Biol. Pharm. Bull. **48**, 86–92 (2025) Vol. 48, No. 1
https://doi.org/10.1248/bpb.b24-00684

Regular Article

Vitamin D Receptor rs2228570 Gene Polymorphism Is Associated with Asthma Severity and Exacerbations

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Received: October 15, 2024; accepted: December 11, 2024

Vitamin D plays a crucial role in immune system function. Several studies have indicated that genetic variations in the vitamin D receptor (VDR) and vitamin D binding protein (VDBP, encoded by *GC* gene) increase the risk of developing asthma. However, the effect of these variations on the prognosis and clinical outcomes of asthma remains unclear. This study, involving 152 adult patients with asthma, aimed to assess the influence of *VDR* and *GC* polymorphisms on asthma severity and its exacerbation. Gene polymorphisms previously associated with asthma risk were analyzed, and *VDR* mRNA expression levels were evaluated in peripheral blood mononuclear cells. The AA genotype of the *VDR* rs2228570 polymorphism was associated with an elevated risk of severe asthma compared to the AG/GG genotype (odds ratio, 3.20; 95% confidence interval [CI], 1.24–8.28). Furthermore, patients with the rs2228570 AA genotype showed an elevated risk of exacerbation during the 1-year follow-up period (hazard ratio, 4.01; 95% CI, 1.75–9.15). *VDR* mRNA expression was significantly reduced in patients with the AA genotype. Furthermore, the mRNA expression levels of *GLCCI1, HDAC2, NR3C1,* and *NFE2L2,* which are associated with steroid response, were reduced in patients with the AA genotype. Our findings indicate that patients with the AA genotype of *VDR* rs2228570 are more likely to experience severe asthma and exacerbations. This polymorphism has the potential to reduce vitamin D efficacy by altering VDR function and expression, potentially resulting in increased inflammation and reduced steroid responsiveness in patients with asthma.

Key words asthma, gene polymorphism, exacerbation, vitamin D receptor

INTRODUCTION

Among patients with asthma, approximately 4–10% have severe asthma, which is associated with a poor prognosis. Despite the use of high-dose inhaled steroids, effective treatment remains challenging.[1)] Several factors influence the severity of asthma. Approximately 50% of patients with asthma exhibit type 2 inflammation involving eosinophils.[2)] Additionally, non-type 2 inflammation, involving neutrophils and T-helper 17 (Th17) cells, is a key factor in the pathogenesis of asthma.[3)] Steroids are typically effective in treating type 2 inflammation and less effective in treating non-type 2 inflammation.[4)] Nonetheless, some patients exhibit residual type 2 inflammation despite treatment with high-dose inhaled steroids.[5)] The various asthma subtypes emphasize the need for identifying each pathological subtype in individual patients and selecting the most appropriate treatment.[6)] However, the complex pathogenesis of asthma remains poorly understood, with no objective indicators existing for subtype-specific treatments. Elucidating its pathology is expected to facilitate the discovery of indicators for optimized treatment, prognostic predictors of asthma, and therapeutic targets.

The regulatory function of vitamin D on the immune system may significantly influence inflammatory processes associated with asthma.[7–9)] Extensive research efforts have explored the potential advantages of vitamin D supplements in managing asthma symptoms.[10,11)] However, some studies have indicated that asthmatic patients with insufficient serum vitamin D levels may experience a decline in pulmonary function and exacerbation of asthma symptoms,[12,13)] while others have yielded conflicting results with no discernible improvement in asthma symptoms following vitamin D administration.[14,15)] In our previous study,[16)] we assessed the relationship between blood vitamin D levels and asthma severity and exacerbation in patients with asthma. However, our results suggested that vitamin D levels did not have a significant effect on the severity or frequency of asthma exacerbations. Consequently, the

role of vitamin D in asthma management remains unclear and requires further investigation to elucidate its association with asthma pathophysiology.

