

A Novel –66T/C Polymorphism in FcεRI α-Chain Promoter Affecting the Transcription Activity: Possible Relationship to Allergic Diseases¹

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We found a novel polymorphism, –66T/C, in the promoter region of human *FcεRIα*, the specific component of the high affinity receptor for IgE (FcεRI), which is essential for the cell surface expression of FcεRI and the binding of IgE Ab. When the effect of the single nucleotide replacement on the promoter function was analyzed, the transcription activity of the T allele promoter was found to be higher than that of the C allele promoter, and was markedly up-regulated by the overexpression of GATA-1 when compared with the C allele promoter. This is probably because the promoter with T at –66 has an additional GATA-1-binding motif in the region, which may assure higher affinity of the transcription factor to the promoter. In accordance with this, EMSA actually indicated that GATA-1 bound to the T allele probe (–80/–59) with the affinity higher than that to the C allele probe. Statistical analysis suggested that a significant portion of nonallergic individuals has heterozygous –66T/C genotype, while most of allergic individuals have homozygous –66T/T genotype in Japanese population. Our findings for the first time demonstrate the presence of *FcεRIα* polymorphism related to the allergic diseases. *The Journal of Immunology*, 2003, 171: 1927–1933.

Allergic (atopic) diseases such as atopic dermatitis (AD)³ and asthma are a major concern as morbidity in developed countries because patients carrying the diseases have been increasing in population. The high affinity IgE receptor, FcεRI, composed of three subunits (α, β, and γ) is mainly expressed on the surface of effector cells such as mast cells and basophils. Cross-linking of IgE Abs bound to FcεRI by multivalent Ags induces activation of these cells, resulting in secretion of allergic mediators and induction of cytokine gene transcription. Thus, FcεRI plays a central role in the induction and maintenance of allergic responses. Until now, a large number of studies on this linkage between the variations in *FcεRIβ* and allergic diseases has been reported by many research groups, because chromosome 11q13 on which *FcεRIβ* is mapped was also assigned to relate to atopic diseases (1, 2). In contrast, there are no reports on the polymorphism of *FcεRIα*, despite that the α-chain is the specific component of FcεRI and directly binds to IgE. Involvement of the α-chain in FcεRI-mediated allergic reaction was definitively proved by the absence of the allergic reaction in α-chain-deficient

mice (3). Recently, the binding itself of monomeric IgE to FcεRI was shown to promote the survival of mast cells without cross-linking of the receptor (4, 5), also suggesting that an increase in FcεRI α-chain on the cell surface accelerates the IgE-mediated allergic reaction. In addition, a genetic linkage to AD was recently assigned on human chromosome 1q21, which is very close to the chromosomal locus where *FcεRIα* is mapped (6). This prompted us to examine that possible polymorphism in *FcεRIα* may associate with allergic diseases.

Materials and Methods

Cell culture

PT18 (mouse mast cell line), RBL-2H3 (rat mast cell line), and KU812 (human mast cell line) were cultured, as described previously (7).

Plasmid construction

As a reporter plasmid carrying luciferase gene under the control of the α-chain promoter (–66T allele), pGV-B2-αNN0.6 (–605/+29) or pGV-B2-αPN0.1 (–90/+29) was used (7, 8). To obtain –66C allele of promoter, site-directed mutagenesis was conducted on –66T allele of the promoter by using a Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA) and synthetic oligonucleotides: 5'-GTAAACCAGATATGACACAGAAAACATTTC-3' and 5'-GAAATGTTTCTGTGTCA TATCTGGTTAAC-3' (Invitrogen, San Diego, CA).

As an expression plasmid for wild-type GATA-1, pCR-GATA-1 (7, 8) was used.

