Influence of glucocorticoid receptor gene NR3C1 646 C>G polymorphism on glucocorticoid resistance in asthmatics: a preliminary study

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Abstract
Background: Glucocorticoid receptor gene polymorphism (NR3C1 646 C>G) may play an important role in the development of severe bronchial asthma and resistance to glucocorticoids (GCs).

Objective: The aim of the present study was to determine the relation between the 646 C>G polymorphism of the glucocorticoid receptor gene (NR3C1) and resistance to GCs with development of severe bronchial asthma.

Material and methods: This case-control study included 40 patients with severe bronchial asthma and 20 apparently healthy controls. Atopic status was determined by skin prick test reaction to the most common locally-encountered allergens. GCs reversibility test was performed to differentiate between GCs sensitive and GCs resistant asthma. For all subjects, analysis of the glucocorticoid receptor gene polymorphism (NR3C1 646 C>G) was done using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP).

Results: The frequencies of NR3C1 646 C>G genotypes and alleles differed significantly between asthmatic patients and controls. The frequencies of the CC genotype and C allele carriers were significantly higher among asthmatics than among controls, and also among GCs sensitive asthmatics than among GCs resistant asthmatics. However, NR3C1 646 C>G genotypes and alleles frequencies did not differ significantly according to the atopic status in asthmatics.

Conclusions: The too small sized of the investigated groups is a shortcoming of this study. Nevertheless, the observed variations demonstrate a marked association of NR3C1 646 C>G CC genotype with the development of bronchial asthma and a higher frequency of the C allele among GCs sensitive asthmatics. Large-scale studies are required to investigate the association between polymorphisms of the NR3C1 gene and GCs resistance among asthmatic patients.

Key words: asthma, glucocorticoid receptor, glucocorticoid resistance, restriction fragment length polymorphism, single nucleotide polymorphism.

Introduction
Asthma is a chronic inflammatory disease of the airways that is characterized by variable airflow obstruction, increased airway responsiveness and inflammation, mucus overproduction, and airway remodeling [1]. Asthma presents clinically as a result of combination of both genetic and environmental factors, and is the result of disrupted immune regulatory mechanisms occurring at the lymphocytic level in coordination with excess production of IgE antibodies resulting in allergic inflammatory reaction [2].

One of the genes involved in the pathogenesis of bronchial asthma is NR3C1; its official name is a nuclear receptor subfamily 3, group C, member 1 (as determined by HUGO Gene Nomenclature Committee) that encodes glucocorticoid receptor (GCR) [3].

The NR3C1 gene is localized on chromosome 5q31.3 and consists of nine exons [3]. Exon 1 has seven basic tran-
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Inflammation of the airways is the primary event of bronchial asthma. GCs are important anti-inflammatory agents for controlling the patient responses to exogenous GCs, which are the most important anti-inflammatory agents for controlling the course of bronchial asthma [8]. As of today, 2571 polymorphisms of this gene are known, but the most common is the NR3C1 646 C>G polymorphism [8]. NR3C1 646 C>G polymorphism was identified as a C/G substitution in the promoter region [20]. NR3C1 646 C>G polymorphism significantly affects the processes of alternative NR3C1 gene splicing and within that mechanism increases the sensitivity to GCs [11, 12]. Other known SNPs of NR3C1 gene included Tth111I and ER22/23EK, N363S polymorphisms, which are also associated with altered glucocorticoid sensitivity. Tth111I polymorphism (rs10052957) is located in the area of the NR3C1 gene promoter, 3807 bp upstream from the mRNA start site. It causes a C>T substitution in the promoter region, that has been associated with enhanced basal cortisol levels [13].

The GCs resistant asthma is a complex problem, and to the present date, it is unknown whether it is inherited or acquired, and whether it is dependent on genetic or environmental factors [2].

The aim of the present study was to determine the association between NR3C1 646 C>G variation and resistance to GCs with development of severe asthma among Egyptians, a previously unstudied ethnic group.

