LETTER TO JMG

Variation in dinucleotide (GT) repeat sequence in the first exon of the *STAT6* gene is associated with atopic asthma and differentially regulates the promoter activity in vitro

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Upregulation of the IL-4/IL-13 mediated Th2 response is a characteristic of allergic diseases such as asthma, a common and often debilitating disease.[1] STAT6 is a critical signalling molecule in the Th2 signalling pathway, and mice lacking STAT6 are protected from allergic pulmonary manifestations.[2] The importance of STAT6 in asthma is also evident from studies showing that *STAT6* gene expression is markedly upregulated in airway epithelial cells in asthma.[3] STAT6 is important in the expression of VCAM-1 in endothelial cells and of chemokines, such as eotaxin, in epithelial cells following stimulation with IL-4 and IL-13.[4] As a consequence, *STAT6* has been considered a strong candidate for predisposition to atopic asthma. Indeed, the human *STAT6* gene is mapped to chromosome 12q13.3−q14.1, a region linked to total serum IgE concentration and atopy in several populations.[5] A number of common polymorphisms have been identified, including a GT repeat in exon 1 and three common SNPs (G4219A, A4491G, and A4671G; GenBank AF067575) in the 3′ untranslated region of the human *STAT6* gene.[6–9] Although all four of these polymorphisms have been shown to be associated with allergic phenotypes in various populations, their functional relevance remains unclear.

Dinucleotide repeats are the most frequent of the simple repeats distributed throughout the human genome, and many of these exhibit length polymorphisms. Investigations into the effect of dinucleotide repeats on gene expression have shown enhanced[10] or decreased transcriptional activity[11 12] in the context of different genes and cell types. These regulatory effects have been proposed to be due, in part, to the fact that dinucleotide repeats have potential to form alternative DNA structures, such as Z-, H- and cruciform DNA.[13] The Z-DNA sequences in human genes tend to be located near transcription sites, which makes it possible that they play a role in the transcriptional control of genes. Because of their close proximity to the transcription start site, we first conducted a case−control association analysis of the GT repeat variants and atopic phenotypes in a white population, and then investigated their potential functional effects on the regulation of the activity of the *STAT6* promoter. We report, for the first time, that the GT_{13} repeat variant in exon 1 of *STAT6* is associated with asthma and total serum IgE level in a white study population, and that variation in the length of repeat variants differentially regulates the promoter activity in vitro.

Key points

- STAT6 is critical in Th2 cytokine signalling. Multiple sequence variants of the *STAT6* gene have been identified, some of which are associated with atopic phenotypes in diverse populations.
- Seven dinucleotide GT repeat variants were identified in the noncoding exon 1 of *STAT6*. Case−control association analysis of 214 white British subjects demonstrated significant association with asthma of an allele with a 13GT repeat sequence (GT_{13}) (OR 1.52, 95% confidence interval 1.02 to 2.28, p = 0.027), whereas the GT_{16} allele showed an inverse association with asthma (p = 0.018). Furthermore, individuals with the GT_{13} allele had a higher mean (SD) level of geometric mean IgE compared with individuals with the GT_{16} allele (480.04 (90.46) kU/l, n = 119 *v* 106.14 (46.12) kU/l, n = 22; p = 0.004).
- Transient transfection assays of different alleles revealed significantly higher transcriptional activity with the GT_{13} allele compared with the GT_{16} allele in Jurkat, HMC-1, and BEAS-2B cell lines.
- Gel shift assays demonstrated a binding complex when six GT alleles were used as a probe. The GT_{13} allele had significantly decreased binding stability compared with the GT_{16} allele in a reciprocal competitive assay.
- These findings suggest that the GT repeat polymorphism of the *STAT6* gene contributes to susceptibility to atopic asthma and total serum IgE levels, and that variation in the length of the GT repeat sequence influences the regulation of promoter activity.

