoblotting analysis. Normal human bronchial epithelial cells were purchased from Takara (Tokyo, Japan) and maintained in an EGM-2-MV BulletKit (Takara) according to the manufacturer's instructions. Asthmatic bronchial epithelial cells were obtained using a standard sterile single sheathed nylon cytology brush (Olympus). Cells were harvested into 5 mL sterile PBS after each brushing, as previously described (15). Asthmatic bronchial epithelial cells were collected before bronchial biopsies on the opposite site of the lung. The epithelial cells from normal controls, and those derived from asthmatic patients were plated at a density of $1x10^5$/ 10-cm dish. When the cultures were subconfluent, the culture medium was removed and the cells were washed twice with and incubated for 24 h in a

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serum-free medium, followed by treatment with fluticasone propionate or budesonide (R&D Systems) at different concentrations and for various times. Cultured bronchial epithelial cells were collected and lysed in TNE buffer as previously described (16). Twenty micrograms of protein were resolved using 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, CA, USA). Each membrane was then incubated with anti-human GLCCI1 (GeneTex, Hsinchu, Taiwan) and GAPDH (GeneTex) antibody. Three separated experiments from each donor were done.

Statistical analysis

Statistical analyses were performed using JMP version 11 (SAS Institute Inc., Tokyo, Japan). All data were expressed as the mean ± standard error of the mean. Comparison of the patients' characteristics between the three groups were performed using one-way ANOVA. Differences in genotype between treatment steps 2-4 and 5 were tested using a chi-square test or two-tailed Fisher's test when the number of expected cases was small. Genetic risks were assessed by calculating odds ratios (OR) with 95% confidence intervals (95% CI). Comparisons of the levels of immunoblotting intensity between the four or five groups were performed using a two-tailed one-way ANOVA. *P* values < 0.05 were considered significant. *Post hoc* multiple comparisons were performed using

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the Tukey-Kramer test for differences among all groups.

Results

Of 405 asthmatic patients screened, 390 of the rs37972 and 398 of the rs37973 patients were successfully genotyped (≥ 94%). We identified 132, 172, and 86 subjects that had CC wild type, CT heterozygotes, and TT homozygotes, respectively, for rs37972 (Table 1). We also identified 122, 184, and 92 subjects that had AA wild type, AG heterozygotes, and GG homozygotes, respectively for rs37973 (Table 2). There were no significant differences in age at study enrolment, sex, eosinophil count status, serum IgE concentration, and pulmonary function among the three groups. There was no trend toward higher IgE level with genotype (Jonckheere-Terpstra test). TT of rs37972 and GG of rs37973 were associated with a higher number of asthma treatment steps (Table 3).

Immunohistochemistry of GLCCI1 expression in the bronchial airways of asthmatic patients

To confirm whether asthmatic patients expressed GLCCI1 protein in the airways, we performed an immunohistochemistry of the bronchial tissues, which were derived from

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asthmatic patients who were using ICS and temporarily stopped for two weeks. The GLCCI1 positive cells were localized to the epithelial cells. As shown in Figure 1, GLCCI1-positive cells were detected in the bronchial epithelial cells of asthmatic patients harboring both *wt* of *GLCCI1* who were using ICS (A), whereas, they were not identified as GLCCI1-positive cells in the airways of the asthmatic patients after they had stopped using ICS (B). The intensity of the staining of the epithelial cells were not significant between genotypes (data not shown).

Influence of fluticasone and budesonide on GLCCI1 expression in bronchial epithelial cells

Having shown the increased protein levels of GLCCI1 protein in the bronchial epithelial cells of the bronchial tissues derived from asthmatic patients using an ICS treatment, we next evaluated the expression of GLCCI1 on cultured human bronchial epithelial cells in the presence of fluticasone indicated concentrations for 24 h. GLCCI1 protein expression by fluticasone was concentration-dependent until a peak was reached at 1 μM for 24 h in normal human bronchial epithelial cells (A). Time-course experiments performed with 1 μM fluticasone showed no significant induction of GLCCI1 within 12 h (B). These concentrations and time-course experiments were similar to the results that

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were derived from budesonide experiments (data not shown).

