Association of *STAT6* and *ADAM33* single nucleotide polymorphisms with asthma bronchiale and IgE level and its possible epigenetic background

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Background. *ADAM33* and *STAT6* belong to the candidate genes that have been commonly associated with asthma, bronchial hyperresponsiveness or IgE levels. Our objective was to assess the association of 11 SNPs of the *ADAM33* and 6 of the *STAT6* and their haplotypes with IgE levels and asthma. We also evaluated the possible role of parental origin of haplotypes on IgE levels.

Methods. We enrolled 109 children with asthma and 45 healthy controls. Genotyping was performed by TaqMan probes and confirmed by sequencing. Haplotype construction was based on the knowledge of parental genotypes and also inferred by using the EM algorithm and Bayes' theorem.

Results. None of the SNPs were associated with elevated IgE level or asthma. We found that the most frequent *STAT6* haplotype ATTCAA (built from rs324012, rs324011, rs841718, rs3024974, rs3024974, rs4559 SNPs, respectively) was associated with elevated total IgE levels ($P=0.01$) and this haplotype was predominantly transmitted paternally ($P<0.001$). We compared our results with those of studies performed on German and Australian Caucasian populations and found that rs324011, rs3024974 and rs4559 SNPs in *STAT6* should have a major effect on IgE levels. Therefore, we suggest the TCA haplotype alone (built from rs324011, rs3024974 and rs4559 SNPs, respectively) in *STAT6* is associated with total IgE elevation.

Conclusions. The influence of paternal origin of the *STAT6* haplotype on IgE levels is surprising but the exact role of possible paternal imprinting in *STAT6* regulation should be investigated and confirmed in future studies.

Key words: *ADAM33*, *STAT6*, IgE levels, asthma, SNP, parental haplotypes

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INTRODUCTION

Asthma is defined as a chronic inflammatory airway disease with recurrent bronchoconstriction caused by stimuli that would otherwise not cause airway narrowing in most individuals. Asthma is a syndrome characterized by airway obstruction that varies both spontaneously and as a result of therapy. The main pathogenic mechanism of bronchial asthma is a special form of chronic inflammation, with many types of cells, in particular eosinophils, and inflammatory mediators influencing the bronchial mucosa. Airway hyperresponsiveness (AHR) is known to be a major risk factor for the development of asthma and to be a surrogate of asthma severity with respect to symptomatic disease. AHR is also reported to be a marker for the subsequent development of asthma in patients with allergic rhinitis. AHR can be defined as an increased sensitivity of the airways to inhaled histamine or methacholine or to exposure to physical stimuli, such as exercise. AHR may represent more than a single entity. It may reflect a common physiological pathway of several mechanisms

leading to a lowered threshold of airway narrowing to bronchoconstrictor stimuli. AHR reflects, and is likely partially caused by, airway inflammation. Measurement of bronchial hyper-reactivity provides quantitative insights into variable airflow obstruction.

Asthma has an important genetic component and complex interactions between environment and multiple genes of small to modest effect give rise to the final disease phenotype. In the last decade, several candidate genes responsible for asthma development have been identified. Van Eerdewegh et al.¹ identified a locus on the short arm of chromosome 20 which was linked to asthma and bronchial hyperresponsiveness. *ADAM33*, a disintegrin and metalloproteinase 33, maps to this region (precisely at 20p13), contains 22 exons (Fig. 1) and was significantly associated with asthma. *ADAM33* belongs to a subgroup of metalloproteinases. These enzymes are involved in cell fusion, proteolysis, cell adhesion and cell signalling and shedding cell-surface cytokines, growth factors and receptors that are linked with inflammation and cell death events. *ADAM33* is expressed by lung fibroblasts and bron-

Fig. 1. Structure of the *STAT6* and *ADAM33*. Upper part shows structure of the *STAT6*, lower part shows *ADAM33* structure – there are exons and SNPs investigated in our study.

chial smooth-muscle cells¹ and according to the site specific expression, *ADAM33* is suggested to be responsible for hypertrophy of airway smooth muscles, subsequently leading to bronchial hyperresponsiveness and activation of the Th₂-mediated allergic reaction. The role of *ADAM33* in inducing myogenesis is supported by structural similarity with *ADAM12* (ref.³). The secreted form of *ADAM12* (*ADAM12-S*) was shown to provoke myogenesis in smooth muscle of airways⁴. Many of the *ADAM33* polymorphisms (SNPs) were studied and several of them were associated with asthma in some studies^{1,2} but not in others^{3,4}. Each of the SNPs probably contributes with their own small effect to overall asthma manifestation and so haplotype association studies are more promising. Eederweh et al. found 8 haplotypes (made of V4, V1, V-1, T1 and S1 SNPs) that were significantly overtransmitted to affected offspring at $P < 0.005$ level¹. Howard et al. evaluated association of *ADAM33* SNPs with asthma in different populations and found that no single haplotype accounted for asthma susceptibility risk across all of the studied groups. However, some of the haplotypes showed significant difference within studied populations. The GGCGTCCG haplotype in the Dutch population, GGAGTCCC haplotype in the US white (Caucasian) population and GCAATCAG in the US African American population were more common in the patients compared with those in the control group². An association study of three SNPs performed on the Chinese Han population didn't find any significant association between the SNPs or haplotypes in *ADAM33* with adult asthma⁵. In contrast, H1, H3, H5 and H8 haplotypes (made of F+1, T+1, T2, T1, V4, and Q-1 SNPs) were strongly associated with child asthma

