

Array-based diagnostic gene-expression score for atopy and asthma

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Whether gene expression is useful in discriminating different atopic phenotypes is unclear. The aim of the study was to evaluate a gene-expression score for the diagnosis of atopy and asthma and to assess disease activity as a guide for therapeutic decisions. Purified mRNA from PBMCs of 18 atopic asthmatic subjects, 8 atopic nonasthmatic subjects, and 14 healthy control subjects was hybridized to cDNA membranes. A composite atopy gene expression (CAGE) score was determined by using 10 genes dysregulated in atopic individuals according to a specific algorithm. The CAGE score was better than total IgE in differentiating atopic from nonatopic subjects (sensitivity, 96%; specificity, 92%). Correlation between the CAGE score and total IgE ($P < .001$) was found, and there was a trend for correlation with asthma severity ($P = .051$). The CAGE score was able to quantify phenotype-specific alteration in gene expression of atopic individuals. The CAGE score might be used as a diagnostic tool or to monitor the effects and side effects of therapy. (J Allergy Clin Immunol 2002;109:271-3.)

Key words: Asthma, respiratory hypersensitivity, health-status indicators, gene expression

Quantifying a complex phenotype like atopy or asthma is difficult. Thus far, there is no consensus score, either in regard to clinical parameters (pulmonary function tests and skin prick tests) or laboratory methods (eg, IgE and IL-5) to define asthma. Even though some clinical scores are widely used,^{1,2} they are very arbitrary and controversial.³⁻⁵

Gene-array technology has recently opened the possibility of screening several hundred gene products. There has been no previous reported attempt to score asthma and atopy with a gene expression-based scoring. A gene expression-based score has several potential advantages: screening of healthy patients at risk of development of disease, early diagnosis, and monitoring of treatment effects and adverse events.

The aim of this study was to develop a gene expression-based score for the diagnosis of atopy and to assess asthma disease activity as a guide for therapeutic decision analysis.

Abbreviations used

CAGE: Composite atopy gene expression score
ROC: Receiver operating characteristic

METHODS

Subjects

Healthy control subjects, atopic nonasthmatic subjects, and atopic asthmatic subjects were recruited. Healthy control subjects were defined as individuals with negative skin prick test results without medication and without lung or systemic disease. Atopy was defined as a positive skin prick test response (wheal of ≥ 3 mm diameter larger than that produced by the negative control wheal) to a range of 4 common aeroallergens: *Dermatophagoides pteronyssinus*, cat, dog, and mixed grasses (ALK). Asthma was defined as symptomatic bronchial hyperreactivity in a sensitized individual with physician-diagnosed asthma. Asthmatic subjects treated with inhaled corticosteroids, but no oral corticosteroids, for at least 6 months were included. The severity of asthma was determined by using the Aas asthma severity score.² The Aas asthma severity score is a clinical score with a 5-step scale, taking events during the previous year into account. Asthmatic individuals were divided into those with mild-to-moderate asthma (Aas asthma severity score ≤ 3) and those with severe asthma (Aas asthma severity score > 3). Smokers were excluded from the study. Subjects were required to give informed consent. The study was approved by the local ethical committee.

PBMC preparation and cDNA hybridization

PBMCs were separated by means of gradient centrifugation and washed in AIM-V serum-free culture medium. The purified mRNA populations (TRIzol, Life Technologies Ltd, and Oligotex, Qiagen Ltd) were reverse transcribed with oligo-dT primer mix and labeled with phosphorous 32-labeled deoxyadenosine triphosphate (Amersham Life Science Ltd). After an overnight hybridization onto membranes with immobilized probe cDNA for 609 gene products in duplicate (Atlas, Clontech: the complete list of genes with accession numbers is published at <http://www.clontech.com>), the quantification was done by means of autoradiography and phosphor imaging.

Data analysis

Data were analyzed with the SPSS for Windows 9.0.0 (SPSS Inc) statistical package. Multiple Mann-Whitney U tests were used to screen for differential gene expression in atopy and asthma after standardization with the geometric mean of the 100 most expressed genes.⁶ A significance level of 5% was taken.

For the design and validation of the CAGE score, the study population was divided, by means of computer-based randomization, in 2 parts. The first half of the study population (atopic asthmatic subjects, $n = 9$; atopic nonasthmatic subjects, $n = 4$; healthy control subjects, $n = 7$) was used to design a gene expression-based score for atopy (CAGE) in a 2-step procedure, which was then tested in the second

