



High-level expression of *early growth response-1* and association of polymorphism with total IgE and atopy in allergic rhinitis adults

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ABSTRACT

Background: Early growth response-1 (*Egr-1*) is expressed in human airways and its polymorphisms have been associated with total IgE and atopy in asthmatic patients. We investigated the effects of Chinese-tagging single nucleotide polymorphism (SNP) of *Egr-1* and its mRNA expression on allergic rhinitis (AR) traits.

Methods: Among 214 Chinese AR adults and 259 controls, tag SNP −4071 A → G was genotyped and mRNA expression in peripheral blood was quantified by real-time PCR.

Results: *Egr-1* mRNA expression was significantly higher in patients than controls (median of 0.23 vs 0.15 fold *GAPDH* expression; $p < 0.001$). Its expression was not associated with −4071 polymorphism. However, significant correlations were found between −4071 A → G with increased plasma total IgE ($p = 0.028$) and atopy ($p = 0.030$) in patients. Logistic regression confirmed the association ($p = 0.034$) with age and gender adjusted. Patients homozygous for the A allele had a 2.3-fold and 1.9-fold risks, respectively of having increased plasma total IgE and atopy than those G allele carriers.

Conclusions: We showed high levels of *Egr-1* mRNA expression and demonstrated a significant association of polymorphism with increased plasma total IgE and atopy in AR patients. It may be useful to explore the pharmacogenetics of *Egr-1* inhibitors.

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1. Introduction

Allergic rhinitis (AR) is a complex inflammatory disease of the upper airway characterized by sneezing, nasal pruritus, rhinorrhoea and nasal obstruction [1]. The pathogenesis of AR and other allergic diseases involves complex interactions of environmental exposure to allergens and respiratory irritants [2,3] as well as genetic predisposition of the individual patients [4,5]. Many susceptibility loci for AR and atopy have been described in the Western populations [4–6]. These genes are responsible not only for causing AR but also affect its severity [7,8].

Abbreviations: AR, Allergic rhinitis; CT, Threshold cycle; *Egr-1*, Early growth response-1; ERK, Extracellular signal-regulated kinase; FcεRI, High-affinity IgE receptor; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; HDM, House dust mite; IFN, Interferon; M-MLV, Moloney murine leukemia virus; PDGF, Platelet-derived growth factor; TGF, Transforming growth factor.

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However, there have only been a few small-scale studies on the AR susceptibility genes in our Chinese population [9]. In Hong Kong, the prevalence rate of school-aged children with AR is known at about 35% to 44% [10] and is increasing [11]. We used the candidate gene approach to study polymorphic markers in susceptibility genes of atopy and airway inflammation [7] in our Chinese adults with AR.

Early growth response-1 (*Egr-1*) is an immediate-early response gene that is rapidly and transiently expressed in many cell types in response to growth factors, cytokines, mechanical stimuli, and oxidative stress [12]. It is a zinc finger transcription factor that can modulate transcription through interactions with coactivators, corepressors, and other sequence-specific transcription factors [13,14]. Studies have been performed on the protein level of *Egr-1* relating to asthma in human and mice. Hjoberg et al. [15] found induction of *Egr-1* by platelet-derived growth factor (PDGF) in human airway smooth muscle. Silverman et al. [16] reported that the *Egr-1* knock-out mice had increased IgE concentrations at baseline and after allergen challenge compared with wild type mice. Recently, Li et al. [17] suggested that *de novo* synthesis of *Egr-1* was required for the full responsiveness of mast cells to produce TNF and IL-13 upon IgE and antigen stimulation. We also first reported significant association of *Egr-1* polymorphisms with plasma total IgE

and atopy in Chinese asthmatics children [18]. The aim of this study was to investigate the correlation between $-4071\text{ A} \rightarrow \text{G}$ polymorphism in the promoter region of the *Egr-1* gene and AR phenotypes, plasma total and allergen-specific IgE concentrations, as well as its mRNA expression in peripheral blood of Chinese AR adults.

