

Different Gene Expressions of Alpha and Beta Glucocorticoid Receptors in Asthmatics

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Abstract

The response to glucocorticoids (GCs) therapy classifies severe refractory asthma (SRA) and mild asthma, so the glucocorticoid receptors (GCRs) gene expression may be involved in SRA pathogenesis. Thus, it is aimed to compare the expression levels of two GCR isoforms (GCR α and GCR β) in SRA, mild asthmatics, and healthy controls. Total RNA was isolated from the peripheral blood mononuclear lymphocytes of 13 SRA patients, 14 mild asthma patients and 30 healthy volunteers. The expression levels of GCR isoforms were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). The expression level of GCR isoforms did not show any significant difference between the cases/control groups. However, the relative expression analysis between asthma/control, SRA/control and SRA/asthma groups was in the order of 0.933, 0.768 and 0.823 for GCR α and 0.697, 1.014 and 1.454 for GCR β , respectively. Also, the expression fold change of GCR α /GCR β in asthma, SRA and control groups was 786.88, 445.72 and 588.13, respectively. The GCR α and GCR β isoforms did not show any correlation in SRA; but they had significant correlation in both healthy volunteers ($r = 0.490$, $P = 0.007$) and mild asthmatics ($r = 0.786$, $P = 0.001$). Also, the GCR α expression level had significant inverse correlation with age in SRA ($r = -0.709$, $P = 0.007$). Glucocorticoid receptors are related to, but not directly responsible for GC resistance. Since the GCR α /GCR β expression ratio decreased in SRA, studies are needed to assess its value in diagnosing GC resistance.

Keywords: Asthma; Severe asthma; Glucocorticoid receptor; Gene expression.

Introduction

Asthma, a chronic inflammatory respiratory disease, is characterized by recurrent attacks of breathlessness and wheezing, which vary in severity and frequency from person to

person. The pathophysiology of asthma is not completely understood; but is now believed that the numerous environmental causative and/or trigger agents (such as allergens, tobacco smoke, chemical irritants, microorganisms, *etc.*) may interact with individual genetics and epigenetic backgrounds to cause asthma or induce its attack (1).

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estimates show that more than 300 million people currently suffer from asthma worldwide and this number is expected to increase to 400 million by 2025. Nearly 250,000 premature deaths occur annually due to asthma. Asthma continues to be a major cause of disability and poor quality of life. It is also a major cause of health resource utilization and a public health concern worldwide (2, 3).

The definition and classification of severe asthma, which comprises nearly 5-10% of asthmatics (4), has been under debate during the past decades. However, the Innovative Medicine Initiative (IMI) published an international consensus statement and clarified a unique definition, classification, and diagnostic algorithm for severe refractory asthma (SRA) as follows:

“The term ‘severe refractory asthma’ should be reserved for patients with asthma in whom alternative diagnoses have been excluded, comorbidities have been treated, trigger factors have been removed (if possible) and compliance with treatment has been checked, but still have poor asthma control or frequent (≥ 2) severe exacerbations per year despite the prescription of high-intensity treatment or can only maintain adequate control when taking systemic corticosteroids and are thereby at risk of serious adverse effects of treatment” (5).

Glucocorticoids (GCs) are among the mainstays of treatment in asthmatics (1). Classification of asthmatics to mild or severe is mainly based on response to GCs (especially the inhaled forms). While systemic GCs are recommended for treatment of severe asthma (4), steroid resistance is being recognized increasingly (6).

Glucocorticoids exert their effects mainly via binding to their intracellular receptors (GCRs). So, among the proposed mechanisms of GCs resistance, the possible role of GCRs in SRA development is prominent (7-9). The resistance varies with disease type, drug regimen, and genetic background, which later may occur due to some mechanisms such as encoding gene polymorphism (*i.e.* *NR3C1* gene), altered expression level of the GCR gene, GCR modification, effects of other transcription factors and defective histone acetylation (10-12).