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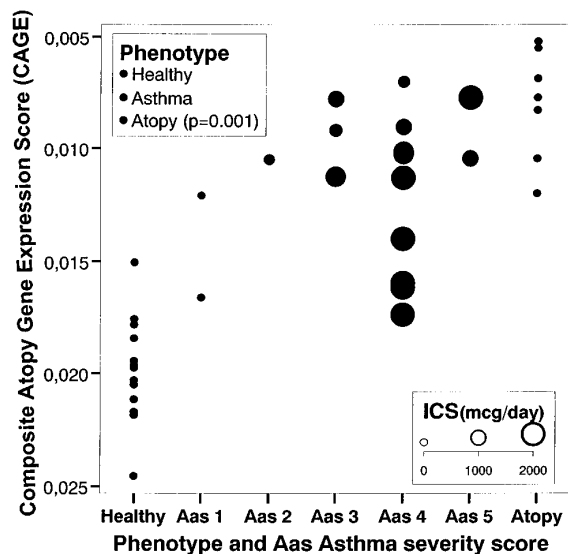


FIG 1. Scatter plot of CAGE scores in healthy individuals and atopic subjects with and without asthma with respect to asthma severity (Aas asthma severity scores) and total daily dosage of inhaled corticosteroids (ICS). Two CAGE clusters can be seen: healthy nonatopic individuals and atopic individuals with and without asthma ($P < .001$, ANOVA). Nonasthmatic atopic subjects clustered tightly together. Within the asthmatic individuals, patients with more severe asthma tended to have more aberrant gene expression. However, the latter did not reach statistical significance because of low numbers.

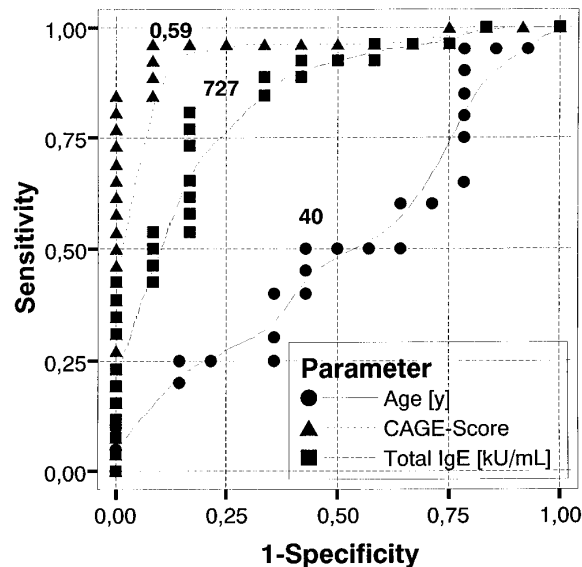


FIG 2. ROC analyses to compare CAGE score, total IgE, and age for diagnosing atopy. The CAGE score (area, 0.96 [0.90-1.00]; $P < .001$) was more able to differentiate atopic from nonatopic individuals than total IgE (area, 0.85 [0.73-0.98]; $P = .001$). The ROC curve for age fluctuated around the diagonal and was not significant (area, 0.50 [0.30-0.71]; $P = .97$). The dots show the relationship between sensitivity and specificity at the respective cut-off values. A smoothing iteration was performed to show continuous ROC curves.

TABLE I. List of the 10 genes identified with the described 2-step procedure and used for CAGE scoring

Gene	Accession No.	Group difference (healthy vs atopy)	Mean gene expression				Expression ratio (atopy/healthy)
			Healthy	Asthma	No asthma	Total	
IL-1 receptor type 1	M27492	.003	0.05	0.14	0.15	0.14	2.85
Proto-oncogene tyrosine protein kinase receptor RET	M31213	.010	0.06	0.11	0.15	0.12	2.17
IL-6 (B-cell differentiation factor)	X04602	.001	0.19	0.38	0.46	0.41	2.15
CD40 receptor-associated factor (CRAF1)	U21092	.008	0.21	0.38	0.48	0.41	2.00
Serine kinase	U09564	.008	0.06	0.11	0.15	0.12	1.96
Proto-oncogene tyrosine protein kinase YES	M15990	.003	0.08	0.13	0.21	0.15	1.79
Integrin $\alpha 6$	X53586	.005	0.28	0.22	0.13	0.19	0.70
60S ribosomal protein L6 (DNA-binding protein TAX)	X69391	.002	11.66	7.15	4.97	6.48	0.56
Transferrin receptor protein (p90, CD71)	X01060	.006	0.21	0.10	0.08	0.10	0.47
IFN- α receptor β -chain	X77722	.003	0.48	0.22	0.15	0.20	0.42

Results of all 14 healthy control subjects and 26 atopic individuals with and without asthma are summarized. Apart from mean gene-expression levels (geometric mean), P values of the comparison between atopic and nonatopic phenotypes and expression ratios are given. The latter represents the ratio between the gene expression in atopic subjects and healthy nonatopic individuals.

half of the subjects. A 2-step procedure was performed to select the genes for the gene-expression score. Analysis in the first half of the patients found a total of 30 different gene products of the 605 genes tested to have a statistically significant differential expression in atopic nonasthmatic and atopic asthmatic subjects when compared with that in healthy control subjects ($P < .01$, Table I). From these 30 genes, only 10 genes for which the geometric mean of the atopic nonasthmatic individuals was more than 2-fold upregulated or down-

regulated compared with the geometric mean in control subjects were taken for the gene-expression score. These 10 genes were then taken as reference genes to calculate the CAGE score. Therefore for each CAGE gene, the specific signal was calculated as follows:

$$\frac{\text{Individual logarithmic expression [gene x]} - \text{Mean logarithmic expression [gene x]}}{\text{SD [gene x]}}$$

The absolute difference of the expression of each of the CAGE-

scored genes between the mean expression in atopic individuals and the tested individual was then added together. This sum, the CAGE score, is a measure of similarity of a gene-expression pattern of an individual to the gene expression in a typical atopic individual. Thus atopic individuals are supposed to have low CAGE scores, whereas nonatopic individuals have high CAGE scores.