Transfection and luciferase assay

Harvested cells were suspended in each culture medium supplemented with additional 10% FCS. The cells (5–10 × 10⁶ cells in 0.5 ml) were cotransfected with 5 μg of the test construct and 25 ng of pRL-CMV (Promega, Madison, WI) by electroporation by using Bio-Rad Gene Pulsar II (Hercules, CA) set at 300 V and 950 μF. On the coexpression analysis, 3 μg of pCR-GATA-1 or pCR3.1-self was added into the cell suspension. In this transient expression system, the amount of exogenous GATA-1 increased 10 times more than that of endogenous GATA-1 by Western blotting analysis (data not shown). The measurement of luciferase activity was performed, as described previously (9, 10).

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³ Abbreviations used in this paper: AD, atopic dermatitis; MFI, mean fluorescence intensity.

EMSA

GATA-1 proteins were synthesized by using pCR-GATA-1 as the template *in vitro*. *In vitro* transcription and translation were performed by using a TNT T7 Quick coupled transcription/translation system, according to the manufacturer's instructions (Promega) (8).

As the probe for EMSA, double-stranded oligonucleotides were prepared by annealing FITC-labeled oligonucleotides: 5'-TTAACCAGATATGATACAGAAA-3' and 5'-TTTCTGTATCATATCTGGTTAA-3' (probe 1, see Fig. 3B); 5'-TTAACCAGATATGACACAGAAA-3' and 5'-TTTCTGTGTATCATCTGGTTAA-3' (probe 2, see Fig. 3C); 5'-TTAACCACCAATGATACAGAAA-3' and 5'-TTTCTGTATCATCTGGTTAA-3' (probe Δ5'T, see Fig. 3D); and 5'-TTAACCACCAATGACACAGAAA-3' and 5'-TTTCTGTATCATCTGGTTAA-3' (probe Δ5'C, see Fig. 3D), respectively. EMSA was performed under the same conditions, as described previously (9, 10), by using 1 μl of reaction product prepared with *in vitro* transcription/translation system, as described above. Anti-GATA-1 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All gels were subjected to a fluorescence detector, FluorImager 595 (Molecular Dynamics, Sunnyvale, CA).

Binding affinities were determined by quantification of free and bound probe on gels with a FluorImager.

Flow cytometric analysis of transfectants

Luciferase genes in plasmids pGV-B2-αNN0.6-66T and -66C were replaced with human FcεRI α-chain cDNA obtained by RT-PCR from KU812 mRNA to generate plasmids p-66T-α and p-66C-α carrying human α-chain cDNA under the control of each allele of α-chain promoter, respectively. Human FcεRI β-chain and γ-chain cDNAs were also cloned by RT-PCR and inserted into pCR-HA (11) (kindly provided from H. Nakano, Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan) to yield pCR-HA-β and pCR-HA-γ, respectively. Nucleotide sequence of each cDNA was determined by an ABI PRISM377 DNA sequencer (Applied Biosystems, Foster City, CA). PT18 cells (2×10^6) were cotransfected with 5 μg of pCR-HA-β, 5 μg of pCR-HA-γ, and totally 10 μg of p-66T-α and/or p-66C-α by electroporation under the same conditions as described above. After 3-wk cultivation in the presence of 0.5 mg/ml of G418, cell surface expression of human FcεRI α-chain was analyzed by flow cytometry with FACSCalibur (BD Biosciences, Franklin Lakes, NJ) after staining with FITC-conjugated anti-human FcεRI α-chain Ab, which is not cross-reactive with mouse α-chain (12) or FITC-conjugated mouse IgG2b (isotype control; BD PharMingen, San Diego, CA).

Accession number

Human FcεRIα, L14075.