**Material and methods**

This study included 40 adult patients with severe bronchial asthma, who were recruited from the Allergy and Chest Diseases outpatient clinics at Ain Shams University Hospitals, Cairo. Asthma diagnosis was established according to GINA guidelines [16]. All patients were subjected to a detailed medical history, clinical examination, and skin prick test (SPT). Atopy was defined by the presence of at least one positive SPT reaction to common environmental allergens including mites, animal epithelia, pollens, and molds. In addition, glucocorticoid (GCs) reversibility test was performed to differentiate between GCs sensitive and GCs resistant asthma.

**Glucocorticoid reversibility test**

This was performed in the Pulmonary Functions Laboratory in Ain Shams University Hospital by spirometry to identify patients with GCs resistant asthma. Spirometry was carried out according to the standards of the European Respiratory Society (ERS) and the American thoracic Society (ATS) [17]. Accordingly, asthmatic patients were subdivided into 2 subgroups: GCs sensitive asthmatic patients (n = 20); GCs resistant asthmatic patients (n = 20).

**GCs resistant asthma** was defined as the failure of asthmatic patients to show an improvement in the forced expiratory volume in the first second (FEV1) by 15% after an adequate course of GCs (oral prednisolone 40 mg/d for 2 weeks) despite showing clear reversibility (> 15%) to β2-agonists. On the other hand, **GCs sensitive asthma** was defined as patients who showed an improvement of 30% in FEV1 measurements after an equivalent dose of GCs.

Individuals who suffered from medication non-compliance, occupational asthma with ongoing antigenic exposure, gastro-esophageal reflux, aspirin or non steroid anti-inflammatory drugs (NSAIDs) sensitivity, food allergy, irreversible airflow obstruction and underlying systemic vasculitis were excluded from the study. Furthermore, patients using drugs which might induce resistance to GCs (including Rifampicin, Phenobarbital, Phenytoin, Ephed-
rine) and subjects with signs of viral infections, either generalized, or affecting the respiratory tract were excluded.

The study also included 20 apparently healthy individuals as a control group. All controls had no history of asthma or other allergic diseases and had no first-degree relatives with bronchial asthma or atopic disorders. In addition, all controls had normal spirometric lung functions and negative SPT reactions. An informed consent was obtained from all subjects, and the study was approved by the Research Ethics Committee of the Faculty of Medicine, Ain Shams University.

The glucocorticoid receptor gene polymorphism NR3C1 646 C>G by PCR-RFLP

This was evaluated for all patients and controls at the Clinical Pathology Department, Ain-Shams University Hospital as follows:

**Sampling**

5 ml of venous blood were collected under aseptic conditions from every patient in a sterile EDTA vacutainer tube.

**DNA Extraction**

It was done using the QIAamp DNA Mini Kit supplied by Qiagen (Hilden, Germany). DNA in the sample was liberated using proteinase K solution and lysis buffer. Released DNA was bound exclusively and specifically to the QIAamp membrane in the presence of binding buffer under appropriate salt and pH conditions. Denatured protein and other contaminants were removed with several washing procedures. The DNA was then eluted from the membrane with elution buffer.

**Polymerase chain reaction**

For detection of NR3C1 646 C>G polymorphism whose amplified product is 418 bp fragment, amplification was performed using a thermal cycler Gene Amp PCR system 9700 Applied biosystems. PCR product were carried out in volume of 50 μl containing 5 μl genomic DNA, 25 μl of the ready to use master mix supplied by Qiagen, 2.5 μl (25 pmol) of forward primer, 2.5 μl (25 pmol) of reverse primer and 15 μl of deionized water. Primers used were forward primer (5’-GAG AAA TTCACC CCT ACC AAC- 3’) and a reverse primer (5’-AGAGCC CTA TTC TTC AAA CTG- 3’). The test primers were chosen according to Pietras et al. [2] and prepared by Promega (Madison Wi, USA). PCR conditions were an initial denaturation step at 94°C for 3 minutes and 30 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 1 minute, then final extension at 72°C for 10 minutes.

**Restriction fragment length polymorphism**

Restriction digestion of the amplified product was performed using 1 μl (10 units/μl) Bcl-1 restriction enzyme supplied by Promega. The enzyme was added to 5 μl of PCR amplified product, 10 μl deionized water, 2 μl of buffer (10 × of 60 mM tris-Hcl) and 2 μl of acetylated bovine serum albumin (BSA) (10 μg/μl), followed by a gentle mix. Then the tubes were placed on a heat block for 2 hours at 50°C. The enzyme was inactivated at 65°C for 15 minutes. As experimental control: no-enzyme “mock” digest was used.