in the Han Chinese population in northern China⁶. The AGCCT, GGCCT, AGACT, GCAGT, GGACT, ACCCC and AGACC haplotypes (made of F+1, S2, ST4, ST5 and V4 SNPs) were positively associated with asthma in the Indian adult population⁷. Many studies have shown linkage of the chromosomal locus 12q13-24 to asthma or associated phenotypes, such as elevated IgE level⁸⁻¹⁰. A signal transducer and activator of transcription 6 (*STAT6*), as one of the asthma candidate genes, was found in this region and some of the association studies focused on the *STAT6* SNPs have shown their linkage to IgE levels^{11,12}. Human *STAT6* is located on 12q13.3-14.1 and contains 23 exons¹³ (Fig. 1). The *STAT6* plays a crucial role in allergic response mediated by IL-4 and IL-13, because of its association with both IL-4 and IL-13 receptors¹⁴. First contact with an allergen leads to stimulation of specific Th₂ lymphocyte clones, they produce several cytokines such as IL-4, which stimulate B lymphocytes to produce IgE. IL-4 signals through two receptors – major IL-4 receptor (type I receptor) and type II receptor (also transmits IL-13 signal) (ref.¹⁵). IL-4 binds to the IL-4R α chain which is common for both of the receptors¹⁶. Next, this complex binds to the γ c chain and creates a complete, active receptor¹⁷. However, IL-4 receptor is unable to transmit a signal by itself, so JAK kinases are required. JAK1 and 3 associate with the IL-4R α and γ c receptor subunit, respectively^{18,19}. Binding of IL-4 to its receptor leads to phosphorylation and transphosphorylation of the associated JAK kinases, which in turn lead to phosphorylation of three Tyr residues Y575, Y603, and Y631 in IL-4R α chain^{20,21}. These phosphotyrosines provide docking sites for *STAT6* monomers, which in turn become phosphory-

Fig. 2. Total IgE levels in patient and control group.

Upper part: distribution of IgE levels. Lower part: box plot and summary statistics.

* Statistically significant difference between the groups (t-test on logarithmically transformed data)

lated by the associated JAK kinases. Activated *STAT6* monomers are able to form dimers and are transported to the nucleus, where they bind to highly conserved DNA motifs - TTC C/A NN G/T GAA and C/T TTC NNN (N) GAA G/A (ref.²²) - found in promoters of IL-4 and IL-13 inducible genes. This leads to Ig class switching and activation of genes responsible for IgE production. IgE production actually requires 2 signals. The first is the above described IL-4 or IL-13 mediated signaling, which promotes switch recombination by activating the *Cε* gene. The second is direct interaction of CD40 surface antigen on B cell with its ligand (CD40L) located on activated T cells. The latter signal leads to deletional switch recombination²³⁻²⁵. The IL-4 driven IgE synthesis is upregulated by IL-6, but IL-6 alone in combination with IL-4 alone are not sufficient to induce IgE production²⁶. Alternatively, IgE production could be regulated via IL-4 signaling in cooperation with CD58 of B cells with its ligand CD2. The CD58 driven IgE production is CD40 and IL-6 independent²⁷. Interaction between cell surface molecules is also an important costimulatory signal for IgE synthesis. The costimulatory signal provided by ligation of CD21, CD54 and CD58 on the B cell with their ligands up-regulates IgE production²⁷⁻³⁰. CD23 is another molecule regulated by factors increasing or decreasing IgE production. CD23 interacts with CD21 on B cells and antibodies to CD23 cause inhibition of IL-4 dependent IgE produc-