2. Materials and methods

2.1. Study population

This study recruited unrelated patients aged over 18 years with AR from the Ear, Nose and Throat (ENT) clinics of the Prince of Wales Hospital, the Alice Ho Mui Ling Nethersole Hospital and the United Christian Hospital in Hong Kong. The diagnostic criteria were according to the guidelines defined by the Joint Task Force on Practice Parameters in Allergy, Asthma, and Immunology [19] and included watery rhinorrhea, nasal itch, sneezing and nasal blockage, with nasal endoscopy being used to exclude other nasal pathologies by experienced ENT specialists. Subjects with a present or past history of asthma or atopic dermatitis were excluded from this study. Patients were categorized as having intermittent, persistent, mild, or moderate-to-severe rhinitis according to the ARIA classification [20]. Both parents of these subjects are ethnic Chinese. Age- and sex-matched Chinese control subjects were recruited from the Hong Kong Red Cross when they came for blood donation. Subjects with any history of allergic diseases were excluded by using the standardized questionnaire. All subjects were free from any self-reported symptoms of infection for 4 weeks before study. Subjects gave written consent, and the Clinical Research Ethics Committee of our university approved this study.

2.2. Plasma total IgE and allergen-specific IgE assays

Plasma total IgE concentration was measured by chemiluminescence immunoassay using the Immulite Analyzer (Siemens Healthcare Diagnostics, Los Angeles, CA) and specific IgE antibodies to crude *Dermatophagoides pteronyssinus*, cat, dog, mixed cockroaches and mixed moulds were measured by fluorescent enzyme immunoassay (ImmunoCAP system, Phadia AB, Uppsala, Sweden). Plasma total IgE results were compared with our in-house upper limits of reference values to determine whether the concentrations were increased. Specific IgE concentration $\geq 0.35\text{ kIU/l}$ was considered positive. Subjects were classified as atopic if they were positive with at least one type of allergen-specific IgE antibody [5].

2.3. Genotyping of *Egr-1* polymorphism

Genomic DNA was extracted from peripheral blood using High Pure Viral Nucleic Acid Kit (Boehringer Mannheim GmbH, Mannheim, Germany). Subjects were genotyped for $-4071\text{ A} \rightarrow \text{G}$ polymorphism by polymerase chain reaction (PCR) amplification of the promoter region of the *Egr-1* gene using the following primers: 5'-GTG ATT CTC ATT GGC CTG GT-3' and 5'-TAC TAT TCC CCA GCC AGC AG-3' according to the published methods [18]. Briefly, PCR reactions were conducted in a total volume of 25 μl containing 60 ng of DNA, 10 mmol/l Tris-HCl buffer (pH 8.3), 1.5 mmol/l MgCl_2 , 200 μM of each deoxynucleotide triphosphate, 50 ng of each primer, and 0.5 U of Taq DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany). PCR products were digested by 5 U of *MseI* (New England Biolabs, Hitchin, Herts, UK) in each digestion. The digested PCR products were resolved on agarose gel containing ethidium bromide. Eight DNA samples of known genotypes were included in every restriction enzyme digestion as positive control together with 88 unknown samples in 96-well plates. Finally, the results of these RFLP assays were validated by direct sequencing of the polymorphisms using Big Dye Terminator Cycle sequencing kits with an ABI-3100 sequencer (Applied Biosystems, Foster City, CA) in 40 randomly selected samples.