**DNA analysis by gel electrophoresis**

Amplified product of DNA samples and restriction fragments were run on 2% agarose gel (Promega) for 30 minutes at 100 V, stained with ethidium bromide (Amresco, Germany). 100 bp DNA ladder (Promega) was also run to identify the site of bands. The gel was examined under an ultraviolet transilluminator (Biometra, Germany).

Digestion of the amplified product resulted in the following products corresponding to the following 3 genotypes defined according to Pietras et al.: homozygous CC genotype if the band was divided into 2 parts: 263 and 155 bp.
bpt homozygous GG genotype if the band was not divided: 418 bp; heterozygous GC genotype if it yielded 3 bands: 418, 263 and 155 bp (Fig. 1) [18].

Statistical analysis
Analysis of data was performed using the SPSS program version 17. Data were expressed as mean ± standard deviation (SD) for quantitative parametric measures and both number and percentage for categorical data. Student t-test was used for comparison between two independent groups for parametric data, whereas the one-way analysis of variance (ANOVA) test was used to compare the means of three groups. Comparison between qualitative variables was carried out by using chi² test and Fisher’s exact test. A p-value < 0.05 was considered significant.

Results
This study included 40 adult patients with severe bronchial asthma (23 females, 17 males). Severe asthmatic patients were subdivided into 2 subgroups: GCs sensitive asthmatic patients, n = 20; GCs resistant asthmatic patients (n = 20). Age, sex, and the atopic status were comparable among both subgroups. Significantly higher percentages of FEV₁ were observed among GCs sensitive asthmatic patients after prednisolone therapy, as compared to GCs resistant asthmatic patients (Table 1).

There was a highly significant difference in frequencies of NR3C1 646 C>G genotypes and alleles between asthmatic patients and controls. The CC genotype was more frequent among asthmatic patients than among controls; the GG genotype was more frequent among controls than among asthmatic patients; whereas the GC genotype did not differ in distribution between asthmatic patients or controls. Moreover, almost all controls were G allele carriers in comparison to about two-thirds of asthmatic patients, whereas almost all asthmatic patients were C allele carriers (Table 2).

Table 3 shows a significant difference in genotype distribution between GCs sensitive and GCs resistant asthmatics, with a greater frequency of the CC genotype distribution as well as C allele carriers among GCs sensitive asthmatics than among GCs resistant asthmatics.

The frequencies of NR3C1 646 C>G genotypes and alleles did not differ significantly according to the atopic status in GCs sensitive asthmatics (Table 4) or in GCs resistant asthmatics (Table 5).

Table 1. Characteristics of the study groups

|                    | GCs sensitive patient (n = 20) | GCs resistant patients (n = 20) | Controls (n = 20) | p       |
|--------------------|-------------------------------|---------------------------------|------------------|---------|
| Age (y)*           | 43 ±8.2                       | 43 ±9.3                         | 40 ±5.3          | 0.410   |
| Sex, n (%)         |                               |                                 |                  |         |
| Male               | 8 (40)                        | 9 (45)                          | 7 (35)           | 0.337   |
| Female             | 12 (60)                       | 11 (55)                         | 13 (65)          |         |
| FEV₁ (%)*          |                               |                                 |                  |         |
| Before prednisolone| 57 ±4.3                       | 54.3 ±4.1                       | –                | 0.06    |
| After prednisolone | 76.2 ±3.9                     | 56.45 ±3.8                      | –                | < 0.001 |
| Atopic status, n (%)|                              |                                 |                  |         |
| Atopic             | 12 (60)                       | 15 (75)                         | –                | 0.311   |
| Non-atopic         | 8 (40)                        | 5 (25)                          | –                |         |

*Values are presented as mean standard deviation; FEV₁ = forced expiratory volume in 1st second; GCs = glucocorticoids