tion. Therefore the CD23/CD21 pair has been suggested to preferentially drive IgE production³¹. Another potent costimulant of IgE synthesis is CD86 of tonsillar B cells. CD86 is a ligand for CD28 expressed on T cells. CD86, together with IL-4 or IL-13, favors CD23-CD21 pairing and provides a selective and potent costimulus for the increase in IgE synthesis³². Contribution of *STAT6* SNPs to the risk of asthma or IgE level has been studied in several populations. The C2892T (rs324011) polymorphism in the second intron of *STAT6* has been repeatedly associated with elevated IgE in the German population¹¹. The T12888C (rs1059513) and A4671G (rs4559) have also showed a significant link with elevated IgE level in the German population^{11,12}. Nagarkatti et al.³³ identified and associated the CA repeating polymorphism (R1 locus) in the promoter region of *STAT6* with atopic asthma in the Indian population. This relationship between SNP and IgE has not been observed in the German population¹¹. As discussed for *ADAM33*, the effect of the *STAT6* SNPs should cumulate in their haplotypes. In the southwestern German population, the main haplotype GCTCAA (made from rs2598483, rs3001428, rs324011, rs3024974, rs1059513, rs4559 SNPs) was seen to significantly increase IgE level, GCCTAG haplotype was a significant risk for the development of specific sensitisation and haplotype ACCCAG was associated with an increased risk of asthma²¹. However, a combined German - Swedish

study assessing the effect of 13 SNPs in *STAT6* on asthma development or elevated IgE level didn't find any association of *STAT6* haplotypes with asthma or IgE level¹¹. The 17_15 and 16_15 haplotypes of CA repeat polymorphisms in R1 and R3 regions (respectively) of the *STAT6* promoter were found to be positively associated with asthma in the Indian population³³. Another study conducted on the German population found haplotype CGTGTT to be associated with elevated IgE (ref.¹²).

Environmental influences and epigenetic mechanisms could pose important modifying factors shaping the phenotype at a clinical level. Risk of asthma development has been associated with antenatal and childhood exposure to tobacco smoke^{34,35} mainly in individually predisposed persons such as carriers of the Arg16 allele in the β 2-adrenergic receptor gene³⁶. Intake of folic acid in supplement form was associated with increased risk of asthma in childhood (at 3.5 years) and with persistent

asthma³⁷. Conversely, maternal exposures to an environment rich in microbial compound may pose a protective effect³⁸. Methylation and histone acetylation belong to the important epigenetic mechanisms of gene expression. Increase in histone acyltransferase with decrease in histone deacetylase was observed in asthma³⁹. Furthermore, inhibition of methyltransferase increased expression of *STAT6* mRNA and protein rather than variants in exon 1 (ref.⁴⁰).

The aim of this study was to find out whether *STAT6* and *ADAM33* SNPs are associated with total IgE levels in our population. We performed genotyping of some previously reported polymorphisms in these genes and examined possible association of these polymorphisms and haplotypes with asthma or atopy in families with children who have asthma. In addition, we also evaluated the possible role of parental origin of haplotypes on total IgE levels.

Table 1. Frequencies of *ADAM33* SNPs. The first nucleotide in each SNP represents the major allele, thus homozygote 1 is homozygote for the major allele.

Patients (n=109)

SNP/ Genotypes	F+1 - G/A rs511898	L-1 - G/A rs2280092	S1 - G/A rs3918396	S2 - C/G rs528557	S+1 - A/T rs2853209	ST+4 - T/C rs44707	T1 - T/C rs2280091	T+1 - C/T rs2280089	V_3 - G/A rs628977	V4 - C/G rs2787094	V5 - A/G rs13527
Homozygote 1	0.29	0.63	0.86	0.45	0.37	0.39	0.61	0.62	0.73	0.60	0.87
Heterozygote	0.53	0.34	0.14	0.42	0.45	0.47	0.35	0.36	0.25	0.38	0.12
Homozygote 2	0.17	0.03	0.00	0.13	0.18	0.15	0.05	0.03	0.03	0.03	0.01
Alleles	G - A	G - A	G - A	C - G	A - T	T - C	T - C	C - T	G - A	C - G	A - G
Major allele	0.56	0.80	0.93	0.66	0.59	0.62	0.78	0.79	0.85	0.78	0.93
Minor allele	0.44	0.20	0.07	0.34	0.41	0.38	0.22	0.21	0.15	0.22	0.07

Control group (n=45)

SNP/ Genotypes	F+1 - G/A rs511898	L-1 - G/A rs2280092	S1 - G/A rs3918396	S2 - C/G rs528557	S+1 - A/T rs2853209	ST+4 - T/C rs44707	T1 - T/C rs2280091	T+1 - C/T rs2280089	V_3 - G/A rs628977	V4 - C/G rs2787094	V5 - A/G rs13527
Homozygote 1	0.33	0.69	0.80	0.47	0.31	0.42	0.69	0.60	0.76	0.60	0.91
Heterozygote	0.49	0.27	0.18	0.40	0.49	0.42	0.27	0.36	0.20	0.38	0.09
Homozygote 2	0.18	0.04	0.02	0.13	0.20	0.16	0.04	0.04	0.04	0.02	0.00
Alleles	G - A	G - A	G - A	C - G	A - T	T - C	T - C	C - T	G - A	C - G	A - G
Major allele	0.58	0.82	0.89	0.67	0.56	0.63	0.82	0.78	0.86	0.79	0.96
Minor allele	0.42	0.18	0.11	0.33	0.44	0.37	0.18	0.22	0.14	0.21	0.04