The vitamin D receptor (VDR) and vitamin D binding protein (VDBP, encoded by *GC* gene) play crucial roles in mediating vitamin D-related functions. Vitamin D forms a complex with VDBP in the plasma and is subsequently transported to the liver where it is converted to 25-hydroxyvitamin D. It is then transported to the kidneys, where it is further converted to 1,25-dihydroxyvitamin D, which is the active form of vitamin D.[17] Upon binding of active vitamin D to VDR in the cytoplasm, the complex translocates to the nucleus and interacts with vitamin D response elements (VDREs) on DNA, thereby modulating the transcriptional activity of specific genes.[7,8] Previous studies indicated a potential association between *VDR* and *GC* gene polymorphisms and asthma susceptibility.[17,18] Several polymorphisms, including rs731276, rs7975232, rs1544410, rs222850, and rs11568820 in the *VDR* gene and rs7041 and rs4588 in the *GC* gene, were associated with an increased risk of asthma.[17,19] However, the relationship between *VDR* and *GC* gene polymorphisms and asthma pathophysiology, including disease severity and prognosis, remains unclear.

Therefore, we investigated the impact of polymorphisms in vitamin D-related genes on the disease's severity and prognosis.

MATERIALS AND METHODS

Study Design and Subjects This single-center, prospective, observational study was conducted at Shizuoka General Hospital.[20] Participants comprised adults with asthma who were diagnosed with asthma according to the Global Initiative for Asthma diagnostic criteria. At baseline, patient blood samples were collected; various laboratory and pulmonary function tests were performed; and the fractional exhaled nitric oxide (FeNO) was assessed. Severe asthma was diagnosed according to the guidelines of the European Respiratory Society and the American Thoracic Society.[21] An exacerbation was defined as an asthma symptom that required systemic corticosteroid treatment for a period exceeding 3 d and was recorded during the 1-year follow-up period. This study was conducted with the approval of the ethics committee of Shizuoka General Hospital (Approval Number: SGH 15-01-55).

Ethics This study was conducted with the approval of the ethics committee of Shizuoka General Hospital (Approval Number: SGH 15-01-55).

Assessment of Gene Polymorphisms Five *VDR* polymorphisms and two *GC* polymorphisms were selected for analysis based on previous reports of asthma and linkage disequilibrium, with minor allele frequencies ≥10%. The PCR-restriction fragment length polymorphism and allele-specific PCR method were used to analyze the single nucleotide polymorphisms (SNPs) on the *VDR* and *GC* genes. The SNPs analyzed included rs731236, rs7975232, rs1544410, rs2228570, and rs11568820 on the *VDR* gene, as well as rs7041 and rs4588 on the *GC* gene. The primers and restriction enzymes used in the genetic polymorphism analysis are listed in Supplementary Table 1. DNA was extracted from whole blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Venlo, the Netherlands). PCR was performed using Taq DNA polymerase, and the genotype was determined by agarose gel electrophoresis after restriction enzyme digestion.

Measurements of Gene Expression Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, U.S.A.) density gradient method, and total RNA was extracted using the NucleoSpin kit (Macherey-Nagel, Düren, Germany). The mRNA expression levels of *VDR*, *NR3C1*, *HDAC2*, *GLCCI1*, and *NFE2L2* in PBMCs were quantified using the intercalation method with Fast SYBR Green Master Mix (Applied Biosystems, Waltham, MA, U.S.A.), after cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was conducted using the 7500 Fast Real-Time PCR System or the QuantStudio 5 Real-Time PCR System (Applied Biosystems), and each sample was analyzed in duplicate. The primers used in this study are listed in Supplementary Table 2. The expression level of each mRNA was normalized to that of *ACTB*, and the resulting values were expressed as log2-transformed values, with the mean value adjusted to zero.