Human GCRs are proteins coded by the nuclear receptor subfamily 3 group C number1 (*NR3C1*) gene on chromosome 5q31.3. The GCR α is one of the four known GCR isoforms generated by alternative splicing. It has been detected in all tissues and binds to GCs. Then, the GCR α -GCs complex translocate to the nucleus and implements its role by two mechanisms. First, as a transcription factor, it binds to promoter regions and down regulates many inflammatory genes (*e.g.* *IL-1*, *IL-2*, *IL-4*, *IL-6*, *IFN γ* , *ICAM-1*, *VCAM-1*, *NO synthase*, *etc.*) or up regulates anti-inflammatory genes (*e.g.* *Lipocortin 1*, *I κ B α* , *etc.*). Second, the complex may interact with other transcription factors like AP-1, NF-AT, NF- κ B or STAT family members (13, 14). The other isoform, *i.e.* GCR β , has been found in different, but not all, tissues and does not bind to glucocorticoids; so it is transcriptionally inactive (12, 15 and 16). However, it seems that GCR β may inhibit the effect of GCR α -GCs complex in a concentration-dependent manner (17). The increase in GCR β expression in steroid resistant conditions indicates that it is associated with GC resistance (4, 6, 17 and 18). Moreover, increased immunoreactivity of GCR β has been demonstrated in bronchoalveolar lavage (BAL) cells, airways tissues (epithelial and submucosal inflammatory cells) and also peripheral blood mononuclear lymphocytes (PBMLs) of SRA patients (19-21). Furthermore, Pietraz *et al.*, and Panek *et al.* reported the association of *NR3C1* gene polymorphisms with asthma and severe asthma in the Polish population (22, 23). However, previous studies, especially limited *in-vivo* studies, have not proven that these receptors can directly cause GC resistance (15). Therefore, controversy regarding these peptides underlies the necessity of evaluation of their accurate expression in clinical cases. Moreover, two other GCR isoforms (*i.e.* GCR γ and GCR δ) are less understood (24, 25).

Accordingly, as the difference in GCR expression levels is a proposed hypothetical mechanisms for occurrence of GC resistance, this study was performed to evaluate the expression level of GCR α and GCR β (from *NR3C1* gene) in PBMLs of SRA patients, in order to investigate differences among asthmatics and healthy volunteers.

Table 1. Designed primers for amplification of GCR α , GCR β and GAPDH.

		Primer sequence	Primer Length (BP)	Product length (BP)
GCR α	F	5'-CTTACTGCTTCTCTTCAGTTCC-3'	24	193
	R	5'-GAGATTTTCAACCACTTCATGC-3'	22	
GCR β	F	5'-CTTACTGCTTCTCTTCAGTTCC-3'	24	198
	R	5'-GGTTTTAACCACATAACATTTTCA-3'	24	
GAPDH	F	5'-CCATGAGAAGTATGACAAC-3'	19	115
	R	5'-GAGTCCTCCACGATACC-3'	18	

Experimental

This cross-sectional study was a joint investigation undertaken by the Tarbiat Modares University (TMU) of Tehran and the National Research Institute of Tuberculosis and Lung Diseases (NRITLD) from 2012 through 2014.

Case Selection and Sampling

Thirteen SRA patients were selected sequentially from asthma clinic of NRITLD, according to the criteria mentioned in the 2011 international consensus for definition of severe asthma (5). The cases were visited by at least one certified pulmonologist. In addition, 14 non-severe asthma patients were enrolled in the study by the same physicians and clinic, according to the criteria of the Global Initiative for Asthma (GINA) (1).

The 30 volunteer participants in the control group had no clinical or paraclinical history of disorders at the time of the study. Demographic and clinical data gathering, together with blood sampling, were performed by a trained expert clinician. The blood samples were immediately incubated at 4 °C for up to two h prior to lab procedures.

All of the participants voluntarily agreed to enroll in the study and signed written informed consent forms. Their demographic and clinical data were kept confidentially and there was no intervention to be applied throughout the clinical management of the cases. The ethical committee of the NRITLD approved all stages of the study under the code sbmu1.REC.1391.1 dated May 14, 2012.

RNA Extraction and cDNA Synthesis

After drawing 5 milliliters of venous blood, PBMLs were separated by Lympholyte[®]-H (Cedarlane Co., Burlington, Ontario, Canada) solution based on its relevant protocol. Using RNX^{Plus} solution (CinnaGen Co., Tehran, Tehran, Iran), total RNA was isolated according to the manufacturer's instructions and kept at -80 °C until molecular analysis. The quality and quantity of the extracted RNA were checked by agarose gel electrophoresis and spectrophotometry, respectively.