MATERIALS AND METHODS

Study population and genotyping

Fifteen randomly selected white American and 214 white British subjects were studied. Details of the British subject selection have been previously described.[6] All the asthmatic subjects had physician diagnosed asthma with (*a*) recurrent breathlessness and chest tightness requiring ongoing treatment, (*b*) documented wheeze, and (*c*) documented labile airflow obstruction with variability in serial peak expiratory flow rates of >30%. Atopy was defined as a positive skin prick test of >5 mm and specific IgE (>3.5 kU/l) against either of the aeroallergens house dust mite or grass pollen. Measurement of total serum IgE was carried out by the CAP ELISA system (Pharmacia, Uppsala, Sweden). DNA samples were extracted using a commercial kit (IsoQuick, Microprobe Corp, Garden Grove, USA), and subjected to PCR amplification of base pairs −72 to +115 (relative to the transcription start site; GenBank AF067575) of the *STAT6* gene. Primers pairs 5′-TCTTCTCTGTGTCGTCAGAG and 5′-CAACACCACCACCACCCCGA were used to amplify the fragment, which was then genotyped by a laser based 377 automated sequencer and GeneScan software (Applied Biosystems).

Reporter gene constructs, transient transfection, and luciferase assay

A genomic DNA fragment (nucleotides −702 to +169; relative to the transcriptional initiation site) containing the GT repeat region in exon 1 of the human *STAT6* gene was amplified and cloned into a TA vector (Invitrogen, San Diego, CA, USA). The reporter constructs used were generated by subcloning the sequences containing GT repeats into the *Hin*dIII site of the pGL2 basic vector with luciferase (Promega, Madison, WI, USA), and the single copy sequence and orientation of each reporter constructs were confirmed by sequencing analysis. Three different human cell lines were used in this study: HMC-1 (a mast cell line), Jurkat (a T cell line) and BEAS-2B (a bronchial epithelial cell line). Cells were transfected with 2 μg of reporter gene constructs or pGL2 basic vector without insertion using SuperFect (Qiagen, Santa Clarita, CA, USA) for HMC-1 and Jurkat cells, and FuGENE 6 for BEAS-2B cells (Qiagen) according to the manufacturer's instructions. Transfections were always performed in duplicate. The luciferase assay used has been described previously.[14]

Electrophoretic mobility shift assay

Preparations of nuclear extracts and DNA binding conditions used for gel mobility shift assays were performed as described previously.[14] The oligonucleotide sequences were: sense 5′-GAGAGGA$(GT)_n$TATGTA, antisense 5′-ATACAT$(AC)_n$TCCTCT, where n ranged from 12 to 17. The complementary oligonucleotides were annealed, and the double stranded oligonucleotides were labelled with [γ-^{32}p]-dATP. Total protein content was determined with Bradford protein assays (Bio-Rad, Hercules, CA, USA). In the absence or presence of varying amounts of unlabelled oligonucleotides (competitor), 20 μg of nuclear proteins were incubated for 20 minutes. Reactions were then incubated with 0.2 ng of GT repeat probes end labelled with [γ-^{32}P] ATP. The complexes were resolved on a nondenaturing 5% polyacrylamide gel (acrylamide:bis-acrylamide 30:1), dried, and subjected to autoradiography. Densitometric scanning of the autoradiographs was also carried out. Each binding was assessed by the ratio of B/F+B after measuring protein−DNA complex (B) and free probe signal (F), and normalised by the control without unlabelled competitor, which was defined as 100%. IC_{50} was defined as the inhibition concentration required to achieve a reduction of 50% of DNA−protein binding.

Statistical analysis

Allele frequency, odds ratios, 95% confidence intervals, and significance values were estimated using SPSS (version 10; SPSS Inc, Chicago, IL, USA). Luciferase activities are expressed as mean (SEM). Differences in the mean luciferase activity for different dinucleotide repeat constructs were compared by using one way analysis of variance. A p value <0.05 was considered significant.

RESULTS

Association of the GT repeats with atopic phenotypes in a British population

GT repeats in exon 1 of *STAT6* were genotyped in 15 unrelated white American and 214 white British individuals using a GeneScan assay. Seven distinct alleles were identified in these 229 subjects, ranging in size from GT_{12} to GT_{17} in the American, and GT_{13} to GT_{18} in the British subjects. Neither GT_{12} nor GT_{18} alleles have been previously found in studies of Japanese[7] and German[8] populations. GT_{13} and GT_{15} appeared to be more frequent in this population than other variants, consistent with the previous reports of Duetsch *et al*, who demonstrated 40.1% GT_{13} and 43.5% GT_{15} in a white population in Germany.[8]