Statistical Analysis In the comparative analysis of the two groups, the Mann–Whitney *U* test was used for continuous variables, while Fisher's exact test was used for categorical variables. Logistic regression analysis was performed with severe asthma as the objective variable and odds ratios (OR) and 95% confidence intervals (CIs) were calculated for each genotype. To evaluate the association between genotype and time to the initial exacerbation of asthma, we used the log-rank test and the Cox proportional hazards regression model to determine the hazard ratio (HR) and 95% CI. All statistical analyses were performed with R version 4.4.1 (R Foundation for Statistical Computing), and a p-value of less than 0.05 was considered statistically significant.

RESULTS

Association of *VDR* Gene Polymorphisms and Severe Asthma The present study included 152 patients with asthma, 25 (16.4%) of whom were classified as having severe asthma. The clinical characteristics of the study subjects and the comparison between patients with severe and non-severe asthma have been described in detail in the literature.[20,22] Table 1 shows the genotype frequencies of the analyzed *VDR* and *GC* gene polymorphisms classified according to asthma severity. The allele frequency of each genetic polymorphism was found to be in accordance with Hardy–Weinberg equilibrium, which is consistent with that previously reported in the Japanese population.[23] The frequency of the AA genotype of the rs2228570 polymorphism was significantly higher in patients with severe asthma than in those with non-severe asthma, suggesting an association between this genetic variant and the severity of asthma. The OR for severe asthma in the AA genotype of the rs2228570 polymorphism was 3.20 (95% CI, 1.24–8.28; $p = 0.017$). This remained statistically significant even after plasma vitamin D (25-hydroxyvitamin D_3) concentration, lung function, and body mass index (BMI) were added as covariates in the logistic regression analysis (OR, 3.43; 95% CI, 1.17–10.08; $p = 0.025$). The *GC* gene

Table 1. Comparison of Genotype Frequencies of *VDR* Gene Polymorphisms in Patients with Severe Asthma and Those with Non-severe Asthma

Gene	SNP ID	Allele 1/2	Severe asthma Genotype frequency			Non-severe asthma Genotype frequency			Overall *p*-value	*p*-Value 1/1 *vs.* 1/2+2/2	*p*-Value 1/1+1/2 *vs.* 2/2
			1/1	1/2	2/2	1/1	1/2	2/2			
VDR	rs731236	A/G	17 (68)	8 (32)	0 (0)	103 (81)	23 (18)	1 (1)	0.308	0.179	1.000
VDR	rs7975232	C/A	10 (40)	11 (44)	4 (16)	64 (50)	51 (40)	12 (9)	0.437	0.387	0.303
VDR	rs1544410	C/T	17 (68)	8 (32)	0 (0)	105 (83)	21 (17)	1 (1)	0.244	0.104	1.000
VDR	rs2228570	G/A	8 (32)	8 (32)	9 (36)	45 (35)	63 (50)	19 (15)	0.046	0.822	0.022
VDR	rs11568820	C/T	10 (40)	12 (48)	3 (12)	49 (39)	58 (46)	20 (16)	0.957	1.000	0.768
GC	rs7041	A/C	10 (40)	13 (52)	2 (8)	74 (58)	42 (33)	11 (9)	0.187	0.123	1.000
GC	rs4588	G/T	14 (56)	11 (44)	0 (00)	73 (57)	41 (32)	13 (10)	0.181	1.000	0.128

Data are presented as frequency (percentage).