Sequencing analysis of human FcεRIα

Genomic DNA was prepared from peripheral blood by a DNA quick (Dainippon Pharmaceutical, Osaka, Japan). PCR was conducted with the human genomic DNA by using Advantage 2 polymerase (Clontech, Palo Alto, CA) with following two oligonucleotides as primers: 5'-TTAGTTGCTGCTGTTTATTCTGCTCTCC-3' (-339/-310) and 5'-TCTAGCTTGGCTCCATCAGAGTCCCTGG-3' (+237/+208). A thermal cycle of 95°C for 30 s, 65°C for 30 s, and 68°C for 30 s was repeated 30 times after 95°C incubation for 1 min and followed by 68°C for 1 min. After the purification of the PCR product by a MinElute Gel Extraction kit (Qiagen, Valencia, CA), the nucleotide sequences of the DNA fragments were determined by using ABI PRISM377 DNA sequencer (Applied Biosystems). An oligonucleotide 5'-GATAGGGAGTGGAGTAAGTG-3' (-182/-163) was used as the sequencing primer.

Subjects

AD individuals were patients of Juntendo University Hospital. The diagnosis was based on the morphological appearance of skin lesions and clinical course. A physician-administered questionnaire, which included the diagnostic criteria for AD of Hanifin and Rajka (13), was completed for each individual. In brief, the presence of at least three basic features from the following four features: 1) pruritus, 2) typical morphology and distribution, 3) chronic or chronically relapsing dermatitis, and 4) personal or family history of atopy, as listed by Hanifin and Rajka.

We selected as controls those who had no symptoms or history of allergic diseases, as written in Table I, based on physician-administered questionnaire.

The ethics committee of Juntendo University School of Medicine approved this study.

Measurement of FcεRI on basophil surface by FACS

Mononuclear cells were enriched from 5 ml of peripheral blood treated with EDTA by using a Percoll-based density-gradient centrifugation technique, as described previously (14). According to the method in a previous report (15), cells were analyzed by flow cytometry with FACSCalibur (BD Biosciences) after staining with PE-conjugated anti-human CD3, CD9, CD14, and CD19 (BD PharMingen); with FITC-conjugated anti-human FcεRI α-chain Ab, which is noncompetitive with IgE (12); or FITC-conjugated mouse IgG2b (isotype control; BD PharMingen), and mean fluorescence intensity (MFI) is calculated. Total serum IgE was measured by enzyme immunoassay (SRL, Tokyo, Japan), and individuals showing low IgE (<250 IU/ml according to a previous report (16)) were used as control.

Statistical analysis

Statistical analysis of reporter assays (Figs. 1 and 2) and of MFI of FcεRI on basophils (Fig. 6A) was performed using the Student *t* test. The association of the polymorphism at -66 with allergic diseases was analyzed by χ^2 test for T/T vs T/C between the controls and allergy groups. Multiple linear regression analysis was used to test for independent predictors of MFI by entering genotypes, and total serum IgE into the model (SPSS version 11.0; SPSS, Chicago, IL). A *p* value less than 0.05 was considered statistically significant. The values of MFI and IgE were shown as mean $\pm$ SEM.

Results

The -66T/C polymorphism affecting the transcription activity of α-chain promoter

We previously determined the nucleotide sequence of a full-length genomic DNA encoding human FcεRI α-chain (17, 18) and analyzed the α-chain promoter (7, 8) with T at position -66. By sequencing analysis of several genomic DNA clones in the present study, we found T/C polymorphism at -66. Because the polymorphism was found at the promoter region, we examined the role of the single nucleotide at -66 for the promoter function. We then constructed two reporter plasmids, each of which carried either of the both types of the α promoter (-605/+29) in front of the luciferase gene, and analyzed their transcription activity by transient