The results of genotyping for the subjects with different phenotypes are presented in table 1. Among all the alleles, the GT_{13} repeat allelic frequency was significantly increased in the atopic asthmatic subjects compared with healthy controls (43% *v* 33%, p = 0.027, OR 1.52, 95% confidence interval (CI) 1.02 to 2.28). In contrast, the GT_{16} allelic frequency was significantly decreased in cases compared with controls (4% *v* 11%, p = 0.018, OR 0.39, 95% CI 0.17 to 0.92). No significant association with these alleles was found when atopy was analysed. A significant association of the GT_{13} allele with the IgE level was demonstrated by comparing the difference in the geometric mean level of total IgE between GT_{13} and GT_{16} alleles (fig 1). Individuals with the GT_{13} allele had a higher level of IgE compared with individuals with the GT_{16} allele (geometric mean (SD) IgE 480.04 (90.46) kU/l, n = 119 *v* 106.14 (6.12) kU/l, n = 22; p = 0.004). A 10 fold difference in geometric mean level of total serum IgE was noted between subjects with homozygous GT_{13} and GT_{16}, but no statistical comparison could be made because of the small number (n = 3) of homozygous GT_{16} available in this population.

Dinucleotide repeat (GT) modulates the promoter activity

To examine the relationship between the length of the dinucleotide repeat and promoter activity, reporter gene assays were performed using luciferase constructs containing two short GT repeats (GT_{12} and GT_{13}) and two long alleles (GT_{16} and GT_{17}). A GT_{18} variant of reporter was not constructed because the frequency of the GT_{18} allele was low (<0.005) in the study populations. The results showed that while the relative transcriptional activity varied in BEAS-2B, Jurkat, and HMC-1 cells, the GT_{13} allele resulted in significantly higher transcriptional activity compared with the other three alleles in all three cell lines tested (fig 2). Most impressively, a mean fivefold greater transcriptional activity was observed in BEAS-2B cells (fig 2A) using the

Table 1 Association of GT repeat alleles with atopy and atopic asthma in the British population

	No. of subjects (%)				No. of subjects (%)			
Allele	Non-atopic	Atopic	OR (95% CI)	P value	Non-atopic asthma	Atopic asthma	OR (95% CI)	P value
GT13	63 (35.2)	94 (37.9)	1.14 (0.76 to 1.69)	0.304	90 (33.2)	67 (43.0)	1.52 (1.02 to 2.28)	0.027*
GT14	8 (4.5)	8 (3.2)	0.71 (0.27 to 1.96)	0.342	11 (4.0)	5 (3.2)	0.79 (0.23 to 2.33)	0.439
GT15	80 (44.7)	116 (46.8)	1.10 (0.75 to 1.61)	0.352	128 (47.2)	68 (43.6)	0.87 (0.58 to 1.30)	0.277
GT16	19 (10.6)	17 (6.9)	0.63 (0.31 to 1.23)	0.118	29 (10.7)	7 (4.4)	0.39 (0.17 to 0.92)	0.018*
GT17	9 (5.0)	13 (5.2)	1.05 (0.44 to 2.50)	0.548	13 (4.8)	9 (5.8)	1.22 (0.51 to 2.94)	0.407

Five common alleles in this study population are shown.
P value for frequency differences of each allele *v* overall; *p<0.05 was considered significant.
OR (95% CI), odds ratio and 95% confidence interval.

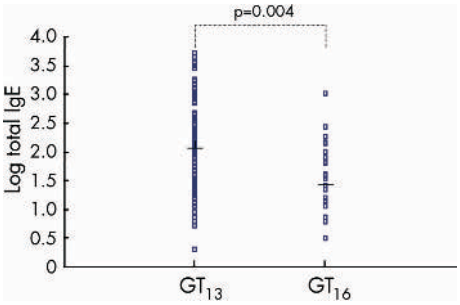

Figure 1 Distribution of total IgE levels in subjects with GT_{13} and GT_{16} alleles. Each point indicates the log transformed total IgE level for each individual. The number of samples: GT_{13} = 119, GT_{16} = 22. P value refers to the significance level comparing the geometric mean IgE levels of GT_{13} versus GT_{16}.

GT_{13} allele compared with the GT_{16} allele. The shortest allele, GT_{12}, showed consistently lower activity compared with the other three alleles in all three cell lines, demonstrating that the transcriptional activity was not proportional to the size of the repeat sequence. The two long repeats, GT_{16} and GT_{17}, demonstrated intermediate levels of transcriptional activity when all four alleles were compared. In HMC-1 cells, the GT_{17} allele showed significantly higher activity compared with GT_{12} and GT_{16}, while the level of transcriptional activity was still lower than that seen for GT_{13} allele (fig 2B). No significant change in transcriptional activity was observed in any of the cell lines following activation with ionomycin (data not shown).