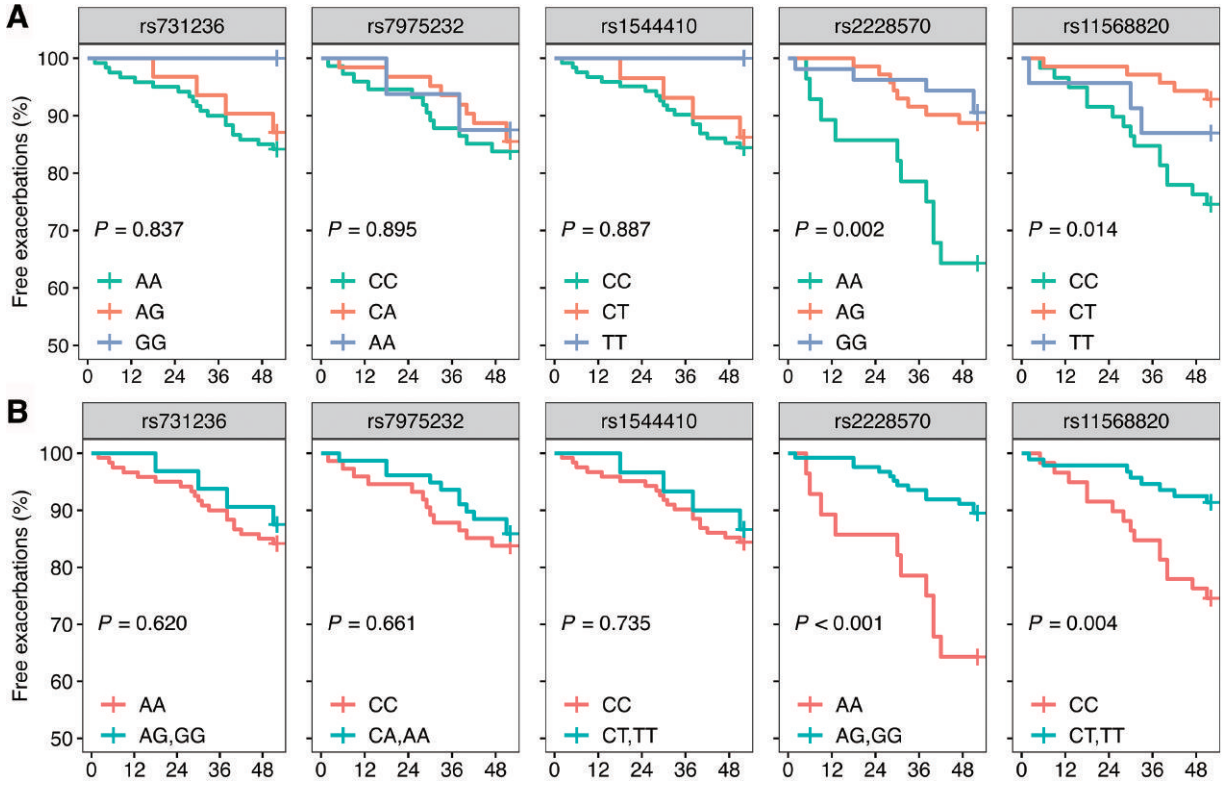

Fig. 1. Kaplan–Meier Curve Showing Time to Initial Exacerbation in Patients, Categorized into (A) Three Groups and (B) Two Groups According to Their *VDR* Gene Polymorphisms

polymorphisms, rs7041 and rs4588, were not significantly associated with severe asthma.

Association of *VDR* Gene Polymorphisms and Asthma Exacerbations A total of 23 patients (15.1%) experienced acute exacerbation within 1 year after inclusion in the study. The relationship between the *VDR* gene polymorphisms and exacerbation is illustrated using Kaplan–Meier curves (Fig. 1). Patients with the AA genotype of the rs2228570 polymorphism demonstrated a markedly elevated risk of exacerbation compared to patients carrying the G allele. The HR for exacerbations among patients with the rs2228570 AA genotype was 4.01 (95% CI, 1.75–9.15; $p<0.001$). This association remained consistent even after adjusting for plasma 25-hydroxyvitamin D_3 concentration, lung function, and BMI as covariates (HR, 3.09; 95% CI, 1.25–7.59; $p=0.014$). Furthermore, the probability of exacerbation was higher in patients with the CC genotype of the rs11568820 polymorphism than in those with the T allele. The unadjusted HR for the rs11568820 CC genotype was 3.25 (95% CI, 1.38–7.66; $p=0.007$), whereas the adjusted HR, which accounted for plasma 25-hydroxyvitamin D_3 concentration, lung function, and BMI, was 3.83 (95% CI, 1.58–9.29; $p=0.003$). By contrast, no association was identified