A receiver operating characteristic (ROC) analysis with calculation of the ROC area under the curve statistic was performed to test the effectiveness of CAGE, total IgE, and age in differentiating atopic from nonatopic individuals in terms of gene expression. The ROC curve gives sensitivity and specificity of a certain test in function of the cut-off value.⁷ The better the diagnostic test, the nearer the curve reaches to the top left corner, which indicates a 100% sensitivity and specificity. The area under the curve of an ROC curve can be calculated and compared with that of other diagnostic tests. Determination of whether CAGE scores correlated with asthma severity and inhaled corticosteroid dosage was performed with the Spearman correlation coefficient.

RESULTS

Fourteen nonatopic healthy control subjects (male/female ratio, 2:12; mean age, 42 ± 13 years; age range, 27-65 years), 8 atopic subjects without asthma (male/female ratio, 7:1; mean age, 36 ± 9 years; age range, 23-49 years), and 18 atopic asthmatic subjects (male/female ratio, 6:12; mean age, 41 ± 14 years; age range, 18-66 years) were included in the study. Sixteen of 18 asthmatic subjects had inhaled corticosteroids and Aas asthma severity scores ranging from 1 to 5 (score 1, n = 2; score 2, n = 1; score 3, n = 4; score 4, n = 9; and score 5, n = 2).

Genes identified with the described 2-step procedure and used for the CAGE score are listed in Table I. Atopic nonasthmatic and atopic asthmatic subjects had significantly different CAGE scores compared with those of the healthy control subjects (ANOVA: $P < .001$, atopic nonasthmatic subjects vs healthy control subjects; $P < .001$, atopic asthmatic subjects vs healthy control subjects, $P < .001$; Fig 1). The CAGE score defined by the first half of the subjects was able to differentiate atopic from nonatopic subjects in the validation experiments. The results of the ROC analysis are given in Fig 2. The CAGE score (area, 0.96 [0.90-1.00]; $P < .001$) was superior to IgE (area, 0.85 [0.73-0.98]; $P = .001$) in differentiating atopic from nonatopic subjects. CAGE had a sensitivity of 96% and a specificity of 92% at its optimal cut-off level. A significant correlation between the CAGE score and total IgE was identified in atopic individuals ($R^2 = 0.75$, $P < .001$). Furthermore, a strong trend for the correlation of the CAGE score and the Aas asthma severity score was found for individuals inhaling less than 500 µg of corticosteroids per day ($P = .051$).

DISCUSSION

In this study we quantified the overall atopy-specific gene-expression activity with the CAGE score. The CAGE score had a sensitivity of 96% and a specificity of 92% for the diagnosis of atopy and thus was superior to total IgE values. The CAGE score correlated with total

IgE levels and a trend for asthma severity was found. Thus the CAGE score provided an overall index of current atopic disease activity.

Gene-expression scores could be used as a clear-cut diagnostic tool for the diagnosis and treatment response in atopy and asthma. Individuals at risk for sensitization and the development of asthma or with preclinical asthma could be identified and lead to preventative measures.⁸ Assessment of disease activity for atopic diseases like asthma is a difficult task for which, thus far, no satisfactory surrogate marker has been identified. Treatment effects and side effects could be monitored with gene-expression scores. The current publication opens the possibility to use specific gene-expression scores, like the CAGE score, as potential outcome measures for clinical trials in asthma.

The correlation of gene expression and asthma severity could even be better, as documented here. The major problem is the fact that there is no gold standard to quantify current asthma severity. The Aas asthma severity score is a subjective assessment of the course of asthma over a 12-month period,² whereas the CAGE score measures the single-time-point gene expression, which is influenced by day-to-day variation in the exposure to allergens and other destabilizing factors in asthma. Furthermore, our study population was rather heterogeneous, with asthma severity and inhaled corticosteroids both possibly interfering with the gene expression score. The proposed approach can easily be adapted for use with modern high-throughput gene chips carrying several thousand genes. In our view applying novel microarray technology is likely to further improve performance of composite gene-expression scores through a better reproducibility of these systems compared with the filter arrays and the possibility of a better selection of genes.

The CAGE score represents a phenotype-specific gene-expression score for atopic diseases. Applications may include determination of patients at risk for asthma, classification of disease severity, and scoring for effects and side effects of treatment. The use of this gene-expression score may also serve as an outcome measure for asthma intervention studies.

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