Impact of fluticasone and budesonide on bronchial epithelial cells harboring genetic variants

We next evaluated the differences of expression of GLCCI1 in the bronchial epithelial cells derived from healthy control subjects with both wild type of rs37972 and rs37973 and from asthmatic patients in the presence of 1 μM fluticasone or budesonide for 24 h. There were no significant differences among the four groups when bronchial epithelial cells were cultured without fluticasone (Figure 3A). Fluticasone treatment for 24 h led to an increase in the expression of GLCCI1 proteins in the epithelial cells derived from the healthy control and wt asthmatic groups compared to the TT of rs37972 and GG of rs37973 groups (Figure 3B). Budesonide also increased the expression of GLCCI1 protein in the healthy control and asthmatic *wt* groups compared to the TT of rs37972 and GG of rs37973 groups (Figure 3C).

Discussion

In this study, we investigated the role of the genetic variants of *GLCCI1*, TT and GG homozygotes, of asthmatic patients in clinical characteristics. Several new findings have

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emerged from this study. First, we have demonstrated that Japanese asthmatic patients following an ICS treatment and having the *GLCCI1* rs37972 TT and rs37973 GG genotypes was not significantly associated with a reduced pulmonary function. Second, we have shown that transcription factor GLCCI1 is increased in the airway epithelium after ICS treatment. Third, the analysis revealed a significant association between the genotype of TT and GG of *GLCCI1*, and the asthmatic treatment steps used with the Japanese population, showing an OR of 2.78 and 2.28, respectively. The two GLCCI1 variants have been shown to have a complete linkage disequilibrium (r^2=0.99) in Caucasian, and this association might reflect these data. Finally, we have observed that fibroblasts harboring the genetic variants of *GLCCI1* have less potential to express transcription factor GLCCI1 after stimulation with fluticasone or budesonide. To date, little is known about the function of the GLCCI1 protein and the *GLCCI1* gene. GLCCI1 had previously been described as a thymocytespecific transcript that is rapidly up-regulated in response to a dexamethasone treatment (17) and represents an early response in steroid-regulated thymocyte apoptosis (18). Apoptosis is a key mechanism through which glucocorticoids resolve lymphocytic and eosinophilic inflammation in asthma. Therefore, decreased GLCCI1 expression, as a result of the presence of rs37972 and rs37973, may reduce inflammatory-cell apoptosis, thus leading to a diminished

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clinical response to inhaled glucocorticoids. It is plausible that the bronchial biopsy specimens exhibited no GLCCI1-positive hemocytes, which had been induced during apoptosis by glucocorticoid inhalation. Previous data reports a significant association between the *GLCCI1* variant and the response to glucocorticoid therapy in patients with asthma (7). A study of Japanese patients showed that *GLCCI1* rs37973 contributed to the annual decline in FEV1 of 30 mL/ yr. or greater, in those receiving long-term ICS treatment (4). The role of the *GLCCI1* gene minor allele has been examined in patients with other chronic inflammatory diseases, rheumatoid arthritis (19), graft-versus-host disease (GVHD) (20), and nephrotic syndrome (21). In terms of sensitivity to GC, genetic variants might be associated with the response to GC therapy in male patients with RA, whereas rs37972 SNP-positive donors and recipients neither increased nor were more likely to have steroid-refractory GVHD. Also, the *GLCCI1* SNP was not associated with steroid sensitivity in pediatric patients with nephrotic syndrome. One explanation for these differences may depend on how GLCCI1 is expressed in the involved tissues. Tantisira *et al*. (7) have demonstrated the relative high level of expression of *GLCCI1* mRNA in the lungs. Little data is known about the GLCCI1 protein expression in the tissues before and after GC treatment, although transcription factor GLCCI1 is induced by GC. Our work shows that GLCCI1 expressed in the

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airways, especially in epithelial cells after ICS treatment, and sequential *in vitro* experiments have shown that the expression was dependent on the genetic variant *GLCCI1* by using cultured epithelial cells of the airways. The different amounts of expression of the GLCCI1 protein induced the varied clinical features. Our data could not detect the differences between the GLCCI1 genotypes and the changes of FEV1, however, there was a noticeable change in the treatment steps. Several factors may explain this discrepancy; foremost, the medications used during the treatment, with the exception of CS, such as tiotropium and anti-IgE, accounts for a large percentage in the treatment step 5 population in this study. Anti-IgE antibody omalizumab treatment significantly improved lung function when added to ICS maintenance therapy in Japanese asthmatic patients (22). Similarly, tiotropium produced a clinically meaningful reduction in the exacerbation rate and an improvement in pulmonary function (23). According to GINA guidelines, estimating asthma severity is based on treatment step, therefore, variants of *GLCCI1* also apply to the predictor of asthma severity.