Dinucleotide repeats bind to nuclear protein(s)

To determine whether the differential promoter activity is due to the difference in transcription factor binding, gel shift assays were performed to investigate the binding patterns of nuclear proteins derived from different cell lines to the GT repeat. A protein−DNA complex formation was observed when all the GT repeat probes (GT_{12}, GT_{13}, GT_{14}, GT_{15}, GT_{16}, and GT_{17}) were examined (fig 3A). For instance, the GT_{12}−protein complex was efficiently inhibited in a concentration dependent manner by the presence of excess unlabelled GT_{12}, but not by a non-specific probe, RANTES (fig 3B). To investigate the difference in DNA—protein binding between GT_{13} and GT_{16}, a reciprocal gel shift competitive assay was performed, and the relative amounts of GT_{16} and GT_{13} binding complex formation in the presence of reciprocal GT_{13} and GT_{16} competitors were assessed (fig 4A). The results showed that IC_{50} for GT_{13} is 10 fold higher than that for GT_{16} (fig 4B), suggesting a relatively more stable binding of GT_{16} to the putative nuclear factor(s). Additional reciprocal gel shift competitive assays were also conducted for the shorter GT repeat allele, GT_{12}, and the longer one, GT_{17} (fig 4C), which gave similar results; IC_{50} for GT_{12} is 12 fold higher than that for GT_{17} (fig 4D). These results imply that the longer GT repeat allele may have a relatively more stable binding to the putative nuclear factor(s) compared with the shorter repeat. Similar results were obtained from assays of nuclear extracts from Jurkat cells (data not shown). Together, the results suggested that the stability of the binding between the GT repeat sequences and protein(s) required for the transcriptional regulation may partially contribute to the functional alteration of *STAT6* transcription.

DISCUSSION

Polymorphic dinuleotide repeats are common throughout the genome and widely used as genetic markers. Some polymorphic dinucleotide repeats have been shown to be functional in the regulation of gene transcription.[10–13] A total of seven alleles of the GT repeats in various populations were detected in the first exon of *STAT6*, ranging in size from 12 to 17 repeat units in white American, and 13 to 18 in white British subjects. The result is somewhat at variance with two previous reports,[7 9] implying that there are clear ethnic differences in allelic frequency of different GT repeats in diverse populations.

In this study, we provide evidence for a significant association of GT_{13} allele with atopic asthma in British whites. In addition, although the GT_{16} allele was not common, a significant association was found, which suggests a lower risk for atopic asthma. Consistent with this result, total serum IgE level, a major phenotypic marker of atopic asthma, was significantly higher for individuals with the GT_{13} allele compared with those with the GT_{16} allele. Of interest is the finding that a 10 fold difference in the geometric mean level of total serum IgE was seen between individuals with homozygous GT_{13} and GT_{16}, but because of the small number of subjects with homozygous GT_{16} in this population, no statistical comparison could be made. Taken together, the GT_{13} allele or homozygous genotype suggests an increased production of total serum IgE, and subsequently contributes to the development of atopic asthma.

As a corollary, the allele frequency of GT_{13} has been reported to be higher in Japanese children with allergic asthma compared with healthy controls.[7] Furthermore, the GT_{13} allele was found to be in significant linkage disequilibrium with a 3′-UTR SNP, G4291A, which has recently been reported to be associated with atopic asthma in another Japanese population.[8] However, in a study of German whites, the GT_{16} allele was determined to be significantly associated with an increased eosinophil cell count, although the significance of this finding is unclear.[9] Taken together, our results and published reports[7 8] suggest that the GT_{13} allele may be a strong predictor for atopic asthma. Further studies including more subjects and independent population are required to verify this genetic association.

Using reporter systems, the results from our present studies demonstrated that GT repeat alleles differentially regulate the constitutive promoter activity of the *STAT6* gene.