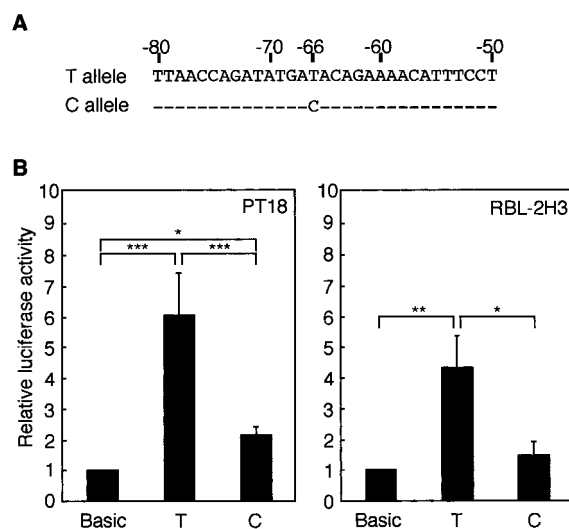


FIGURE 1. Effect of -66T/C polymorphism on the transcription activity of human FcεRI α-chain promoter. **A**, Nucleotide sequences of human FcεRI α-chain promoter (-80/-50). **B**, PT18 cells or RBL-2H3 cells were transiently transfected with pGV-B2-αNN0.6-66T, pGV-B2-αNN0.6-66C, or pGV-B2 (=pGL3-Basic). The relative luciferase activity is represented as the ratio of the activity to the luciferase activity in the cells transfected with the empty control vector pGV-B2. Each experiment was conducted in duplicate for each sample, and the results are expressed as mean $\pm$ SE for three independent experiments in this figure and Fig. 2. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.005, as determined by a paired *t* test.

reporter assay in the α -chain-positive cells, PT18 and RBL-2H3. As shown in Fig. 1, luciferase activity derived from the $-66T$ promoter was significantly higher than that from the $-66C$ promoter in both of the cell lines. Similar results were obtained, when human cell line KU812 was used for the transient reporter assay, although the promoter activity detected in KU812 was lower than those in other two cell lines (data not shown).

The above-mentioned results suggested that nucleotide T at -66 was critical for showing the strong transcription activity of the Fc ϵ RI α -chain promoter. Interestingly, in the promoter with $-66T$, an additional GATA-1-like motif (3'-GATA) is present at $-68/-65$, although the sequence, TGATAC, is not completely identical with the optimum sequence (A/T)GATA(A/G) for GATA-1 binding (19). To confirm the involvement of GATA-1 in α -chain promoter activity, we performed coexpression experiments. The luciferase activity derived from $-66T$ promoter was ~ 4 -fold higher in the presence of overproduced GATA-1, and that from $-66C$ promoter was increased about only 2-fold (Fig. 2). Thus, $-66T$ promoter responded to GATA-1 better than $-66C$ promoter.

Binding profile of GATA-1 to DNA around -66

We next performed EMSA using several labeled and nonlabeled oligonucleotides. Specific binding between GATA-1 protein and probe ($-80/-59$) was observed with both probe 1 ($-66T$ allele, lane 2 in Fig. 3B) and probe 2 ($-66C$ allele, lane 2 in Fig. 3C). When nonlabeled wild-type ($-66T$) double-stranded oligonucleotide, competitor 1, was added as the competitor, 50-fold excess of the competitor was sufficient for disappearing the specific bands (lane 6 in Fig. 3, B and C). In contrast, both the competitors, competitor 2 lacking 5'-GATA motif and competitor 3 lacking 3'-GATA motif, inhibited the binding between GATA-1 and the wild-type probe, but the inhibitory activity was obviously lower than that of competitor 1. It should be noted that the band intensity in the presence of competitor 2 or 3 was always weaker for probe 2 (Fig. 3, B and C). This suggests that GATA-1 has higher affinity to probe 1 than to probe 2. To confirm the possibility that 3'-GATA can be also recognized by GATA-1, we performed EMSA by using probes $\Delta 5'T$ and $\Delta 5'C$, which carried GATA and GACA at 3'-GATA site, $-68/-65$, respectively, but lacked 5'-GATA motif. As shown in Fig. 3D, shift band was observed with probe $\Delta 5'T$ (lane 3), but not with probe $\Delta 5'C$ (lane 4). From these observations, it was concluded that 3'-GATA-1 motif of $-66T$ allele