Using Oligo-dT, random hexamer and reverse transcriptase enzyme (Fermentas, Thermo Fisher Scientific Co., Waltham, Massachusetts, USA), cDNAs were synthesized from 3 micrograms of isolated RNAs. The prepared cDNAs were checked by simple PCR on glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene and the qualified ones were kept at -80 °C to be studied by qRT-PCR.

Primer Design and qRT-PCR

The designed primers for amplification of GCR α and GCR β and their specifications are shown in Table 1. Unfortunately, similar to other studies (26), it was not technically possible to design a pair of primers to distinguish the two variants GCR α and GCR- γ by qRT-PCR, because GCR- γ has only three more sequential nucleotides (TAG, codes for arginine) after nucleotide 1844 of the GCR α mRNA variant (27, 28). In order to normalize expression of our desired genes (GCR α and β), the expression of the housekeeping gene of *GAPDH* was evaluated simultaneously by qRT-PCR (as the endogenous control) for each sample (Table 1).

Table 2. Demographic data of participants.

	Severe asthma	Asthma	Healthy	Total	Total <i>P</i> -value
Gender					
- Male	8	9	27	44	
- Female	5	5	3	13	
- Total	13	14	30	57	0.52
Age (years)					
- Mean ± SE	50.23 ± 3.80	54.71 ± 4.31	39.17 ± 1.54	45.51 ± 1.80	<0.001
- 95% CI	41.95-58.51	45.39-64.4	36.01-42.33	41.89-49.13	
- Min.-Max.	21-76	24 -80	23-60	21-80	
BMI (kg/M2)					
- Mean ± SE	26.39 ± 1.25	26.41 ± 0.81	26.49 ± 0.70	26.45 ± 0.50	0.99
- 95% CI	23.65-29.14	24.65-28.16	25.04-27.93	25.44-27.45	
- Min.-Max.	19.53-35.49	20.05-30.12	18.65-34.09	18.65-35.49	

Using an Applied Biosystems 7500 sequence detection system (Applied Biosystem, Foster City, CA, USA) and Power SYBR Green I PCR Master Mix (Takara, Japan), qPCR was done according to the manufacturer's protocol. The qRT-PCR was started by a preliminary denaturing stage at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing at 60 °C for 20 sec and extension at 72 °C for 20 sec.

Statistical Analysis

The mean Ct of at least duplicate tests for each sample was used to make a comparative analysis of gene expression for GCR α , GCR β and GAPDH genes in all three groups of participants. The difference of the mean Cts between the desired genes of GCR α or GCR β and GAPDH gene has been defined as delta Ct (Δ Ct), which was used for further analysis. Independent sample *t*-test and one-way ANOVA with a Tukey's HSD post-test were used to seek statistical differences between the mean Δ Cts of the asthma, SRA, and control groups. Also, considering the optimal efficacy of the designed primers (97.2% for GCR α and 94.3% for GCR β), the $2^{-\Delta\Delta Ct}$ method (29) was used to study any fold change in gene expressions. The Pearson's test was used to evaluate correlations.

Statistical analysis was done using the Statistical Package for Social Sciences (SPSS) Version 21.0 (Microsoft, Chicago, IL, USA) at a *p*-value of 0.05.

Results

Participant Characteristics

Demographics of subjects are summarized in Table 2. Although it was desired to have matched groups, due to voluntary case selection, some inevitable differences were seen; *i.e.* ANOVA (Tukey's HSD) test showed that the mean age of the control group was significantly different from that of asthma and SRA groups ($P = 0.001$ and $P = 0.02$, respectively) and males participated more in the control group. The body mass index (BMI), which may represent a general index for nutritional condition and physical health of participants, had no significant differences among the three groups. The ethnicity of participants was nearly the same in the three groups (60% Fars, 8% Turks, 12% Lores and 20% Kurds).

Comparison of Gene Expressions

Based on the results of the test of homogeneity of variances, Tamhane's and Tukey's HSD ANOVA tests were used to analyze GCR α

Table 3. Data of GCR α and GCR β gene expressions.