MATERIALS AND METHODS

Subjects

Our study was performed on a population of 109 children with bronchial asthma and their relatives (parents and sibs), overall 375 persons in 108 families, and 45 controls from the general population. The study was approved by the Palacky University Hospital Ethics Committee. The parents of all children, who participated in this study, gave their signed informed consent. The diagnosis of persistent mild atopic bronchial asthma (a positive skin prick test) was established, using the GINA 2002 guidelines, in 109 children (76 boys and 35 girls) aged 0.4–20 years (median 11 years), referred to the Department of Allergology of the Department of Paediatrics in 2003–2005. In all patients, atopic status was evaluated by skin prick test-

ing using common allergen extracts (grass and tree pollen, house dust mite, moulds, cat/dog extracts). IgE level was determined in both the patient and control group with a highly significant difference between the groups ($P < 0.01$, Fig. 2).

Genotyping and haplotyping

DNA samples were isolated from peripheral blood by standard salting out method. Genotypes in patients and their relatives and controls were identified by using allele discrimination/SNP's real time PCR. The probes for discrimination/SNP's real time PCR were obtained from the Applied Biosystem database of TaqMan SNP genotyping assays or designed by the manufacturer according to our specifications. The PCR reaction was carried out in overall volume of 10 μ l with these components: 6.28 μ l

Table 2. Frequencies of *STAT6* SNPs. The first nucleotide in each SNP represents the major allele, thus homozygote 1 is homozygote for the major allele.

Patients (n=109)

SNP/ Genotypes	IN2-SNP2 - G/A rs324012	IN2-SNP3 - C/T rs324011	IN17 - T/C rs841718	IN18 - C/T rs3024974	3UTR-SNP3 - A/G rs 1059513	3UTR-SNP4 - A/G rs4559
Homozygote 1	0.28	0.37	0.30	0.81	0.88	0.34
Heterozygote	0.48	0.42	0.53	0.18	0.11	0.54
Homozygote 2	0.25	0.21	0.17	0.01	0.01	0.12
Alleles	G - A	C - T	T - C	C - T	A - G	A - G
Major allele	0.51	0.58	0.57	0.90	0.94	0.61
Minor allele	0.49	0.42	0.43	0.10	0.06	0.39

Control group (n=45)

SNP/ Genotypes	IN2-SNP2 - G/A rs324012	IN2-SNP3 - C/T rs324011	IN17 - T/C rs841718	IN18 - C/T rs3024974	3UTR-SNP3 - A/G rs 1059513	3UTR-SNP4 - A/G rs4559
Homozygote 1	0.31	0.31	0.42	0.89	0.89	0.44
Heterozygote	0.53	0.56	0.47	0.11	0.11	0.51
Homozygote 2	0.16	0.13	0.11	0.00	0.00	0.04
Alleles	G - A	C - T	T - C	C - T	A - G	A - G
Major allele	0.58	0.59	0.66	0.94	0.94	0.70
Minor allele	0.42	0.41	0.34	0.06	0.06	0.30

Table 3. Frequency of *ADAM33* and *STAT6* haplotypes in individuals with determined haplotypes.

	Haplotype	Code	Patients [%]	Controls [%]	p-value
<i>ADAM33</i> n=140 haplotypes in patients n=20 haplotypes in controls	1111211111	a1	27.1	30.0	0.792
	1111121111	a2	22.9	25.0	0.783
	2212112211	a3	16.4	10.0	0.742
	1111121112		8.6	15.0	0.405
	2111121111		6.4	10.0	0.630
<i>STAT6</i> n=168 haplotypes in patients n=22 haplotypes in controls	221111	s1	39.9	50.0	0.368
	112112	s2	27.4	9.1	0.071
	111111	s3	11.9	4.5	0.477
	212212		4.8	0.0	0.600

ADAM33**STAT6**

1 – G	2 – A	1 – G	2 – A
1 – G	2 – A	1 – C	2 – T
1 – G	2 – A	1 – T	2 – C
1 – C	2 – G	1 – C	2 – T
1 – A	2 – T	1 – A	2 – G
1 – T	2 – C	1 – A	2 – G
1 – T	2 – C		
1 – C	2 – T		
1 – G	2 – A		
1 – C	2 – G		
1 – A	2 – G		

The table displays only haplotypes with a frequency rate > 4% (in patients). Legend shows alphanumerical code for haplotype denotation, 1 stands for major allele and 2 stands for minor allele of the studied SNPs. The haplotypes are made from F+1, L_1, S1, S2, S+1, ST+4, T1, T+1, V_3, V4 and V5 SNPs (respectively) in *ADAM33* and IN2-2, IN2-3, IN17, IN18, 3'UTR-3, 3'UTR-4 (respectively) in *STAT6*. Differences between patients and controls in haplotype frequencies were computed with Fisher's exact test (2 sided).