2.4. mRNA extraction and quantitative real-time PCR

Total RNA was isolated from Trizol preserved whole blood using a commercial available kit (RNeasy Plus Mini Kit, Qiagen Inc., Valencia, CA) and stored at $-70\text{ }^\circ\text{C}$. Reverse transcription was performed with the First-Strand cDNA Synthesis Kit (Invitrogen Corporation, Carlsbad, CA) containing oligo (dT) primers and Moloney murine leukemia virus (M-MLV) reverse transcriptase. Primers for *Egr-1* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were listed in Table 1. Amplifications of *Egr-1* and *GAPDH* were performed in a 96-well reaction plate. The 25- μl reaction mixture in each well contained 1 μl of total cDNA, 200 nmol/l of sequence-specific primers, and 12.5 μl of qPCR Super Mix (SYBR® GreenER, Invitrogen Corporation, Carlsbad, CA, USA). A negative PCR control without template and a positive PCR control with a template of known amplification were included. All samples and controls were duplicated. Real-time PCR was performed using an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). After amplification, the relative gene expression was determined for each sample. For each target gene, a threshold was set above the mean baseline value for fluorescence determined on the basis of the first 15 cycles. Amplification reactions in which the intensity of fluorescence exceeded the threshold were defined as positive reactions [21]. The threshold cycle (CT) is the PCR cycle at which an increase in reporter signal above the baseline signal can first be detected. A comparative CT method was used for quantification of *Egr-1* mRNA expression as previously reported [22]. Briefly, the *Egr-1* specific signal was normalized by constitutively expressed *GAPDH* signal using the formula $2^{-\Delta\text{CT}} = 2^{-(\text{CT}_{\text{GAPDH}} - \text{CT}_{\text{Egr-1}})}$, resulting in the evaluation of the samples as n-fold difference relative to that of *GAPDH* mRNA.

3. Statistical analysis

The results of plasma total IgE and allergen sensitization as risk factors for the development of AR between patients and controls were compared using Student's *t* test or χ^2 as appropriate. Hardy-Weinberg equilibrium for the *Egr-1* $-4071\text{ A} \rightarrow \text{G}$ polymorphism in each group and the associations between *Egr-1* genotypes with AR and increased plasma total IgE and atopy were analyzed using χ^2 test. Because only 6 patients and 6 controls with the GG genotype of $-4071\text{ A} \rightarrow \text{G}$ had allergen-specific IgE data available, AG and GG genotypes were combined into a single category for the statistical comparisons. Multivariate logistic regression analysis was used to evaluate the effects of different covariates on increased plasma total IgE concentrations. Kruskal-Wallis test was used for comparing *Egr-1* expression in all groups while subgroup analyses were followed by Dunn's Multiple Comparison test. All comparisons were made 2-sided. A $P < 0.05$ was considered significant.

4. Results

4.1. Demographic data

Two hundred and fourteen AR patients and 259 non-allergic healthy control subjects were recruited. The mean (SD) ages in patients and controls were 39.2 (13.7) and 37.2 (11.3) years, respectively ($p = 0.083$),

Table 1
Sequences of primers for quantitative real-time PCR.

Gene	PCR primers	References
<i>Egr-1</i>	F: 5'-CAC CTG ACC GCA GAG TCT TT-3' R: 5'-GTG GTT TGG CTG GGG TAA CT-3'	NM_001964
<i>GAPDH</i>	F: 5'-ATG GGG AAG GTG AAG GTC G-3' R: 5'-GGG GTC ATT GAT GGC AAC AAT A-3'	NM_002046

Abbreviation: F, forward primer; R, reverse primer.

Table 2

The demographic data and clinical characteristics in our 214 AR patients.

Characteristics	Result*
Background information	
Age at evaluation (y)	39.2 ± 13.7
Sex (male)	100 (46.1%)
Classification	
Intermittent	84 (39.3%)
Persistent	130 (60.7%)
Severity	
Mild	64 (29.9%)
Moderate–severe	150 (70.1%)
Anti-allergic rhinitis treatment	
Steroid nasal spray	128 (59.8%)
Anti-histamine	138 (64.5%)
Immunosuppressant	3 (1.4%)
Others (antibiotic)	2 (0.9%)

*Expressed as mean ± SD for age while the rest as number (%).

with 46% of patients and 54% of controls being males ($p=0.092$). The demographic and clinical characteristics of the patients are summarized in Table 2. Among the patients, 84 cases (39%) were intermittent and 130 (61%) were persistent. Based on the classification proposed by Bousquet et al. [21], 64 cases were mild while the rest of 150 were moderate–severe. There were 128 (60%) patients using steroid nasal spray together with or without other medications.