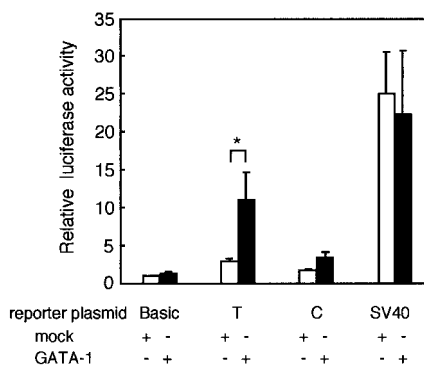


FIGURE 2. Effect of overexpression of GATA-1 on T allele or C allele promoter activity. A total of $5 \mu\text{g}$ of each of reporter plasmids, pGV-B2, pGV-B2 α PN0.1-66T, pGV-B2 α PN0.1-66C, or pGV-P2 (=pGL3-Promoter (SV40)), was transiently transfected with $3 \mu\text{g}$ of pCR3.1-self (mock) or pCR-GATA-1 to KU812 cells. The relative luciferase activity is represented as the ratio of the activity to the luciferase activity in the cells transfected with the empty control vector pGV-B2 and pCR3.1-self. *, $p < 0.001$.

had a potential to be recognized by GATA-1, although the affinity was lower, compared with that of 5'-GATA. Actually, Scatchard plot analysis (20, 21) using the data of EMSA indicated that the binding affinity of GATA-1 for T allele probe was about 2 times higher than that for C allele probe, and the affinity for T allele probe lacking 5'-GATA was significantly lower than that for T allele probe, but still retained in the same order of magnitude (1/4 for T allele probe). These results indicate that 3'-GATA itself possesses the binding potential to GATA-1 and increases the binding affinity of GATA-1 for the Fc ϵ RI α -chain promoter.

Effect of T/C polymorphism for cell surface expression of Fc ϵ RI α -chain

To confirm whether $-66T/C$ polymorphism affected cell surface expression of Fc ϵ RI, we introduced the plasmids p-66T- α and p-66C- α , which carry $-66T$ and $-66C$ promoters followed by human α -chain cDNA, respectively, along with expression vectors for human β - and γ -chains into PT18 cells, and analyzed cell surface expression of Fc ϵ RI by an anti-human Fc ϵ RI α -chain Ab, which recognizes human α -chain specifically, but not rodent α -chain. By FACS analysis, slight, but apparent expression of human α -chain protein was detected on surface of PT18 cells transfected with these plasmids (Fig. 4). The highest expression of human α -chain was observed when only a plasmid carrying $-66T$ promoter was transfected, whereas introduction of a plasmid carrying $-66C$ promoter resulted in faint expression of the α -chain. Cotransfection with the plasmids carrying $-66T$ and $-66C$ promoters gave intermediate expression in comparison with the cases of $-66T$ promoter alone and $-66C$ promoter alone. These results indicated that T/C polymorphism actually affected the cell surface expression of Fc ϵ RI α -chain.

Polymorphism in Fc ϵ RI α -chain gene regulatory region

As described above, the transcription activity of Fc ϵ RI α -chain promoter was influenced by a single nucleotide substitution at -66 . To elucidate possible relationship between $-66T/C$ polymorphism and atopic diseases, we next preliminarily analyzed the population with $-66T/C$ polymorphism in Japanese individuals (Fig. 5). We sequenced ~ 350 bp of Fc ϵ RI α , in which we found most of all the elements responsible for Fc ϵ RI α -chain gene expression in our previous study (7, 8, 22), and found polymorphism only at -66 (Fig. 5A). To elucidate the relationship between the polymorphism and allergic diseases, statistic analysis was performed. As shown in Table I, T/T was major (46 of 54, 85.2%), while T/C was minor (8 of 54, 14.8%) in population. Most (96.2%) of the patients with AD carried T/T at -66 . Surprisingly, a significant portion (25.0%) of control individuals without allergic diseases, such as AD, atopic asthma, and allergic rhinitis, had T/C at this position. Frequency of heterozygous $-66T/C$ was further increased (33.3%), when individuals without any symptoms or history of allergic diseases for allergic conjunctivitis, pollinosis, contact dermatitis, and family history in addition to above three allergic diseases were chosen. In both calculations, significantly small p values (< 0.05) were obtained. These observations suggest that this polymorphism is possibly related to allergic diseases, and that the individuals (in Japanese population) carrying the α -chain promoter with C allele at -66 are apt to be resistant to allergy.