	GCR α (Δ Ct)				GCR β (Δ Ct)			
	Mean \pm SE	95% CI	Min.-Max.	P-value	Mean \pm SE	95% CI	Min.-Max.	P-value
Gender								
- Male	2.46 \pm 0.16	2.12-2.79	0.63-5.01	0.77	11.72 \pm 0.34	11.02-12.42	5.69-15.78	0.85
Female	2.57 \pm 0.48	1.50-3.64	-0.32-5.83		11.58 \pm 0.70	10.05-13.12	5.78-14.73	
Groups								
Severe asthma	2.75 \pm 0.42	1.82-3.68	-0.32-4.71	0.65	11.55 \pm 0.58	10.27-12.82	8.37-15.37	0.77
Asthma	2.47 \pm 0.44	1.50-3.44	-0.12-5.83		12.09 \pm 0.61	10.75-13.43	8.38-15.73	
Healthy	2.37 \pm 0.14	2.07-2.67	0.65-3.56		11.57 \pm 0.45	10.63-12.51	5.69-15.78	
Total	2.48 \pm 0.16	2.16-2.81	-0.32-5.83		11.69 \pm 0.31	11.07-12.31	5.69-15.78	

and GCR β gene expressions (mean Δ Cts), respectively. In general, GCR α and GCR β expressions were not significantly different between genders. As indicated in Table 3 and Figure 1, there were no significant differences in gene expressions among the three groups for both isoforms. The $2^{-\Delta\Delta C_t}$ values (*i.e.* expression ratio) of asthma/control, SRA/control and SRA/asthma groups for GCR α were in order of 0.933, 0.768, and 0.823. Similarly, these values for GCR β were calculated to be 0.697, 1.014, and 1.454, respectively. These findings indicated that GCR α expression in SRA was lower than controls and asthma cases (ratio < 1) and vice versa for GCR β (Figure 1). On the other hand, the expression fold changes between the GCR isoforms in asthma, SRA, and control groups were calculated to be 786.88, 445.72, and 588.13, respectively. It means the GCR α /GCR β expression ratio in asthmatics was nearly 1.8 times that of SRA cases (786.88/445.72) and almost 1.3 times that of controls (786.88/588.13). Also, this ratio in severe asthma cases was lower than controls (detailed calculations in Supplementary file).

Correlation with Gene Expressions

There was no significant correlation (Pearson's test) between age of the participants and the Δ Cts of GCR α ($r = -0.314$, $P = 0.17$) and GCR β ($r = 0.003$, $P = 0.98$). Additionally,

Table 4 shows the correlation of age and BMI with GCR α and GCR β gene expressions (Δ Cts) in the mild asthma, severe asthma, and healthy groups. Although, GCR α Δ Cts had non-significant mild positive correlation with age in controls; it was moderately correlated (negative, near to significant) in mild asthma group and had significant negative high correlation in SRA group. Meanwhile, the age did not show any similar pattern of correlations with GCR β Δ Cts. The correlations of GCR α Δ Cts and GCR β Δ Cts were different in the three groups. Their Δ Cts were significantly correlated in both healthy volunteers ($r = 0.490$, $P = 0.007$) and mild asthmatics ($r = 0.786$, $P = 0.001$). However, there were no significant correlation between the expression of GCR α and GCR β isoforms in SRA. The dot plot diagrams are presented in Supplementary file.

Discussion

The underlying mechanisms of GC resistance are poorly understood and may vary with disease type, treatment regimen, and the genetic background of the patient. However, some reports indicate that changes in the relative expression levels of alternatively spliced variants of GCR (like GCR β) may account for GC resistance (4, 12).