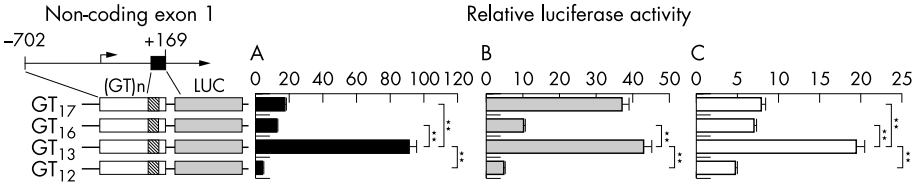

Figure 2 Transcriptional activities of reporter constructs containing each of the four GT repeat alleles in (A) BEAS-2B, (B) HMC-1, and (C) Jurkat cell lines. Cells (5×10^5/1.5 ml) were transiently transfected for 24 hours with each of the luciferase reporter constructs, pGL2 basic (negative control, not shown), GT_{12}, GT_{13}, GT_{16}, and GT_{17} (each *STAT6* promoter allele bearing base pairs −702 to +169 relative to the transcription start site of the human *STAT6* gene). Results are expressed as relative luciferase activity, the average value obtained from pGL2 basic transfected cells being taken as 1. Numbers reflect mean (SE) of five independent experiments. **p<0.001.

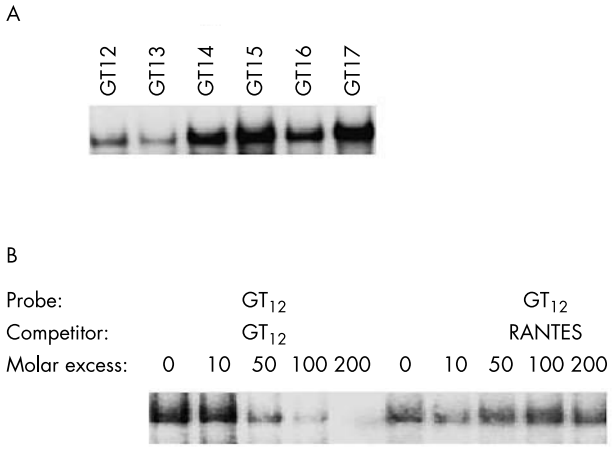

Figure 3 Binding of GT repeats to nuclear extracts. (A) ^{32}P-ATP labelled probes from GT_{12} to GT_{17} spanning the region from +126 to +169 bp were used in EMSA with nuclear extracts from HMC-1 cells; (B) 20 μg of nuclear extracts from HMC-1 cells were incubated with ^{32}P labelled GT_{12} in the absence or presence (10, 50, 100, and 200 fold excess) of cold competitor GT_{12} or a non-specific probe, RANTES.

In agreement with the data from the association analysis, the luciferase activity assays consistently showed that the GT_{13} allele has significantly increased promoter activity compared with the GT_{16} allele in the HMC-1, BEAS-2B, and Jurkat cells. These data are consistent with previously described findings showing allele size dependent modulation of transcriptional activity in vitro in several human genes encoding, for instance, human type I collagen α2,[15] human growth hormone receptor,[16] and the matrix metalloproteinase-9 enzyme.[17]

The effect of these repeats on promoter activity could be explained by the formation of non-B-form DNA confirmations that are involved in transcription, such as Z-, H- and cruciform DNA.[18] Moreover, these repeat sequences capable of forming non-B-form DNA are known to occur with high frequency in eukaryotic genomes, particularly around promoter regions, and have been shown to be involved in many biological events, such as DNA replication, site specific recombination, and transcription.[18] It has been shown by helical conformation analysis that the dinucleotide repeat is highly flexible, and that the longer the dinucleotide repeat, the longer the highly bendable section becomes.[19] Because the GT repeat in exon 1 of *STAT6* is in close proximity to the transcription start site, it is tempting to speculate that these GT repeat sequences may favour a DNA secondary structure, Z-DNA, which modulates the binding of transcription factors to the neighbouring transcriptional element. It is noted, however, that like other repeat sequences in a few genes,[12 16 20] the influence on the transcriptional activity is not proportional to the size of the repeat sequences (fig 2). In addition, the level of transcriptional activity varies in different cell types, suggesting a cell type specific regulation and a complex modulatory mechanism.