Table 2. Comparison between patients and controls regarding NR3C1 646 C>G genotypes and alleles

| NR3C1 646 C>G polymorphism | Patients (n = 40) | Controls (n = 20) | p       |
|----------------------------|------------------|-------------------|---------|
| Genotypes                  |                  |                   |         |
| GG                         | 5 (12.5)         | 9 (45)            | 0.007   |
| GC                         | 22 (55)          | 10 (50)           |         |
| CC                         | 13 (32.5)        | 1 (5)             |         |
| G allele carriers*         | 27 (67.5)        | 19 (95)           | 0.023   |
| C allele carriers¶          | 35 (87.5)        | 11 (55)           | 0.009   |

*Subjects with GG or GC genotypes; ¶Subjects with CC or GC genotypes

All values are presented as number (percentage)

Table 3. Comparison between GCs sensitive and GCs resistant asthmatic patients regarding NR3C1 646 C>G genotypes and alleles

| NR3C1 646 C>G polymorphism | GCs sensitive patients (n = 20) | GCs resistant patients (n = 20) | p       |
|---------------------------|--------------------------------|--------------------------------|---------|
| Genotypes                 |                                |                                |         |
| GG                        | 0                              | 5 (25)                         |         |
| GC                        | 11 (55)                        | 11 (55)                        | 0.028   |
| CC                        | 9 (45)                         | 4 (20)                         |         |
| G allele carriers*        | 11 (55)                        | 16 (80)                        | 0.176   |
| C allele carriers§         | 20 (100)                       | 15 (75)                        | < 0.001 |

*Subjects with GG or GC genotypes; §Subjects with CC or GC genotypes

All values are presented as number (percentage); GCs, glucocorticoids
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Table 4. Comparison between atopic and non-atopic GCs sensitive asthmatic patients regarding NR3C1 646 C>G genotypes and alleles

| NR3C1 646 C>G polymorphism | Atopic patients (n = 12) | Non-atopic patients (n = 8) | p |
|---------------------------|------------------------|----------------------------|---|
| Genotypes                |                        |                            |   |
| GC                       | 6 (50)                 | 5 (62.5)                   | 0.362 |
| CC                       | 6 (50)                 | 3 (37.5)                   |   |
| G allele carriers*       | 6 (50)                 | 5 (62.5)                   | 0.362 |
| C allele carriers         | 12 (100)               | 8 (100)                    | 1.0 |

*Subjects with GG or GC genotypes; ‡Subjects with CC or GC genotypes
All values are presented as number (percentage); GCs, glucocorticoids

Table 5. Comparison between atopic and non-atopic GCs resistant asthmatic patients regarding NR3C1 646 C>G genotypes and alleles

| NR3C1 646 C>G polymorphism | Atopic patients (n = 15) | Non-atopic patients (n = 5) | p |
|---------------------------|------------------------|----------------------------|---|
| Genotypes                |                        |                            |   |
| GC                       | 4 (26.7)               | 1 (20)                     | 0.645 |
| GC                       | 7 (46.7)               | 4 (80)                     |   |
| CC                       | 4 (26.7)               | 0                          |   |
| G allele carriers*       | 11 (73.3)              | 5 (100)                    | 0.530 |
| C allele carriers‡       | 11 (73.3)              | 4 (80)                     | 0.999 |

*Subjects with GG or GC genotypes; ‡Subjects with CC or GC genotypes
All values are presented as number (percentage); GCs – glucocorticoids

Discussion

Despite the fact that GCs are widely used with great success in many inflammatory disorders including bronchial asthma, some patients show poor and sometimes no response to high doses. Difficult-to-treat asthma or GCs resistant asthma is explained by mutations of h-GCR gene, causing impairment of one or more of the molecular mechanisms of h-GR action leading to tissue non-responsiveness to GCs [19].

Polymorphisms present within the h-GR/NR3C1 gene may inhibit formation of GR/GCs complexes, reduce transcription and cause transrepression of the genes encoding proteins synthesized within the framework of cellular response to GCs and decreased expression of GR that leads to a reduced response to GCs and impairment of GCR [20]. GCs resistance is the main obstacle in many inflammatory diseases and therefore makes clinical management difficult [21].