Table 4. Parental origin of s1 haplotype related to the difference in total IgE levels.

	At least one s1	Level of IgE [IU/ml]	Paternal s1	Level of IgE [IU/ml]	Maternal s1	Level of IgE [IU/ml]
All (N=84)	<i>Yes (59.5 %)</i>	612 (19; 2450)	<i>Yes (40.5 %)</i>	814 (39; 2590)	<i>Yes (39.3 %)</i>	642 (19; 2590)
	<i>No (40.5 %)</i>	309 (18; 1220)	<i>No (59.5 %)</i>	269 (18; 1150)	<i>No (60.7 %)</i>	390 (18; 1220)
	<i>P-value</i>	0.014	<i>P-value</i>	<0.001	<i>P-value</i>	0.218
Female (N=24)	<i>Yes (54.2 %)</i>	768 (54; 2800)	<i>Yes (37.5 %)</i>	923 (102; 2800)	<i>Yes (33.3 %)</i>	866 (54; 2800)
	<i>No (45.8 %)</i>	376 (18; 2060)	<i>No (62.5 %)</i>	388 (18; 2060)	<i>No (66.7 %)</i>	450 (18; 2060)
	<i>P-value</i>	0.022	<i>P-value</i>	0.018	<i>P-value</i>	0.106
Male (N=60)	<i>Yes (61.7 %)</i>	557 (19; 2450)	<i>Yes (41.7 %)</i>	774 (39; 2450)	<i>Yes (41.7 %)</i>	571 (19; 2450)
	<i>No (38.3 %)</i>	277 (34; 1150)	<i>No (58.3 %)</i>	218 (18; 1150)	<i>No (58.3 %)</i>	363 (19; 1220)
	<i>P-value</i>	0.191	<i>P-value</i>	<0.001	<i>P-value</i>	0.759

The table shows values of IgE of patients according to parental origin of s1 haplotype in males and females.

IgE: mean values (5th-95th percentiles)

P-value of Mann-Whitney U test comparing IgE levels in patients with and without haplotype s1

H₂O, 0.02 µl reference dye Rox 50X (Finnzymes), 4.0 µl DyNamo™ Probe qPCR Kit F-450S/L (Finnzymes), 0.2 µl SNP genotyping assay (Applied Biosystem) and 0.5 µl DNA (100 ng/µl). Allele discrimination PCR was run on Stratagene MxPro3005 thermal cycler under the following conditions: starting denaturation at 95 °C for 15 min and then 55 cycles including denaturation at 95 °C for 15 s followed by annealing/synthesis step at 60 °C for 1 min. The dRn values (baseline subtracted fluorescent reading normalized to the reference dye) were used to determine fluorescence c_T values. Difference in c_T values and shape of curves from fluorescence readings (dRn data) were used to determine genotypes.

Observed genotypes were confirmed by direct sequencing of the SNPs of interest in 15 patients and their relatives, overall 50 persons of 15 families. The sequencing reaction was carried out in a volume of 10 µl with reagents obtained from Applied Biosystems, reaction mixture contained: 2 µl of primer, 2 µl of PCR water (Top-Bio), 2 µl of purified PCR amplicons and 4 µl of ABI PRISM® Big Dye® Terminator v 3.1 (4-times diluted by 5x sequencing buffer). Sequence reaction ran under the following thermal conditions: denaturation at 94 °C for 2 min and 34 cycles of 96 °C for 10 s, with gradual 1 °C per 1s temperature decreasing to 50 °C, 50 °C for 5 s and 60 °C for 4 min. After the sequencing reaction and products purification (ethanol precipitation), capillary electrophoresis was performed on ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

Haplotypes and their parental origin were determined according to genotypes of patients' parents. Nonconclusive haplotypes were inferred by using expectation maximization algorithm modified by partition ligation mode⁴¹ and also using Bayes' theorem⁴².

Statistics

Hardy – Weinberg equilibrium (HWE) was tested with the χ^2 -test between patients and controls separately. Standard non-parametric statistics were used to describe primary data, i.e. absolute and relative frequencies, median and 10th-90th percentile range. ML- χ^2 test and Fisher's exact test were applied to compare differences among variants in categorical variables. Content of IgE as the only quantitative variable in the study revealed asymmetric sample distribution of log-normal type. Therefore, the data were log-transformed, $X_{tr} = \ln(X+1)$ and comparisons of groups (one-way ANOVA, t-test) were performed on the transformed data. The transformation proved effective in normalizing the IgE distribution (Shapiro-Wilk's test). The transformed IgE values revealed homogeneous variance in groups of patients and controls (Levene's test). Final assessment of the relationship between IgE levels and haplotypes was performed by non-parametric Mann-Whitney test using primary IgE data.