Cell surface expression of Fc ϵ RI on basophils from peripheral bloods of T/T and T/C individuals

To evaluate the polymorphism in cell surface expression of Fc ϵ RI, we measured the amount of Fc ϵ RI on cell surface of basophils from 14 volunteers (6 T/C and 8 T/T) of nonatopic individuals and 5 patients of AD, all of which are T/T genotype. No basophils from

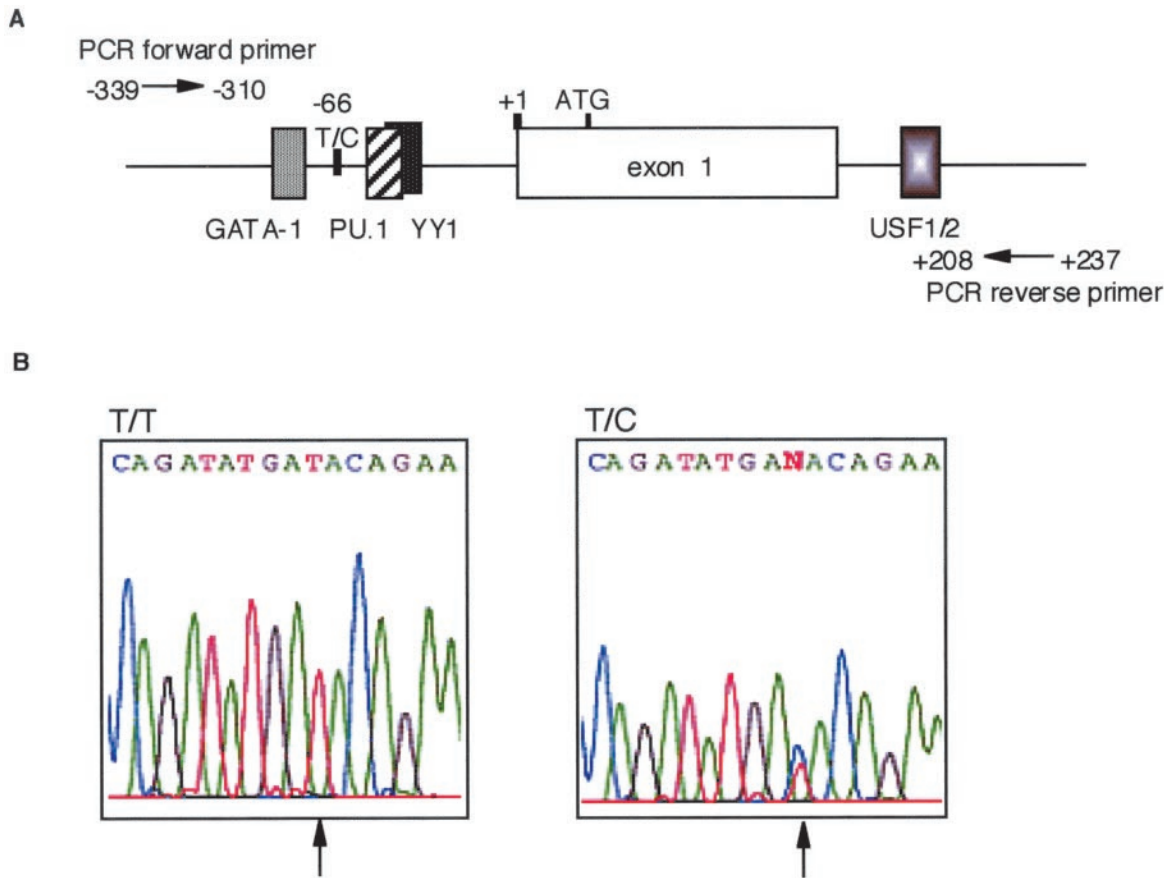


FIGURE 5. Sequencing analysis of human *FcεRIα*. *A*, Nucleotide sequencing analysis was performed on 5'-flanking region, exon 1, and a part of intron 1. *B*, DNA sequence electrophoregrams of the polymorphism at -66 of *FcεRIα* with T/T and T/C. Arrows indicate the polymorphic sites.

of T/T allele control express higher amount of cell surface FcεRI than those of T/C allele control, independently of serum IgE level.

Discussion

Among three subunits, α , β , and γ , the gene encoding β is mapped on chromosome 11q13. Because this region was also assigned to be related to atopic diseases (1), a large number of studies on this linkage between the variations in β -chain gene and allergic diseases has been performed by many research groups (2). In contrast, there are no reports on the polymorphism of *FcεRIα*. In this study, however, we found a T/C polymorphism in the promoter at the

nucleotide position of -66, and demonstrated that the promoter with -66T had stronger activity than that with -66C and the allelic frequency was associated with allergic diseases in the statistic analysis. Considering that α -chain is the FcεRI-specific molecule and essential for both functional expression of FcεRI on the