Of interest, our experiments showed a DNA–protein binding complex with a similar binding pattern of nuclear proteins to different GT repeat alleles using gel shift assay. However, reciprocal gel shift competitive assays demonstrated that GT_{16} competes with the formation of the binding complex more efficiently than GT_{13}, indicating that the GT_{13} allele has reduced stability compared with GT_{16}. Consistently, a longer repeat allele, GT_{17}, competes better than a shorter one, GT_{12}. It is postulated that these GT repeats may either bind to transcriptional inactivator(s) (silencers), thus modifying the gene expression, or form non-B-form DNA conformations that differentially modulate the binding of transcription factors to the neighbouring transcriptional element. Moreover, the GT_{12} allele showed a reduced binding stability but with a decreased transcriptional activity, which is distinct from that seen for the GT_{13} allele. We therefore hypothesised that the effects of these GT repeat alleles are more likely due to the combination of binding to suppressive element(s) with various non-B-form DNA conformations. In fact, it is probably far more complex than this. At present, the nature of the putative binding factor(s) to double stranded simple repetitive DNA remains to be defined. Therefore, better understanding of the regulatory mechanism of the GT repeat sequence in transcription and, perhaps, in RNA stability and translation, awaits the identification of the GT repeat binding protein(s).

In summary, the data in this report illustrate that GT_{13} and GT_{16} repeat alleles in exon 1 of the human *STAT6* gene may be crucial factors in the pathogenesis of atopic asthma and in the regulation of total serum IgE. We demonstrate that

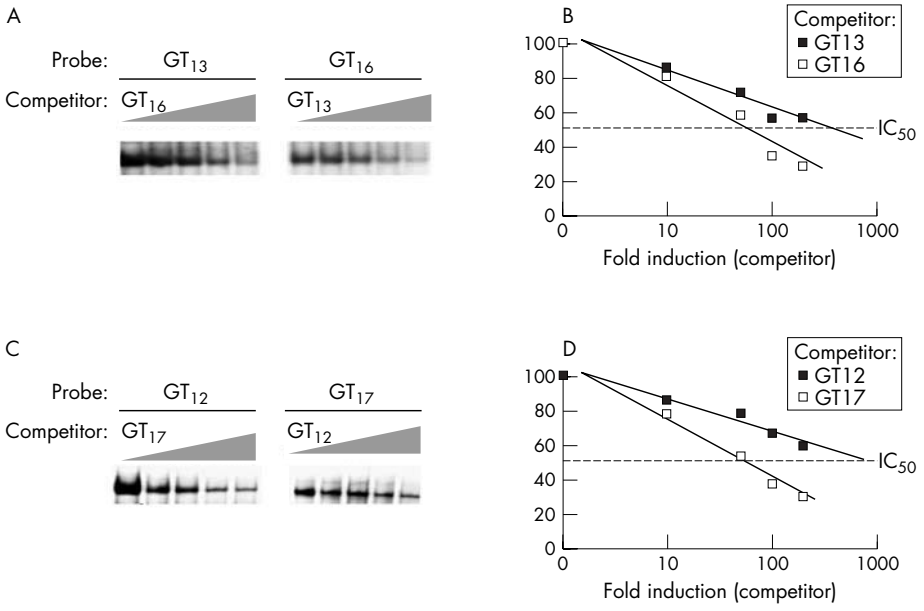

Figure 4 Autoradiograph of the gel shift competitive assay with reciprocal competitors. We incubated 20 μg of nuclear extracts from the HMC-1 cell line with ^{32}P-labelled GT_{13} and GT_{16}, or GT_{12} and GT_{17} in the absence or presence (10, 50, 100 or 200 fold) of unlabelled reciprocal probe, GT_{16} and GT_{13} (A), or GT_{12} and GT_{17} (C) (B, D). Quantification of the binding complex formation by scanning densitometry was presented by the intensity ratio of B/F+B and normalised by the control without competitor. IC_{50} was defined as the inhibition concentration required to achieve a reduction of 50% of DNA−protein binding. The graph represents the mean (SEM) of the values obtained from three independent experiments.

variation in the length of the GT repeat sequence contributes to the regulation of gene transcriptional activity. The greater promoter activity of reporter constructs containing the GT_{13} variant is in agreement with the association of this variant with both high IgE levels and allergic asthma, and suggests that the associations with IgE and disease may result from higher transcriptional activity of promoters containing the GT_{13} variant. Further experiments are needed to provide a more detailed view on the mechanisms of transcriptional activation exerted by the GT repeat. These results provide a basis for continued investigation of the association of inherited polymorphisms of *STAT6* with the pathogenesis of STAT6 mediated allergic diseases such as asthma.

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