To determine whether there is a fundamental difference in epithelial transcription factor productions in asthma, we have established primary cultures of bronchial epithelial cells from normal subjects and asthmatic subjects with and without variants of *GLCCI1*. Cultured epithelial cells harboring a GLCCI1 variant expressed modest GLCCI1 protein

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compared to the *wt GLCCI1*. As infected bronchial epithelial cells with a *GLCCI1* variant had a reduced apoptosis, it seems plausible that virus-induced injury, specifically to epithelial cells and leading to an enhanced release of IL33, IL25, and thymic stromal lymphopoietin (24-26), might drive an acute exacerbation of allergic asthma *via* activation of ILC2 as type 2 cytokine-producing cells.

Several potential limitations should be indicated in this study. We did not examine the initial responsiveness to ICS and annual changes in FEV1 in this cohort. However, we assume that FEV1, %predicted is also important as a hallmark of the effects of ICS and airway remodeling. The number of bronchial epithelial cells obtained from asthmatic participants was small. The main reason is based on the genetic reason of why the patients harboring the combination of genetics, such as homozygous of rs37972 and *wt* of rs37973 or *wt* of rs37972 and homozygous of rs37973, were few.

In conclusion, we demonstrate an important role for *GLCCI1* TT of rs37972 and GG of rs37973, which increased treatment steps for bronchial asthma. These genetic biomarkers might transform disease management, especially if these are upstream factors in the pathogenetic sequence and closer to the disease origins where stratified medicine is most effective.

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Conflict of interests

The authors declare no conflicts of interest.

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Figure legends

Figure 1. Immunostaining examination of the expression of the GLCCI1 positive cells in the airways. Representative photomicrographs show bronchial mucosal biopsy specimens stained with anti-GLCCI1 antibody. Bronchial tissues obtained from asthmatic patients without genetic variants of *GLCCI1* 2 weeks after cessation of inhaled corticosteroid treatment (A). Bronchial tissue derived from asthmatic patients without genetic variants of *GLCCI1* and continued inhaled corticosteroid treatment (B).

Figure 2. Concentration-change and time course of GLCCI1 induction by fluticasone. Normal human bronchial epithelial cells were treated at the indicated concentration by fluticasone, and the lysates were subjected to immunoblotting. A representative immunoblotting GLCCI1 expression in the normal human bronchial epithelial cells after 24 h exposure to fluticasone at varying concentrations (left in A) and

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GAPDH-normalized band intensity quantification for GLCCI1 were shown (right in A). A representative immunoblotting GLCCI1 expression in the normal human bronchial epithelial cells after indicated times at 1 μM of fluticasone stimulation (left in B) and GAPDH-normalized band intensity quantification for GLCCI1 were shown (right in B). The basal expression in non-treated cells was set to 1.0. Pooled data, quantitating the immunoblot signals using infrared imaging, and the defining for each antibody is shown. Densitometry was significantly increased when cells were cultured at 1μM and 1mM of fluticasone concentration for 24 h compared to those without fluticasone. Also, densitometry showed no significant differences when cells were cultured in the presence of fluticasone within 12 h. The whiskers indicate the standard errors of the mean. *$P <$ 0.05 compared with the means calculated for the no-treatment expression.

Figure 3. Effects of glucocorticoid on GLCCI1 expression in cultured human bronchial epithelial cells. Epithelial cells were treated without corticosteroid (A), with 1 μM fluticasone (B), or with budesonide (C) for 24 h to examine the GLCCI1 expression. Using immunoblotting, cells were harvested, and the cell lysates were then analyzed in immunoblots probes with antibodies directed against GLCCI1 and GAPDH. Representative immunoblotting (left), which was repeated five times, showed

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equivalent results, and GAPDH-normalized band intensity quantification (right) for GLCCI1 expression in the epithelial cells after no-exposure (A), exposure to fluticasone (B), and to budesonide (C). The basal expression in the cells obtained from normal healthy control was 1.0. Pooled data, quantitating the immunoblot signals using infrared imaging, and the defining for each antibody is shown. Densitometry showed no significant differences between the four groups without stimulation, however, densitometry was significantly decreased in rs37972 and rs37973 compared to healthy controls when bronchial epithelial cells were cultured in the presence of fluticasone or budesonide. The whiskers indicate the standard errors of the mean. *$P < 0.05$ compared with the means of the normal control expression.