Table 2. Comparison of Clinical Characteristics between Patients with the AA Genotype and Those with the G Allele of the *VDR* rs2228570 Polymorphism

Characteristics	AA genotype ($n=28$)	AG+GG genotype ($n=124$)	p-Value
Sex, female	14 (50)	67 (54)	0.834
Age (years)	65 (51, 74)	66 (50, 74)	0.844
BMI (kg/m^2)	24.5 (22.9, 27.2)	22.7 (20.6, 25.0)	0.015
FEV_1 (%predicted)	81.1 (66.1, 93.8)	86.9 (73.1, 98.7)	0.106
FEV_1/FVC (%)	68.1 (63.6, 73.7)	70.5 (62.5, 77.1)	0.427
Pack-years	0 (0, 24)	0 (0, 15)	0.878
Peripheral blood cells (/μL)			
Neutrophil	4168 (3104, 5439)	3501 (2930, 4446)	0.075
Lymphocyte	1695 (1451, 1938)	1689 (1351, 2209)	0.807
Monocyte	373 (298, 463)	359 (299, 455)	0.892
Eosinophil	304 (210, 600)	324 (186, 479)	0.966
Basophil	51 (36, 67)	43 (31, 62)	0.422
Neutrophil-to-lymphocyte ratio	2.45 (1.83, 3.07)	2.01 (1.56, 2.78)	0.078
FeNO (ppb)	44 (26, 108)	28 (17, 45)	0.016
Serum total IgE (IU/mL)	229 (126, 616)	238 (85, 636)	0.930
Plasma 25-hydroxyvitamin D_3 (ng/mL)	14.1 (10.9, 17.1)	13.4 (10.6, 16.9)	0.585
Concomitant drugs			
ICS use	27 (96)	118 (95)	1.000
ICS dose (μg/d)	500 (0, 500)	500 (400, 500)	0.226
Oral corticosteroid	5 (18)	5 (4)	0.019
LABA	26 (93)	100 (81.1)	0.167
LAMA	4 (14)	8 (6)	0.235

Data are presented as median (interquartile range) or frequency (percentage). BMI: body mass index; FeNO: fractional exhaled nitric oxide; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; LABA: long-acting β2 agonist; LAMA: long-acting muscarinic antagonist.

between *GC* gene polymorphisms rs7041 and rs4588 and an elevated risk of asthma exacerbation (Supplementary Fig. 1).

Association of *VDR* Gene Polymorphisms and the Pathophysiology of Asthma The AA genotype of the *VDR* rs2228570 polymorphism increases the risk of severe asthma and exacerbations. Consequently, we conducted a more detailed investigation into the relationship between this gene polymorphism and asthma pathophysiology. Table 2 presents a comparison of the clinical characteristics of patients with the AA genotype of the rs2228570 polymorphism and those with the G allele. Patients with the AA genotype had significantly elevated levels of FeNO, a marker of eosinophilic airway inflammation, and a trend toward a higher neutrophil-to-lymphocyte ratio (NLR). Furthermore, *VDR* mRNA expression levels, extracted from PBMCs, were significantly lower in patients with the AA genotype compared to those with the G allele (Fig. 2). Furthermore, building on previous research suggesting that elevated inflammatory conditions decrease steroid responsiveness,[24] we postulated that *VDR* gene polymorphisms alter the expression levels of molecules involved in steroid response. Specifically, the expression level of the glucocorticoid receptor *NR3C1* is associated with steroid responsiveness.[24] Additionally, the significance of *GLCCI1*, *HDAC2*, and *NFE2L2*, which are also involved in steroid responsiveness, has been suggested in patients with asthma.[22] Therefore, we focused on these steroid-responsive molecules and analyzed their expression levels. Our findings revealed that the expression levels of *GLCCI1*, *HDAC2*, *NFE2L2*, and *NR3C1*, which are molecules related to steroid responses, decreased in patients with the *VDR* rs2228570 AA genotype (Fig. 3).