Table 5. Frequencies of the most common haplotypes in our study compared to results from two other studies on Caucasian population.

The haplotypes were constructed from rs324011, rs3024974, rs4559 SNPs (respectively) of *STAT6* SNPs. TCA = s1, CCG = s2, CCA = s3 haplotype.

Haplotype	Our population	Weidinger	Duetsch
TCA	43.68	37.51	42.82
CCG	26.84	26.00	15.87
CCA	20.52	25.04	23.30

RESULTS

Genotypes and allele frequencies of the studied genes *ADAM33* and *STAT6* SNPs were successfully determined in all studied patients, their relatives (parents and sibs) and in the control group (45 children) as well. All of the studied SNPs were in HWE in both patients and controls ($P>0.05$). We found some differences in allele frequencies of studied SNPs between patients and control group, but these differences didn't reach a level of statistical significance (Tables 1,2).

According to knowledge of parental genotypes in studied *ADAM33* and *STAT6* SNPs, we constructed haplotypes in patients. The haplotypes were successfully determined in 64.2% (N = 70) for *ADAM33* and in 77.1% (N = 84) for *STAT6* in the patient group and in 22.2% (N = 10) for *ADAM33* and in 24.4% (N = 11) for *STAT6* in the control group. For each of the studied genes we found three of the most common haplotypes (with frequency above 10.0% in patients); they were marked as a1-3 for *ADAM33* and s1-3 for *STAT6*, in descending order according to their frequency rate. The frequency of the most common haplotypes was not significantly different between patients and control group (Table 3). We were unable to analyze minor haplotypes, but their mild contribution (together as a group) to final asthma phenotype can't be ruled out.

We tested association of *ADAM33* and *STAT6* haplotypes with IgE levels. All of the three most common haplotypes (a1-3, s1-3) showed a tendency to be linked to increased IgE levels. Statistical significance was only reached in case of *STAT6* haplotypes (Fig. 3). An elevated level of IgE is linked mainly to s1 haplotype (*STAT6*) and moreover there were differences in s1 haplotype distribution according to parental origin. Seventy-three and one-half percent of patients with IgE levels > 200 IU/ml shared haplotype of paternal origin vs. 57.6% of patients with maternal origin of s1 haplotype. This relationship of s1 haplotype to total IgE levels was significant for haplotype of paternal origin ($P<0.01$) and marginally for haplotype from maternal line, too ($P=0.08$).

Fig. 3. *STAT6* haplotypes in relation to total IgE levels.

Upper part documents the relation of *STAT6* haplotypes to IgE levels and influence of maternal and paternal origin of haplotypes to IgE levels. Lower part describes the link of haplotypes to total IgE levels in presence of one certain haplotype (without consideration of its parental origin).

A, B/a, b: marks of homogeneous groups; groups marked by the same letter are not mutually statistically different ($P < 0.05$; pairwise multiple comparison with Bonferroni correction)

We performed an analysis of combined paternal and maternal haplotypes in an attempt to assess their contribution to IgE levels (Table 4). Patients with increased IgE levels shared at least one s1 haplotype ($P = 0.01$) and the subgroup of females with at least one s1 haplotype showed differences in IgE levels ($P = 0.02$). We subsequently analyzed the effect of parental origin of s1 haplotype on total IgE levels. The median value of total IgE was higher in patients with paternally transmitted s1 haplotype ($P < 0.01$, 618 IU/ml in patients with s1 haplotype versus 115 IU/ml without). The difference in total IgE distribution related to paternal s1 haplotype was also observed when evaluating males and females separately. Paternal origin of s1 haplotype showed a tendency to elevate IgE levels in males ($P < 0.001$), as well as in females ($P = 0.02$). Differences in IgE levels were not observed when evaluating transmission of s1 haplotype from maternal line. The other *STAT6* haplotypes also correlated with IgE levels, although their linkage is not as significant as for s1, and so the effects on IgE levels by them can't be distinguished.

ADAM33 haplotypes did not reveal such significant linkage to elevated IgE levels, as was seen in *STAT6* haplotypes. There was observed increased IgE variation among patients with particular *ADAM33* haplotypes, therefore haplotype effect on IgE levels can't be significantly determined. The a2 haplotype represents the only exception showing slightly elevated IgE levels, but without exact statistical significance ($P = 0.26$). Also analysis of combined *ADAM33* haplotypes did not reveal significant relationship with IgE levels.