There four groups can be indicated as follows: control: normal human bronchial epithelial cells harboring CC (*wt*) of rs37972 and AA (*wt*) of rs37973; wt: asthmatic patients harboring CC (*wt*) of rs37972 and AA (*wt*) of rs37973; TT: asthmatic patients harboring TT (homozygous) of rs37972 and AA (*wt*) of rs37973; GG: asthmatic patients harboring CC (*wt*) of rs37972 and GG (homozygous) of rs37973.

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Table 1 Patients characteristics according to the rs37972 genotype GLCCI1

rs37972 genotype	CC (n=132)	CT (n=172)	TT (n=86)	*P* Value
Age, yr	58.2±1.5	58.1±1.2	56.1±1.9	NS
Sex, n (%)	55 men (42)	75 men (44)	34 men (40)	NS
Eosinophil count /μL	309.4±30.5	314.0±19.7	265.1±28.6	NS
IgE level, IU/mL	412.1±58.0	435.2±77.9	565.4±233.2	NS
pulmonary function				
FVC, L	3.0±0.1	2.9±0.1	2.9±0.1	NS
%FVC	97.4±3.0	101.1±2.7	98.7±3.5	NS
FEV1, L	2.2±0.1	2.1±0.1	2.2±0.1	NS
%FEV1	95.1±2.6	96.0±2.4	96.0±3.3	NS

Data are expressed as the mean ± standard error of the mean. FVC, forced vital capacity FEV1, forced expiratory flow volume in one second, NS, not significant among the three groups

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Table 2 Patients characteristics according to the rs37973 genotype GLCCI1

rs37973 genotype	AA (n=122)	AG (n=184)	GG (n=92)	*P* Value
Age, yr	58.7±1.6	58.1±1.2	55.9±1.8	NS
Sex, n (%)	48 men (40)	90 men (49)	37 men (40)	NS
Eosinophil count /μL	323.2±32.8	302.5±18.8	321.3±27.4	NS
IgE level, IU/mL	407.5±58.0	439.4±74.9	562.2±221.2	NS
pulmonary function				
FVC	3.0±0.1	2.9±0.1	2.9±0.1	NS
%FVC	99.2±3.2	98.0±2.3	99.6±3.4	NS
FEV1	2.2±0.1	2.1±0.1	2.2±0.1	NS
%FEV1	95.6±2.8	94.3±2.4	96.9±3.1	NS

Data are expressed as the mean ± standard error of the mean. FVC, forced vital capacity FEV1, forced expiratory flow volume in one second. NS, not significant among the three groups

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Table 3 Analysis for the association of each GLCCI1 SNPs on treatment steps GINA 2-4 vs. 5

	n	OR (95%CI)	*P* Value
TT rs37972	86	3.53 (1.9-7.3)	*P*<0.001
GG rs37973	92	2.78 (1.3-5.8)	*P*<0.001

OR, odds ratio, CI, confidence interval

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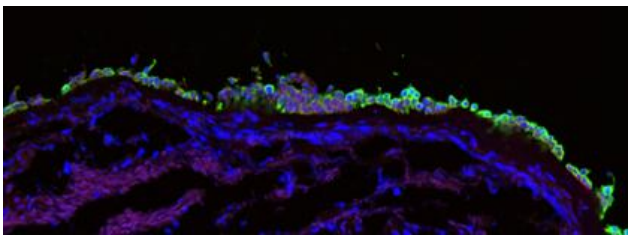
A