The detailed differences in structure, function,

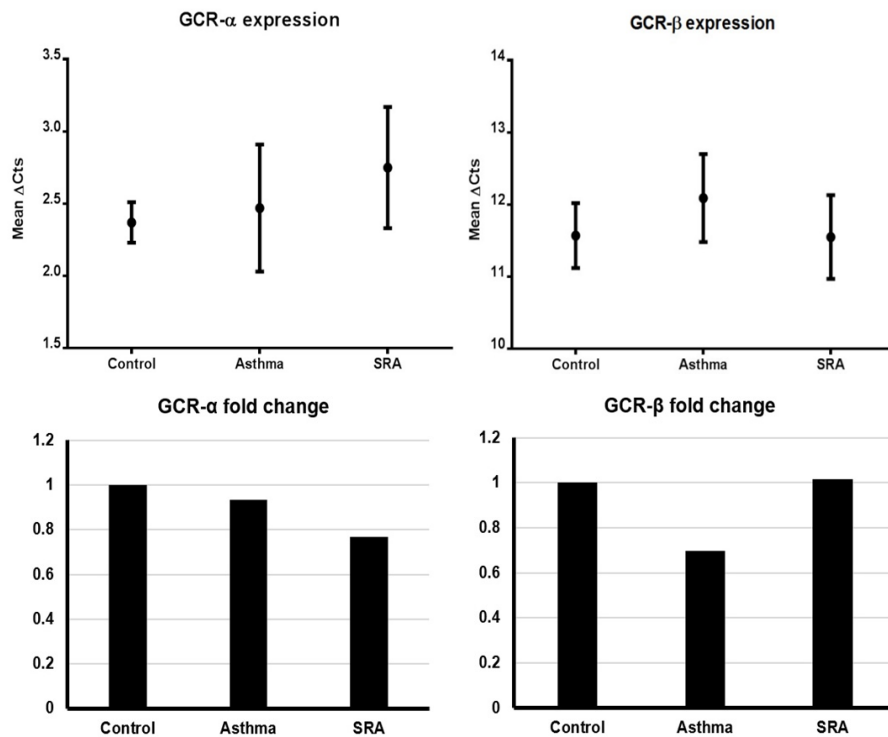


Figure 1. The GCR α and GCR β expression levels and their fold changes in the three groups. Left up: GCR α expression, Right up: GCR β expression, Left down: GCR α fold change, Right down: GCR β fold change. (Note: the controls are adjusted to 1 in fold change graphs).

and localization of the GCR α and GCR β explain why GCR β , in contrast to GCR α , does not bind to ligand (GC) and cannot affect gene expression by itself. Instead, as a dominant negative, GCR β may represses the transcriptional activity of GCR α (12). While some experiments denote that changes in relative expression of GCR α and GCR β may develop GC resistance in diseases (30, 31), they do not prove that the higher expression of GCR β is directly a causative factor of GC resistance (12). In this study, we aimed to determine the relative expression of GCR α and GCR β in the PBMLs of healthy controls, mild asthmatics, and severe asthmatics.

Totally for all participants, the GCR α and GCR β expressions (Δ Cts) were neither associated with age nor significantly different in sex groups. So, it seems that the significant difference of age and gender between the control and case groups (asthma and SRA) did not affect the interpretation of the results. Also, the lack of correlation between BMI and other variables (Table 4) shows that participants' physical

conditions did not affect the results.

The steroid regimens and pulmonary function evaluations of all cases were variable (during the time period, by methods, *etc.*); hence, there was the possibility of confounding in the results. Thus, these factors were not directly included in the analysis. Furthermore, to prevent bias, it was defined that the cases had to have the sampling criteria (according to GINA for asthma and IMI for SRA) for more than two years. Also, the cases were clinically controlled by the least corticosteroid regimens for at least two months. On the other hand, it seems that prescription of Inhaled Corticosteroids (ICS) had negligible systemic effects (32) while prescription of systemic corticosteroids only in SRA cases may alter GCR expressions. Thus, we suggested that in current study, the ICS prescription in all studied cases had minimal systemic effects on GCR expression of PBMLs, while systemic corticosteroids, inevitably altered GR expression in SRA.

Our results showed that age and GCR α Δ Cts

Table 4. Correlation of GCR α and GCR β gene expressions.

		Ager (<i>P</i> -value)	BMI r (<i>P</i> -value)	GCR α Δ Cts r (<i>P</i> -value)
Severe Asthma	Age	-		
	BMI	-0.125 (0.685)	-	
	GCR α Δ Cts	-0.709* (0.007)	-0.160 (0.602)	-
	GCR β Δ Cts	-0.081 (0.793)	0.072 (0.816)	0.533 (0.61)
Asthma	Age	-		
	BMI	0.083 (0.778)	-	
	GCR α Δ Cts	-0.509 (0.063)	-0.246 (0.397)	-
	GCR β Δ Cts	-0.404 (0.171)	-0.232 (0.446)	0.786* (0.001)
Healthy	Age	-		
	BMI	0.116 (0.542)	-	
	GCR α Δ Cts	0.139 (0.464)	-0.043 (0.821)	-
	GCR β Δ Cts	0.257 (0.178)	-0.089 (0.646)	0.490* (0.007)