Among the several known functionally relevant alleles of the GCR are the alleles that comprise the NR3C1 646 C>G. First discovered as a restriction fragment length polymorphism after digestion of GR DNA with Bcl-1, these alleles are determined by a single nucleotide variation – cytosine (most common) or guanine – in intron 2, 646 nucleotides downstream from exon 2. The allele containing cytosine is referred to as allele C, and the allele containing guanine as allele G, resulting in three possible genotypes at this position: homozygous CC, heterozygous GC and homozygous GG [22].

NR3C1 646 C>G polymorphism significantly affects the process of alternative NR3C1 gene splicing and within that mechanism increases the sensitivity to GCs [18]. However, Manenschijn et al. observed that polymorphism is intronic, and that its location does not involve a coding, regulatory or splicing part of the GCR gene. Additionally, they suggested that this polymorphism is in linkage with other variations, e.g., in the promoter region, or linked to other functionally important polymorphisms [23].

The aim of the present study was to determine the correlation between NR3C1 646 C>G single nucleotide polymorphism of h-GR/NR3C1 gene and resistance to GCs therapy with the development of severe form/difficult-to-treat asthma. We demonstrated a statistically significant difference in genotype frequencies (GG genotype and CC genotype) of the NR3C1 646 C>G polymorphism in all severe asthmatic patients compared with healthy controls; the CC genotype was more frequent in asthmatic patients compared with control subjects, while the GG genotype was less frequent in patients compared with control subjects. This finding is in disagreement with that observed by Pietras et al. who studied a group of Polish patients with severe asthma and observed significant differences between the investigated genotypes with a significant increase in the GG genotype among severe asthmatic patients compared to controls [2].

Our findings are in line with Panek et al. who demonstrated a lower frequency of the NR3C1 646 C>G GG homozygote and a higher frequency of the CC homozygote among asthmatics (which included mild, moderate, and severe asthmatics) in comparison to the control group. However, the authors observed no statistically significant differences in frequencies of NR3C1 646 C>G polymorphism when severe asthma was compared with the control group [18]. The discrepancy between their findings and our results may be attributed in part to our relatively smaller sample size.

In the present study, there was a significant difference in allelic frequencies of the NR3C1 646 C>G polymorphism in asthmatic patients compared to controls. C allele carriers were more frequent among asthmatic patients, whereas G allele carriers were more frequent among healthy controls. This finding is in contrast with that observed by Pietras et al. who observed a significant difference in allelic frequency regarding the G allele between asthmatic patients and controls but no significant difference in allelic frequency regarding the C allele between asthmatic patients and controls.

In addition, the current study showed a significant difference in NR3C1 646 C>G genotypes and alleles frequencies between GCs sensitive and GCs resistant asthmatics. We demonstrated that GCs sensitive asthmatics had a greater frequency of the CC genotype distribution as well as C allele...
carriers than GCs resistant asthmatics. This finding is also in contrast with that observed by Pietras et al., who demonstrated that allele G is particularly associated with sensitivity to GCs, and that it increases the cellular response to GCs and occurs less frequently than allele C [2]. This discrepancy could be attributed to different population ethnicity.

Atopy is considered one of the important risk factors in the pathogenesis of asthma. In turn, antigen-specific IgE produced in a sequence of immune processes is essential for atopy to occur. One review explained the role of environmental agents in the modulation of these immune processes, and therefore, modulation in asthma development [24]. The few studies available on the association between NR3C1 646 C>G single nucleotide polymorphism of h-GR/NR3C1 gene promoter and resistance to GCs therapy with development of severe form/difficult-to-treat asthma have not addressed the relationship with atopy. To our knowledge, we report here for the first time that there is no relationship between atopy and this gene polymorphism. Further large-scale studies on larger numbers of atopic asthmatics should be carried out to investigate this relationship.

Conclusions

The presented study is a preliminary stage for more profound analysis of association between the prevalence of the particular allelic variants of the h-GR/NR3C1 gene and the development of bronchial asthma, including severe, treatment-resistant forms of the disease. It should be emphasized that no studies concerning the frequency of h-GR/NR3C1 glucocorticoid receptor gene polymorphisms in atopic asthmatics patients have been conducted to date.

The authors declare no conflict of interest.

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