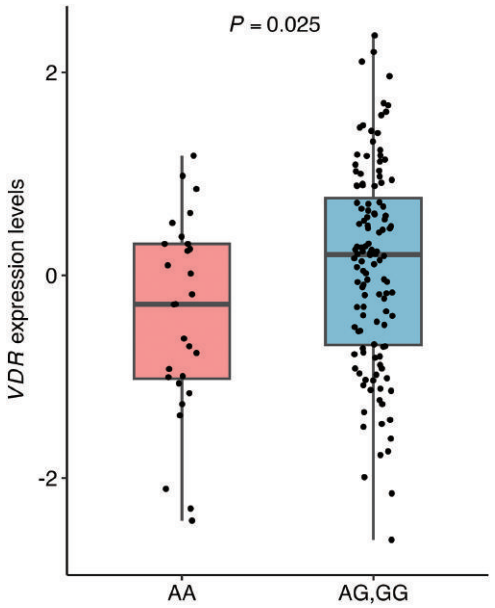

Fig. 2. Comparison of *VDR* mRNA Expression Levels between Patients with the AA Genotype and Those with the G Allele of the *VDR* rs2228570 Polymorphism

DISCUSSION

The study shows that the *VDR* rs2228570 SNP is associated with poor clinical outcomes, including asthma severity and exacerbations, in adult patients with asthma. The rs2228570 AA genotype has been implicated in the intensification of

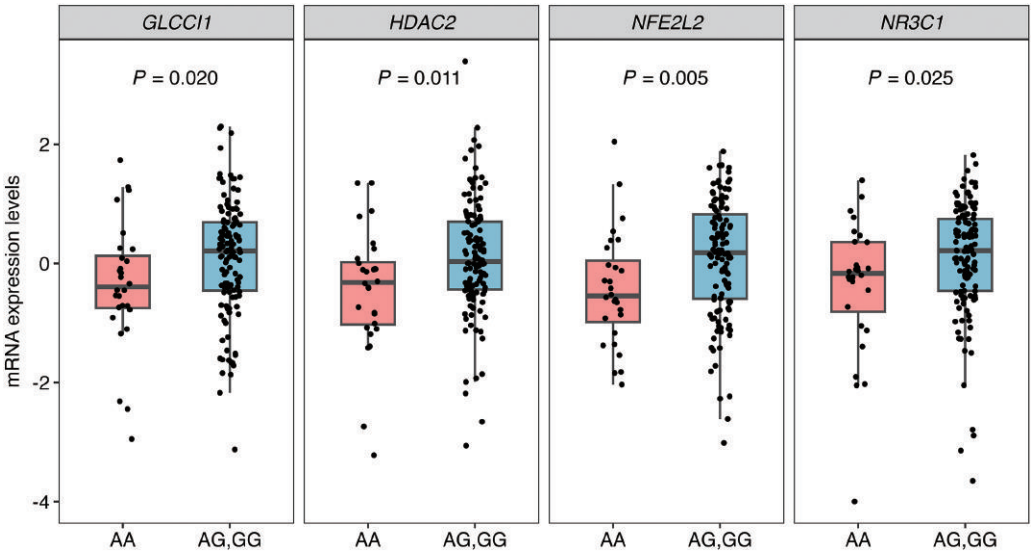

Fig. 3. Comparison of mRNA Expression Levels of Steroid-Responsive Related Molecules between Patients with the AA Genotype and Those with the G Allele of the *VDR* rs2228570 Polymorphism

inflammatory responses, and its presence has been shown to decrease the expression of genes encoding molecules associated with the glucocorticoid response. These findings suggest that patients with the AA genotype of the *VDR* rs2228570 polymorphism experience reduced vitamin D efficacy due to impaired *VDR* functionality and expression. This diminished effect of vitamin D contributes to increased inflammation and, in turn, reduced steroid efficacy, thereby potentially exacerbating asthma severity. Therefore, the *VDR* rs2228570 polymorphism may serve as a valuable biomarker for predicting poor prognosis in patients with asthma.