*Correlation is significant at the 0.01 level (2-tailed).

were negatively correlated in asthma cases (more in SRA), while they were not correlated in controls. This means that direct correlation between age and GCR α expression, seen in asthmatics, increased with disease severity. Also, correlation between GCR α Δ Cts and GCR β Δ Cts in asthmatics was higher than in controls; but this correlation was not seen in SRA. Thus, some affecting factors (*e.g.* medications, other cellular pathways, *etc.*) may interfere in the mechanism of GCR expression in mild and severe asthmatics.

The preliminary review of our findings showed that there were no significant differences in GCR α and GCR β expressions (mean Δ Cts) among the cases and controls. Meanwhile, more detailed analysis by $2^{-\Delta\Delta C_t}$ method indicated the followings.

The comparison of GCR α fold changes (expression ratios) between the ratios of asthma/control (0.933), SRA/control (0.768), and SRA/asthma (0.823) showed that GCR α mRNAs are produced in decreasing order in controls, asthma, and SRA. Also, these figures for GCR β were 0.697, 1.013, and 1.453, respectively. It means the decreasing order of GCR β expression is SRA, control, and asthma (Figure 1). The latter means that GCR β nearly expresses 1.5 times more in SRA than in asthma. Also, it was found

that the GCR α /GCR β expression ratio decreases in the order of asthma, control, and SRA groups (786.88, 588.13 and 445.72 respectively).

These figures show that a decrease in GCR α expression occurred in all asthma cases, and even more in SRA. Meanwhile, GCR β expression increased in SRA and decreased in asthma, compared to the controls. The reduction of GCR α expression and rise in GCR β expression in SRA group (in comparison to controls) explain the decrease of GCR α /GCR β expression ratio; the findings that have been showed by IHC study previously (21). But for asthma, it seems that the decrease in GCR β expression is greater than GCR α reduction; hence the GCR α /GCR β expression ratio rises. It seems that in cases of asthma and SRA, the observed discrepancies in GCR α and GCR β expressions occurred due to pathogenesis and/or other interfering factors. Although there were some differences in sampling and methodology, our finding partly documents the study of Goleva *et al.*, (2012) who tried to introduce a marker for GC resistance in asthma. Our results were comparable with their findings that in PBMLs of SRA cases, the GCR β expression increased while the GCR α /GCR β expression ratio decreased (6).

Using different methods, several studies

on different airway diseases have showed controversies in GCRs expressions (33). An *in-vitro* study showed that corticosteroids down regulate GCR α expression in respiratory cell lines dose-dependently (15). So, our finding of lower expression of GCR α in PBMLs of the SRA cases was probably due to prescription of systemic GCs; although it does not explain low expression of GCR α in asthma cases who had not received systemic GCs. Regarding these findings and in contrast to the proposed role of GCR α in GC resistance (34), it seems that decrease in GCR α expression occurs in every asthma cases (both mild and severe), and its higher reduction in SRA cases may be due to systemic GC treatment. Thus, decline in GCR α expression is not the main cause of GC resistance; as it has been seen in non-severe forms as well.

On the other hand, Pujols *et al.* showed that in cell lines, GCs down regulate GCR β in a short period of time (15) while we found the opposite in SRA; similar to what the others have shown (12, 30 and 31). Furthermore, there was down-regulation of GCR β in asthmatics that were not on systemic GCs. Thus, it seems that in non-severe asthma, down-regulation of GCR α and GCR β occurs, with a higher degree for GCR β . But in SRA, systemic GC treatment decreases GCR α expression more, while expression of GCR β increases; in contrast to the expected effect of GCs on the cell lines. These expression changes describe why the GCR α /GCR β expression ratio increased in non-severe asthma and decreased in SRA; the finding which previously mentioned as a critical criterion for definition of GC sensitivity and resistance (12). Also, GC prescription cannot merely explain the mechanism(s) of GC resistance, unless other possible mechanisms (*e.g.* transcriptional, post-transcriptional and post-translational) are involved. It worth to be noted that we studied clinical expression where many factors can interfere. For example, the stability of GCR mRNA may be influenced by estrogens and iron. Also, some cytokines (*i.e.* *IL-2* and *IL-4*) can decrease GCR α expression without affecting GCR β (15, 18).