B

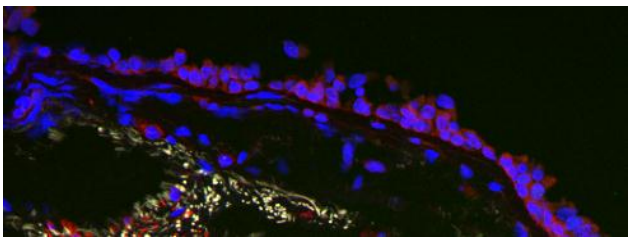


Figure 1

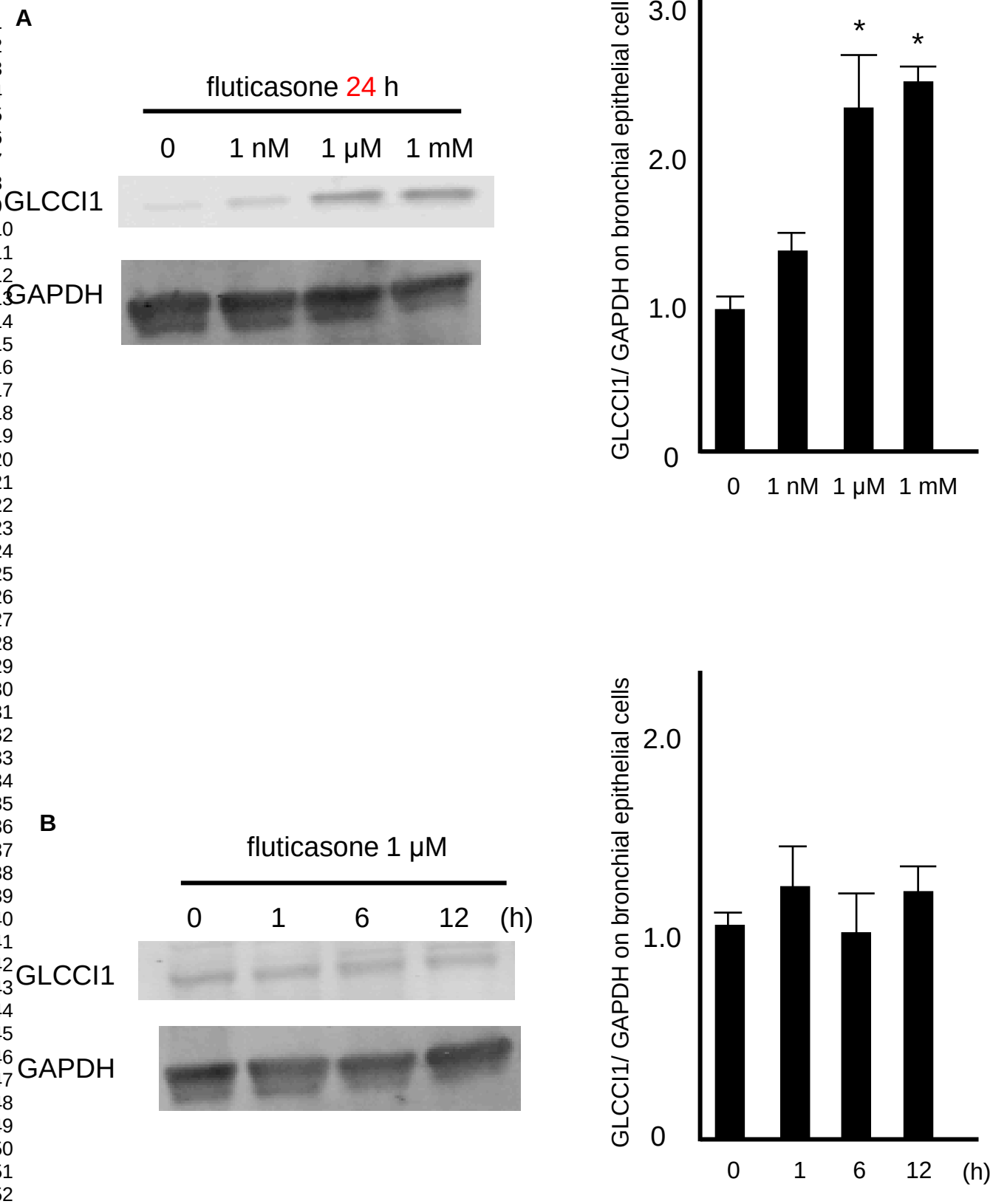

A
fluticasone 24 h
0
1 nM
1 μM
1 mM
GLCCI1
GAPDH
GLCCI1/ GAPDH on bronchial epithelial cells
3.0
2.0
1.0
0
*
*
0
1 nM
1 μM
1 mM
B
fluticasone 1 μM
0
1
6
12
(h)
GLCCI1
GAPDH
GLCCI1/ GAPDH on bronchial epithelial cells
2.0
1.0
0
0
1
6
12
(h)