Table I. -66T/C polymorphism in *FcεRIα* in Japanese populations

Group Studied	Number of Individuals	Number of Individuals with Each Allele (allelic frequency, in %)		
		T/T	T/C	C/C
Total	54	46 (85.2)	8 (14.8)	0 (0.0)
Patients with AD	26	25 (96.2)	1 (3.8)	0 (0.0)
Control ^a	28	21 (75.0)	7 (25.0)	0 (0.0)
Control ^b	21	14 (66.7)	7 (33.3)	0 (0.0)

^a As control, individuals without allergic diseases (AD, atopic asthma, and allergic rhinitis) were selected. For AD patients vs control, $\chi^2 = 4.78$, $p = 0.029$. AD patients and control group were aged 27.1 ± 9.1 and 28.9 ± 6.7 years, respectively.

^b As control, individuals without allergic diseases (AD, atopic asthma, allergic rhinitis, allergic conjunctivitis, pollinosis, contact dermatitis, and family history) were selected. For AD patients vs control, $\chi^2 = 7.15$, $p = 0.008$. Control group was aged 28.5 ± 6.7 years.

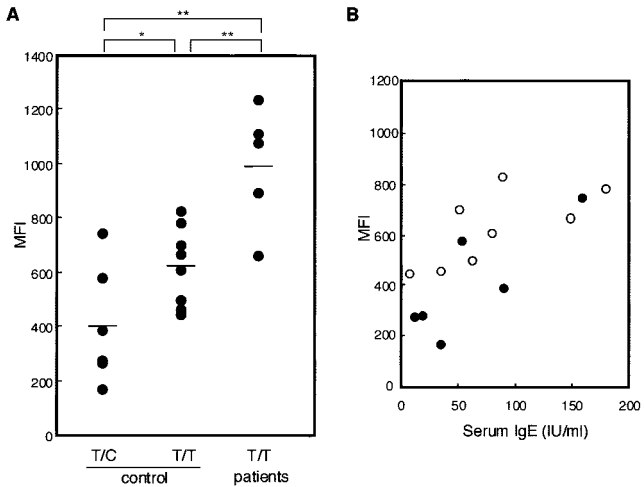


FIGURE 6. Cell surface expression of FcεRI on basophils from peripheral bloods. *A*, Basophils from peripheral bloods of T/C control ($n = 6$), T/T control ($n = 8$), and T/T patients ($n = 5$) were stained with FITC-conjugated anti-human FcεRI α -chain Ab, which is noncompetitive with IgE (see *Materials and Methods*). *, $p < 0.05$; **, $p < 0.005$, as determined by a paired t test. *B*, MFI vs concentration of serum IgE of each individual. Open circle, T/T control; closed circle, T/C control.