DISCUSSION

In this study, we investigated an influence of 6 SNPs of *STAT6* on asthma and total IgE levels, all of them in intronic or UTR regions. Hence none of the SNPs reached significant level (Table 2), some of the SNPs (such as In2 - SNP3) were more often present in patients with asthma or with increased total IgE levels. This result was

not surprising because some of these SNPs (such as In2-SNP3, In17, In18, 3UTR-SNP4) were associated with elevated total IgE levels, or with decline in lung function (FEV₁) in some studies^{11,12,43} but not in others^{12,44}. This difference could be explained by different patient group size in the studies, however, international studies working with hundreds/thousands of asthma patients of Caucasian origin didn't find statistically significant differences in the distribution of investigated polymorphisms between the group of asthmatics and the control group^{43,44}. This fact points to the small contribution of the SNPs to the studied phenotype and possibly to individual differences in SNPs distribution inside the examined populations as well. The group size was relatively small but the number of subjects was sufficient to reach statistical significance, as seen also in similar studies^{11,45}. Frequencies of genotyped SNPs were similar to SNP frequencies reported in similar studies (In2-SNP3, In18 and 3UTR SNP4 polymorphism investigated in this study as well as in German studies), differences in allelic frequencies were up to 5% (ref.^{9,45}) and the allelic frequencies were also similar compared to the population of asthmatics in a study of 1500 subjects by Schedel et al.¹².

Haplotype analysis revealed that the most common *STAT6* haplotype – s1 haplotype (ATTCAA) – is significantly associated with the elevation of total IgE levels. To compare our results with published data, we focused on SNPs common to our and other (German) studies (In2-SNP3, In18 and 3UTR SNP4). We used these three SNPs to determine haplotypes – the frequencies of the most frequent haplotypes were almost the same as the frequencies

of haplotypes built from all examined SNPs, so we used the same names for these “refined” haplotypes (s1, s2 and s3). The same procedure was applied in published data. Although our population is different from the German one, we found that both SNPs frequencies and haplotype frequencies in our population were also similar to published German populations. Our s1 and s2 haplotypes had similar frequencies as the haplotypes no. 1 and 5 respectively published by Weidinger et al.⁴⁵ and haplotypes no. 1 and 3 published by Duetsch et al.¹¹ (Table 5). One small exception was s3 haplotype with greater difference in frequency in our and German studies.

In light of these findings, we suggest that the following SNPs have the greatest effect on IgE levels: rs324011, rs3024974, rs4559, respectively. Haplotype TCA built up from these SNPs is associated with IgE levels. Of these three SNPs, the first one is probably the most “potent” one, since Schedel et al. in 2004 (ref.¹²) proved its influence on elevated total IgE levels. They revealed two putative transcription factor binding (TFB) sites – nuclear factor κ B (NF- κ B) by computer analysis of the *STAT6* second intron sequence. Both the *STAT6* and the NF- κ B binding to target sequence is required to induce IgE production. The two TFB sites in the second intron are very close to each other and the T allele of rs324011 gives rise to the distal one. Sequence at the site of this SNP is strongly conserved among primates¹². In a functional study, Schedel et al.⁴⁶ confirmed that the T allele of rs324011 increases *STAT6* promoter activity by creating a new site for NF- κ B specific binding. In expression analysis, they discovered two new *STAT6* isoforms – *STAT6d* and *STAT6e*. Patients with asthma bearing the TT genotype in rs324011 had a mild, but not significant, increase of the known *STAT6* form and significantly increased level of *STAT6d* and *STAT6e* mRNA. However, the role of the two isoforms on IgE production is still unclear. According to their results, they predicted physical interaction of intron 2 with the promoter region and subsequent change of the *STAT6* 3D structure or its epigenetic signature caused by NF- κ B binding⁴⁶.

In the last years, researchers pointed out that environmental and epigenetic factors have an important impact on the final atopy or asthma phenotype⁴⁷. The main epigenetic mechanisms are histone acetylation and DNA methylation^{47,48}. For example, the DNA methylation has been implicated in Th2 cell differentiation. Hence the third CpG residue in *rad50* hypersensitive site 7 (RHS7) of the Th2 LCR (locus control region) in naïve T cells were almost fully methylated (96%), the Th2 cells showed complete demethylation in the RHS7 and this occurred in a *STAT6*-dependent manner⁴⁹.

The parental origin of atopy or asthma has been discussed for a long time. Reduced risk of asthma and AHR in children has been associated with Val105 polymorphism of GSTP1 (glutathione S-transferase) in the mother⁵⁰. Transmission of atopy from maternal line was observed for locus 11q13 (ref.⁵¹). Infants with elevated cord blood IgE had a higher risk of developing asthma and elevated IgE in cord blood was associated with mater-

Table 6. Frequencies of the most frequent *ADAM33* haplotypes in patient groups from our and German⁵⁹ studies. The haplotypes are built from SNPs common for the studies: F+1, S1, S2, ST+4, T1 and V4.

Haplotype	German Caucasian	Czech Caucasian
GGCTTC	31.59	23.16
GGCCTC	16.63	20.00
AGGTCC	14.00	14.74
GGCCTG	11.22	7.89

Table 7. Frequencies of the most frequent *ADAM33* haplotypes in patient groups from our and Australian⁵⁸ studies. The haplotypes are built from SNPs common for the studies: F+1, S1, ST+4, V4 and V5.