4.2. Atopic status

Table 3 summarizes results of the plasma total and allergen-specific IgE measurements. The IgE_{log} in our patients was significantly higher than in the healthy controls ($p=0.006$). Atopy was defined by the presence of $\geq$ one specific IgE to inhalants occurred in 105 (49%) patients and 71 (27%) age- and sex-matched controls (odds ratio 2.55; $p<0.001$). The major environmental allergens in our patients was house dust mite (HDM, 48%), mixed cockroaches (12%) and cat (7%). The same patterns of allergic sensitization were found in the control group. Sensitization to HDM was significantly more common in patients as compared with the controls (Table 3).

4.3. Association of AR and atopy with *Egr-1* –4071 genotypes

The minor allele frequency for *Egr-1* –4071 A→G in patients and controls were 0.17 and 0.18, respectively. The genotyping was validated by direct sequencing in 40 randomly selected samples and the completion rate for the SNP was 100%. The distributions of genotypes and alleles for the polymorphism followed Hardy–Weinberg equilibrium. There was no significant association between *Egr-1* –4071 polymorphism with the diagnosis of AR as shown in Table 4. Significant

Table 3

The pattern of allergen sensitization as determined by plasma total and specific IgE assays.

Characteristic	AR patients	Controls	Odds ratio (95% CI) [‡]	P value
Plasma total IgE_{log}^*	1.78 ± 0.65 [†]	1.61 ± 0.67	NA	0.006
Atopy ($\geq$ 1 positive specific IgE)	105 (49)	71 (27)	2.55 (1.74–3.74)	<0.001
Positive specific IgE				
<i>D. pteronyssinus</i> (%)	103 (48)	68 (26)	2.61 (1.77–3.83)	<0.001
Cat (%)	16 (7)	9 (3)	2.25 (0.97–5.19)	0.053
Dog (%)	4 (2)	1 (0)	4.91 (0.55–44.30)	NS
Mixed cockroaches (%)	25 (12)	25 (10)	1.24 (0.69–2.23)	NS
Mixed moulds (%)	1 (1)	12 (5)	0.10 (0.01–0.75)	0.006

Abbreviation: CI, confidence interval; IgE_{log} , log-transformed total IgE; NA, not applicable; NS, not significant.

*Expressed as mean ± SD.

[†]Comparison made by Student's t test.[‡]Odds ratios were calculated by χ^2 .**Table 4**Association between AR phenotypes and –4071 genotypes in the *Egr-1* gene.

Phenotypes of AR	–4071 genotypes				P value [†]
	n	AA	AG	GG	
All physician-diagnosed AR patients*	211	147 (70)	58 (27)	6 (3)	
With control adults	259	171 (66)	82 (32)	6 (2)	NS
Atopic patients [‡]	103	79 (77)	21 (20)	3 (3)	
With non-atopic patients	108	68 (63)	37 (34)	3 (3)	0.030 [‡]
With non-atopic controls	188	126 (67)	58 (31)	4 (2)	NS
Patients of increased total IgE [§]	54	44 (81)	10 (19)	0	
With patients of normal total IgE	157	103 (66)	48 (31)	6 (4)	0.029 [‡]

Abbreviation: NS, not significant.

*Three patients without DNA sample.

[‡]Atopy defined as $\geq$ 1 positive result for allergen-specific IgE. Since only 6 patients and 6 controls possessed the GG genotype of –4071 had allergen-specific IgE data available, AG and GG genotypes were combined into a single category for the statistical comparisons.[†]Comparison made by Pearson χ^2 test.[§]Odds ratio (95% confidence interval) of A vs G for atopy was 1.94 (1.06–3.53); Odds ratio (95% confidence interval) of A vs G for increased plasma total IgE was 2.31 (1.08–4.94).

association was found between –4071 A→G and atopy ($p=0.030$, Table 4) among patients. Patients homozygous for the A allele of –4071 A→G had a 1.9-fold (95% CI: 1.06–3.53) risk of having atopy than those with the G allele. Besides, significant correlations were also found between increased plasma total IgE concentration and –4071 A→G in AR patients ($p=0.028$) but not in controls ($p=0.969$). Patients homozygous for the A allele of –4071 A→G had a 2.3-fold (95% CI: 1.08–4.94; $p=0.029$; Table 4) risk of having increased plasma total IgE than those with the G allele. Multivariate logistic stepwise regression analysis confirmed the significant association between –4071 A→G polymorphism and increased plasma total IgE concentration with a regression coefficient of 0.389 (95% CI: 0.163–0.930; $p=0.034$) in AR patients after adjustment for age and gender. We could not find any significant association between this polymorphism with disease's severity nor classification.