cell surface and binding to IgE Ab, it is probable that the polymorphism found in the α-chain gene is related to allergic diseases. Recently, a genetic locus related to AD was mapped on chromosome 1q21, which is very close to 1q23 on which FcεRI α-chain gene is mapped (6). In that study, Cookson et al. also mapped determinants for the amount of total serum IgE on chromosome 16q-tel and weakly on chromosome 5 as well. These observations suggest that the increase in the amount of total serum IgE is not always a direct cause of the AD, but that possible increase in the FcεRI α-chain expressed on the cell surface may enhance the response to IgE Abs.

Recently, a single nucleotide polymorphism was found in the proximal CD14 promoter that is related to the expression level of total serum IgE. In that case, the promoter activity was modified by affecting the binding affinity for the promoter of Sp3, which regulated CD14 promoter repressively (23). The polymorphism in the promoter region of the α-chain gene may become a similar example found in allergy-related genes. For further study of the relationship between T/C polymorphism and allergic diseases, it is necessary to analyze the polymorphism in a large number of individuals with allergic diseases in various countries, because the allele frequency of the α-chain promoter may vary in other countries, as is the case for -590C/T polymorphism of IL-4 promoter (24).

The promoter with T at -66 displayed increased activity to initiate transcription, compared with the promoter with C at -66. Because GATA-1 binds to T allele promoter with the affinity higher than that to C allele promoter, it is likely that the difference in GATA-1-binding affinity is attributed to the difference in the promoter activity. Palindromic or repetitive GATA sites were found in several promoters and were shown to have higher GATA-1-binding affinity than a promoter with a single GATA site (20). Even in such cases, however, only a single GATA-1 protein could bind to the promoter, probably because of steric hindrance of the GATA-1 to its neighboring GATA motif. Then, a question is evoked; that is, how two GATA-1 binding sites serve to increase the affinity and up-regulate the FcεRI α-chain promoter activity. Recently, it has been shown that N-finger that has been thought to facilitate the formation of specific and stable DNA/C-finger complex has the DNA-binding ability (25). Based on the available information on structure and function of GATA-1, we speculate that specific binding of C-finger to 5'-GATA site could spatially dispose N-finger near 3'-GATA site to bind, and the possible weak binding between N-finger and 3'-GATA could be sufficient to enhance the affinity of GATA-1 to the promoter DNA. For elucidation of the mechanism for GATA-1-mediated up-regulation of the T allele promoter, three-dimensional structure of whole GATA-1/DNA (-66T) complex will be obviously required.

Comparison of cell surface expression of FcεRI on basophils using MFI as the index revealed that AD patients possessed higher degree of cell surface FcεRI than those of control individuals, which is consistent with other studies showing the elevated cell surface expression of FcεRI in allergic patients (26). To eliminate the possible effect of IgE for increasing expression of FcεRI, we compared cell surface expression of FcεRI between T/C and T/T allele in nonatopic individuals with low IgE (<250 IU/ml) and found apparently higher surface expression in T/T control (620 ± 52) than that in T/C control (399 ± 89). Furthermore, the multiple linear regression analysis revealed that -66T/C polymorphism is actually related to cell surface expression of FcεRI, irrespective of serum IgE level. All these data suggest that -66T/C polymorphism at FcεRIα, which affects the expression of FcεRI α-chain, could be one of the determinants for allergic diseases. To clarify

the relationship between -66 polymorphism and atopic diseases, further detailed analysis with a numerous number of individuals including atopic patients of T/C genotype will be required. We also think that it is necessary to find polymorphism in other loci of the genome and analyze its association with -66T/C polymorphism for further elucidation of genomic background for allergic diseases.

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