Haplotype	Australian	Czech
GGTCA	34.00	27.1
GGCCA	17.00	23.6
AGTCA	13.00	20.0
GGCGA	10.00	8.6

nal atopy (ref.⁵²). At the present time the maternal influence is believed to have the greatest influence on atopy or asthma development. In our study, we also attempted to examine the role of parental origin of *STAT6* haplotypes on total IgE levels in the patients. Surprisingly, we observed that s1 haplotype (associated with elevation of total IgE levels) was significantly more often transmitted from the paternal line. The mean IgE value in asthma patients was slightly higher in females. The paternally transmitted s1 haplotype showed a higher tendency to increase the total IgE level than maternally inherited haplotype and this effect was stronger in males (Table 4). This different quantity of haplotype effect according to parental origin may be due to a distinct imprinting pattern of the alleles. Some genes are able to fully or partially escape epigenetic reprogramming during prenatal development^{47,53} and so they can preserve their special DNA methylation pattern. Moreover, the specific epigenetic pattern can be transmitted to the descendant, in other words the epigenetic changes may be transmitted transgenerationally^{53,54}. These processes could at least partially explain the difference in total IgE levels. We hypothesize that paternally inherited s1 haplotype by itself confers greater risk to the elevation of total IgE. This effect is modulated *in utero* by the mother, as e.g. maternal behaviour and diet could influence epigenetic programming of the fetus⁵³. The gender related difference in distribution of total IgE levels would probably be attributed to other loci than *STAT6* as suggested by some reports^{50,55-57}. Although the observed s1 parental origin related to the difference in total IgE levels according to gender is interesting, the size of subgroups (patients were subsequently divided according to their gender) is relatively small and the observed results must be evaluated to rule out an influence of small numbers.

We attempted to assess the possibility of association of 11 SNPs in *ADAM33* with atopy or asthma in our population. We observed differences in SNP distribution between asthmatics and control group, but they were not significant. We compared allele frequencies and frequencies of the most frequent haplotypes (constructed from SNP common for evaluated studies) from our study with published data from Caucasian populations^{58,59} and we found that the frequencies of SNPs were similar (data not shown). As seen for *STAT6* haplotypes, some of the *ADAM33* haplotypes had a greater frequency difference in our and the two studies (Tables 6,7) (ref.^{58,59}). The haplotype analysis didn't find any association with asthma. Three major haplotypes (named a1-3) showed a relation to elevated total IgE levels, but without statistical significance. The evidence may reflect the role of *ADAM33* in intercellular adhesion because its disintegrin domain supports $\alpha 9\beta 1$ – integrin dependent leukocyte adhesion⁶⁰ and expression of integrin in mesenchymal cells (such as fibroblasts and smooth muscle cells) (ref.⁶¹). The combinations of SNP genotypes in haplotypes could influence disintegrin domain function in intercellular adhesion and therefore possibly partially influence the quantity of leukocytes (and thus the inflammation reaction itself) in airways. This observation points to the fact that *ADAM33* has an important role in the development of non-atopic asthma.

However, studies with a large size of investigated subjects (at least thousands) and meta-analysis are required to confirm the exact role of *ADAM33* SNP and haplotype role in disease development.

The investigated genes are part of a signalling pathway or are connected onto greater pathways in which they are complexly interacting together. Undermaa et al.⁶² investigated the reproducibility of the 23 most promising asthma and asthma-related candidate genes in the Japanese population. They found that interactions between *IL4RA* and *C3*, and *TBXA2R* and *ADAM33* were suggested to increase the risk for childhood and all asthma (childhood and adult asthma together). In the middle Chinese population, significant gene-gene interaction was detected between *IL-4* -C33T, *IL-13* R130Q, *IL-4Ralpha* I75V, *IL-4Ralpha* Q576R, *STAT6* C2892T, and *CD14* -C159T in the risk of pediatric asthma⁶³. Therefore assessing gene-gene interaction or interaction of SNPs in genes implicated in asthma or IgE level is a very important task for further investigation.

CONCLUSIONS

Results of our study, as well as results of similar studies on persons of Caucasian origin, indicate that polymorphisms rs324011, rs3024974 and rs4559 in *STAT6* have a major influence on total IgE level. Haplotype analysis revealed that the TCA haplotype alone (constructed from those polymorphisms) in *STAT6* is associated with total IgE elevation. Moreover, the haplotype predominantly originated from the fathers of investigated patients. The influence of paternal origin of the *STAT6* haplotype on IgE levels is therefore surprising but the exact role of possible paternal imprinting in *STAT6* regulation should be investigated and confirmed in future studies.

CONFLICT OF INTEREST STATEMENT

Author's conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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