4.4. Association of *Egr-1* mRNA expression with AR and clinical characteristics

Expression of mRNA for *Egr-1* in peripheral blood was observed in >98% of the samples. Values of $2^{-\Delta\Delta CT}$ were summarized in Table 5. The expression of *Egr-1* was significantly higher in AR patients than in non-allergic healthy controls (median of 0.23 vs 0.15 fold *GAPDH* expression; $p<0.001$). Patients with persistent or moderate–severe symptoms had a significantly higher level of *Egr-1* transcripts than controls ($p<0.001$) but not to those patients with intermittent or mild symptoms. However, there was no significant difference in *Egr-1* expression with disease's classification or severity. *Egr-1* expression was not associated with anti-rhinitis treatments, –4071 polymorphism, plasma total IgE nor specific IgEs (data not shown).

Table 5Association of *Egr-1* mRNA expression with clinical characteristics.

Characteristics	<i>Egr-1</i> expression ($2^{-\Delta\Delta CT}$) [†]	P value [‡]
Controls* (n = 257)	0.15 (0.07–0.28)	
With all rhinitis patients* (n = 208)	0.23 (0.09–0.57)	<0.001
With intermittent patients (n = 79)	0.17 (0.07–0.61)	NS
With persistent patients (n = 129)	0.26 (0.11–0.57)	<0.001
With mild patients (n = 62)	0.21 (0.05–0.44)	NS
With moderate–severe patients (n = 146)	0.26 (0.09–0.59)	<0.001

Abbreviation: NS, not significant.

*Six patients and 2 controls were excluded as they did not have *Egr-1* mRNA expression data.[†]Expressed as median (interquartile range).[‡]Kruskal–Wallis test was used for comparing *Egr-1* expression in all groups ($P<0.001$) while subgroup analyses were followed by Dunn's multiple comparison test.

5. Discussion

Our study demonstrated that the mRNA expression of *Egr-1* in peripheral blood was significantly higher in AR patients than non-allergic healthy controls. Patients with persistent or moderate–severe symptoms had a significantly higher level of *Egr-1* transcripts than controls, but not to those patients with intermittent or mild symptoms. *Egr-1* mRNA expression may be affected by presence of certain specific cells such as eosinophils in AR patients. To overcome this problem, we had the *Egr-1* mRNA expression signal normalized by constitutively expressed housekeeping gene, *GAPDH* and reported the results as n-fold difference relative to that of *GAPDH* mRNA in whole blood. Elevated expression of *Egr-1* had been found in emphysema [23], atherosclerosis [24] and chronic obstructive pulmonary disease (COPD) [25]. *Egr-1* expression was especially evident in smooth muscles of bronchial and vascular walls, in alveolar macrophage, and some vascular endothelium [23]. Hjoberg et al. [15] found that PDGF induced *Egr-1* in human airway smooth muscle through a signal transduction pathway involving extracellular signal-regulated kinase (ERK) 1/2. They speculated that *Egr-1* would be increased in airway smooth muscle of asthmatic patients compared with non-asthmatic controls. Though AR is an inflammatory disease of the upper airway tissue while asthma is an inflammatory medical condition of the lower airways [26], we found that elevated expression of *Egr-1* in AR demonstrating the importance of *Egr-1* in allergy.