Vitamin D modulates the immune system by regulating the transcription of specific molecules after binding to VDR. VDR is expressed in various immune cells, including mast cells, dendritic cells, and granulocytes, such as neutrophils and eosinophils. Although the precise mechanisms by which vitamin D influences the immune system remain unclear, previous studies have suggested that it may play a role in suppressing the production of inflammatory cytokines and promoting the differentiation of regulatory T cells.[25] Consequently, the reduced effects of vitamin D may exacerbate asthma-related inflammation. These effects are likely attenuated by insufficient serum vitamin D concentrations and impaired VDR function. *VDR* gene polymorphisms alter its function and affect its transcriptional activity. Numerous studies have investigated the association between *VDR* gene polymorphisms and the prevalence of asthma.[18] Several studies and meta-analyses have shown that rs2228570, the focus of this study, is associated with an increased risk of asthma.[26] However, the relationship between the rs2228570 polymorphism and the pathology and prognosis of asthma is yet to be extensively investigated. In a previous investigation, we examined the relationship between plasma 25-hydroxyvitamin D_3 levels and asthma severity and exacerbations in the same patient cohort as in the present study.[16] However, our findings did not reach statistical significance. Given the inter-individual variability in plasma 25-hydroxyvitamin D_3 levels, we incorporated this variable into the multivariate analysis as a covariate to assess the association of *VDR* gene polymorphisms. Additional covariates known to be associated with asthma severity and exacerbations, such as lung function and BMI, were included in the analysis. Notably, even after considering these covariates, rs2228570 was still shown to be significantly associated. Consistent with our findings, some studies involving pediatric patients with asthma have suggested that individuals with the AA genotype of rs2228570 experience a more severe form of the disease.[27] Our study showed that patients with the AA genotype of rs2228570 had significantly elevated levels of FeNO, a marker of eosinophilic airway inflammation, and tended to have higher levels of NLR, a marker of systemic inflammation. These results suggest that the AA genotype of rs2228570 is associated with increased inflammation, possibly due to reduced vitamin D efficacy, which contributes to severe disease and a poor prognosis in patients with asthma.

The rs2228570 SNP represents a genetic variation located at the initiation codon of the *VDR* gene. The A allele functions as an initiation codon, while the G allele does not. In the presence of the G allele, the position of the initiation codon is shifted downstream by nine base pairs. Substituting alleles A to G resulted in a reduction in the length of the VDR protein from 427 to 424 amino acids. Shorter VDR proteins produced by the G allele show greater stability and higher transcriptional activity.[28] Accordingly, the function of *VDR* among patients with the AA genotype is considered inferior to that observed in the GA and GG genotypes, consistent with our findings that indicated a relationship between the rs2228570 polymorphism and *VDR* mRNA expression levels which were lower in the AA genotype. However, the relationship between this polymorphism and gene expression remains unclear, and contradictory results have been reported.[29] The AA genotype may impair VDR function and reduce *VDR* expression. However, further validation is required. The frequency of the A allele of the rs2228570 polymorphism is higher in East Asians than in other racial groups, highlighting

it as a potential factor to be considered when investigating individual differences in the pathophysiology of asthma.