Figure 2

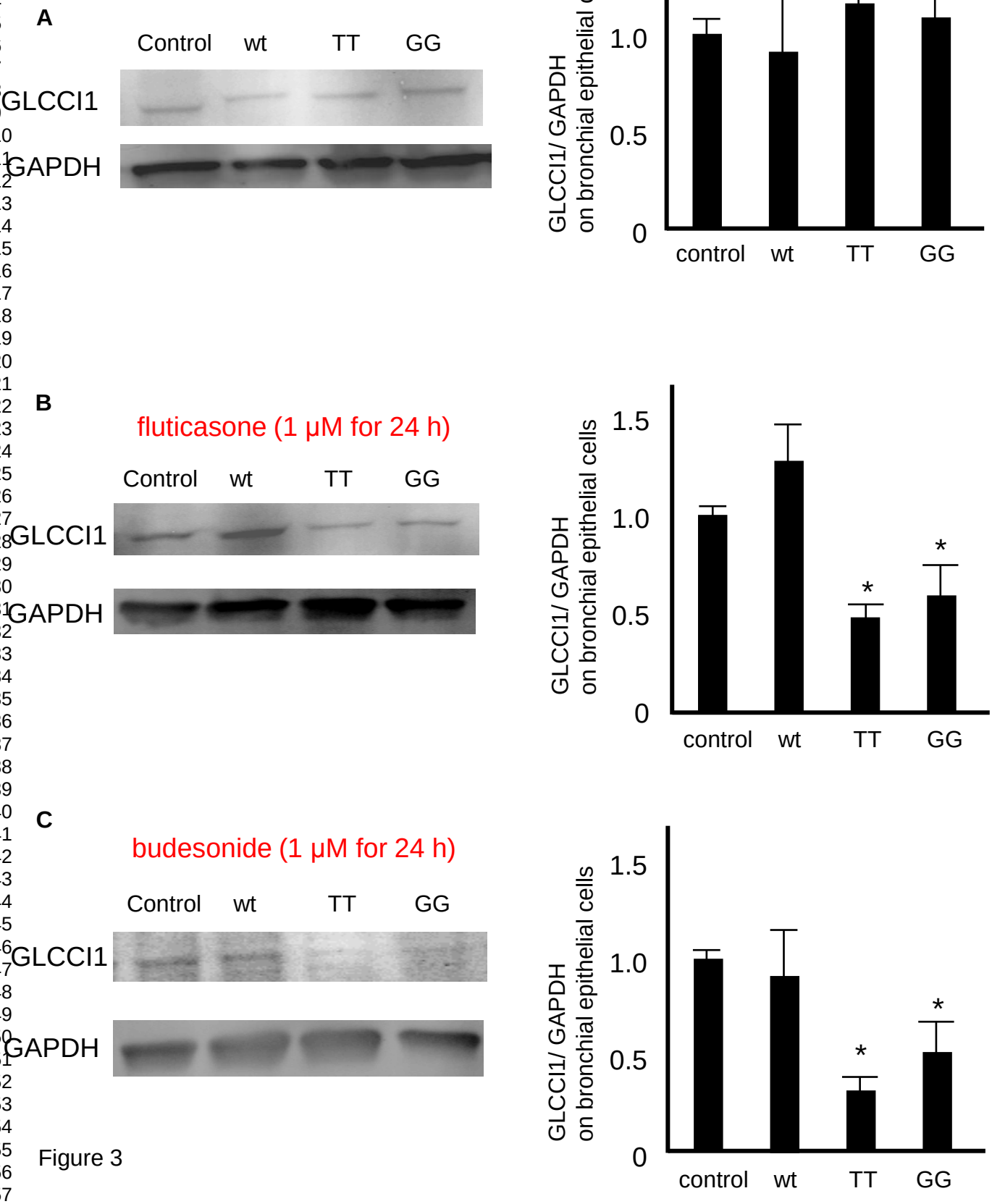

A
Control
wt
TT
GG
GLCCI1
GAPDH
GLCCI1/ GAPDH
on bronchial epithelial cells
1.5
1.0
0.5
0
control
wt
TT
GG
B
fluticasone (1 μM for 24 h)
Control
wt
TT
GG
GLCCI1
GAPDH
GLCCI1/ GAPDH
on bronchial epithelial cells
1.5
1.0
0.5
0
*
*
control
wt
TT
GG
C
budesonide (1 μM for 24 h)
Control
wt
TT
GG
GLCCI1
GAPDH
GLCCI1/ GAPDH
on bronchial epithelial cells
1.5
1.0
0.5
0
*
*
control
wt
TT
GG

Figure 3