We found that patients with persistent or moderate–severe AR symptoms had a significantly higher level of *Egr-1* expression than controls, but not to those patients with intermittent or mild symptoms. The classification of allergic rhinitis determines the necessary treatment [27]. Most of our AR patients were old cases and if under successful treatment, their initial class of persistent and/or moderate–severe may change to intermittent and/or mild. That means those in the groups of intermittent and/or mild may consist of initial intermittent and/or mild cases, and those changed from persistent and/or moderate–severe after treatments. Therefore, the groups of intermittent and mild may not be homogeneous in this situation. Thus, we could not deduce the role of *Egr-1* as AR causing or modifying here. However, Zhang et al. [24] found that treatment of cultured lung-derived human fibroblasts and bronchial and pulmonary artery smooth muscle cells with a cocktail of cytokines including IL-1 α , TNF- α , IFN- γ and TGF- β 1 resulted in enhanced *Egr-1* transcripts. These data together with our findings suggest the relevance of examining the contribution of *Egr-1* to the biology of tissue remodeling and active airway destruction.

Although we could not find any correlation between *Egr-1* mRNA with plasma total IgE level in this study, Li et al. [17] suggested that *de novo* synthesis of *Egr-1* was required for the full responsiveness of mast cells to produce TNF and IL-13 upon IgE and antigen stimulation. Besides, Silverman et al. [16] reported that the *Egr-1* knock-out mice had increased IgE concentrations at baseline and after allergen challenge compared with wild type mice. It is possible that *Egr-1* directly alters immunoglobulin gene transcription or isotype switching, or regulates these events indirectly through the regulation of other genes. Li et al. [17] demonstrated that IgE and antigen stimulation induced a rapid expression of *Egr-1* mRNA in mouse bone marrow-derived mast cells. They also found that high-affinity IgE receptor (Fc ϵ RI)-induced TNF- α and IL13 expression was reduced at both mRNA and protein levels in *Egr-1* deficient mast cells when compared with that in wild-type mast cells. Their data suggested that Fc ϵ RI aggregation induces the new synthesis of *Egr-1*, which in turn amplifies TNF- α and IL13 gene expression by mast cells.

In this study, we also found the significant association of *Egr-1* polymorphism with increased plasma total IgE and atopy. This is not surprising as Marsh et al. reported a relationship between markers in 5q31.1 and the presence of elevated serum IgE antibody concentration [28]. It happens that *Egr-1* is also a gene located in the “cytokine-gene cluster” (q31–33) of chromosome 5. Besides, in our previous study of

childhood asthma, we also first reported the association of *Egr-1* polymorphism with plasma total IgE and atopy. The only difference was that in adults with AR, patients homozygous for the A allele had a 2.3-fold of increased plasma total IgE concentration and a 1.9-fold risk of having atopy than those G allele carriers. But in childhood asthma, children homozygous for the A allele had a lower plasma total IgE level and risk of having atopy than those G allele carriers. Reasons for the discrepant result may due to different pathophysiology of AR in the upper airways while asthma is inflammation in the lower airways. Moreover, there may be pleiotropic effects of *Egr-1* at different stages or etiologies of disease. Indeed, Srivastava et al. [29] found an age-specific association of CCR5 in asthma. It may be erroneous to assume that allergy across a wide range of ages, especially in the transition from childhood to adulthood. The contribution of different candidate genes to different allergy phenotypes may therefore change with advancing maturity. Furthermore, there are atopic candidate genes such as *IL-4* and *IL-13* [30] on chromosome 5 that could be in linkage disequilibrium with *Egr-1* and explain the association.

A major drawback of this study is that our AR patients were selected from a hospital cohort and this approach generally identified a more severe group of patients compared with the general population as shown by several epidemiological studies [31,32]. Leung et al. [32] showed in a study involving 1062 schoolchildren in Hong Kong that 58% of the subjects were atopic as defined using skin prick test. In our study, atopy was found in 48% of AR patients and 26% of age-matched controls. Although our findings may not be generalized to the general population, this study does suggest that –4071 A $\rightarrow$ G polymorphism in the *Egr-1* gene may be important predictors of plasma total IgE and atopy in Chinese AR adults.

In summary, although the role of *Egr-1* in conferring AR susceptibility remains obscure, our study suggests that there is a clinical correlation between *Egr-1* mRNA expression, –4071 A $\rightarrow$ G polymorphism and an increased risk of developing atopy in Chinese AR adults. This may also help to explore a possible role for the pharmacogenetics of *Egr-1* inhibitors in anti-rhinitis therapy.

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