Among the genetic polymorphisms examined in this investigation, an association with exacerbation for rs11568820 was identified. However, we did not find an association between this polymorphism and severe asthma. Further research is required to elucidate its effects on the pathophysiology of asthma. The rs11568820 polymorphism is located in the promoter region of the *VDR* gene and influences variations in promoter activity.[30] Previous studies have suggested that this polymorphism plays a role in the development of asthma.[31] Furthermore, meta-analyses and other reports have indicated that the relationship between *VDR* polymorphisms and asthma onset may vary depending on an individual's racial background.[32,33] This study did not identify any association between the three *VDR* gene polymorphisms (rs1544410, rs731236, and rs7975232) and two *GC* gene polymorphisms (rs7041 and rs4588) and asthma severity or prognosis. However, the possibility that these SNPs may affect the pathophysiology of asthma cannot be ruled out. More studies are required to elucidate this potential association. Asthma is widely recognized as a heterogeneous disease.[4] It may be beneficial to assess the relationship between vitamin D function and asthma pathogenesis in each pathological subtype.

The findings of this study indicated that patients with the AA genotype of the rs2228570 polymorphism showed lower expression levels of *NR3C1*, *HDAC2*, *GLCCI1*, and *NFE2L2*, which are molecules involved in the response to steroids. Steroids bind to the glucocorticoid receptor encoded by the *NR3C1* gene. This complex then moves into the nucleus, where it performs its function through the action of HDAC2, a histone deacetylase enzyme.[24] Steroid responsiveness is linked to *GLCCI1*,[22,34] while *NFE2L2*, which encodes Nrf2, exhibits anti-inflammatory and antioxidant properties and is related to *HDAC2*.[35] Given that patients with the AA genotype of *VDR* rs2228570 show reduced expression of these genes, it is plausible that their responsiveness to steroids is impaired. The efficacy of steroid treatment decreases under conditions of increased inflammation.[24] It is reasonable to suggest that the elevated inflammatory state observed in individuals with the AA genotype contributes to this reduced response. Furthermore, vitamin D affects steroid responsiveness,[36] and evidence indicates that vitamin D may enhance the diminished steroid responsiveness observed in patients with asthma.[37,38] The findings of this study are significant because they elucidated the potential impact of *VDR* gene variations on drug responses in patients with asthma. This suggests a reduction in the functionality of VDR and vitamin D efficacy, which are critical for the advancement of asthma treatment strategies.

There are some limitations to this study. First, it was a single cohort study conducted in a single center and the results have not been verified in another cohort. Second, although we confirmed that the participants did not receive the active form of vitamin D_3, we did not investigate their dietary vitamin D intake. Third, we were unable to directly assess the sensitivity of steroids.

In conclusion, our findings indicate that the rs2228570 polymorphism in *VDR* is associated with the severity and exacerbation of asthma. The rs2228570 polymorphism diminishes the effects of vitamin D by impairing function and expression, thus promoting increased inflammation and reducing the response to steroids in patients with asthma. In asthmatic patients, the AA genotype of the rs2228570 polymorphism has been identified as a risk factor for severe disease and exacerbations and can also serve as a prognostic indicator and a potential marker for optimizing treatment.

Acknowledgments We would like to thank the patients for their participation in this study, and the medical staff for their cooperation. The authors thank the Nagai Memorial Research Scholarship from the Pharmaceutical Society of Japan for providing scholarship support to Sekiko Uehara.

Funding This study was supported by Grants-in-Aid for Scientific Research (Grant numbers: 16K18949 and 22K06772) from the Japan Society for the Promotion of Science (to K. Hirai).

Author Contributions K.H. conceptualized the study. S.U., K.H., T.S., and K.I. designed this study. T.S. and T.A. recruited the patients for the study. S.U., K.H., T.S., and T.A. collected clinical data. S.U., K.H., and H.O. performed the experiments. S.U. and K.H. analyzed the data. S.U. and K.H. drafted the first draft. All authors have revised the manuscript accordingly. All the authors approved the submitted version of the manuscript and agreed to be accountable for any part of the work.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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