The increase of GCR β expression in SRA has been reported in some studies and many investigators believe that due to the negative effect of this isoform on GCR α activity, it may

be a notable cause in SRA pathophysiology. This hypothesis has been proposed in other GC resistance conditions like leukemia, ulcerative colitis, and nasal polyps, too. (12). However, some authors doubt it and mention GCR β as a very low-expressed intranuclear peptide (compared to GCR α); hence, it has less effect on GCR α activity (12, 35 and 36). Furthermore, as far as our knowledge extends, no evidence has been published so far to show either GCR β overcome GCR α or its upregulation suppresses the anti-inflammatory actions of GCR α in clinical samples of SRA (33).

Similar to the study of Jakiela *et al.*, the current study showed that GCR β is expressed hundreds times less than GCR α in all disease/health conditions (36); so logically, the beta isotype either has no role on GC resistance or should compete or affect GCR α potently, in order to influence its activities. Furthermore, the effect of GCR β may be non-competitive or unknown. For instance, there is an evidence that GCR β can disrupt GCR α nuclear translocation and it may constitute the underlying mechanism (35). This finding has been confirmed in smooth muscles of airways in severe asthma cases, too (37). As the other mechanism, it is also proposed that GCR β controls the expression of histone deacetylase 2 through inhibiting GC response elements in its promoter (38), while it is not confirmed by the others (39).

The lack of correlation between GCR α and GCR β in SRA (Table 4) has been supported by some studies. For example, the microarray study of Kino *et al.* indicated that GCR β can implement intrinsic gene-specific transcriptional activity, in a GCR α independent way. The ability of GCR β to negatively or positively regulate a large number of genes may be a hypothesis for its role in GC resistance (40). Also, some other GCR β -related metabolic pathways may be responsible for SRA. For example, Vazquez-Tello *et al.* showed that some asthma-related cytokines (like *IL-17* and *IL-23*) can significantly increase GCR β expression (18).

Thus, all together, it is not wise to consider GCRs as the main causes of GC resistance and the other proposed genetic and acquired factors (41) should be considered simultaneously. However, up-regulation of GCR β in SRA is

an actual finding, which has no relation to GC treatment. It seems that in collaboration with other molecular factors and mechanisms, GCR β is related to GC resistance; however, it is not exactly clear whether or not increased GCR β is a causative factor in SRA pathogenesis. Nevertheless, its very low expression level means that competence with GCR α is less likely and the other mechanisms are involved. Furthermore, it should be emphasized that increased GCR β expression is just one of the several proposed mechanisms for GC resistance (42).

The current cross-sectional study was performed on PBMLs, which represent systemic data; however, different cells of the immune and respiratory systems are involved in the pathogenesis of asthma. Thus, the simultaneous expression study of the candidate genes at the pulmonary and immune levels will provide more comprehensive and applicable data in this regard. But according to ethical issues, it was not possible for us to use invasive procedures (like surgery or bronchoscopy) to obtain pulmonary samples. Furthermore, the restricted inclusion criteria made our sample size limited and it may be partly the reason of non-significant results. Furthermore, asthma and its severe form are complex multifactorial disorders, which have no unique definition. Thus, various sampling criteria and bioenvironmental factors may yield in different results of various studies. It seems that well-defined longitudinal comprehensive studies of clinical, molecular, and bioenvironmental factors with reasonable sample size will provide more valid and reliable results.

It should be noted that this study just assessed the GCR activity at the transcriptional level of SRA cases. While some studies at the proteomic level have supported our findings (43, 44), many other factors at the post-transcriptional and protein levels may change the expression results. So, comprehensive proteomics studies will provide more valuable information in this respect; although, it was not possible for us to perform that, technically.

Finally, we would like to re-emphasize that there are other isoforms of GCRs, which may play roles, especially when GCR α and GCR- γ expressions are inevitably evaluated altogether.

Conclusion

The present *in-vivo* quantitative study indicates that GCRs are related to, but not directly responsible for SRA pathogenesis and their expression evaluation is not valuable for diagnosis of GC resistance. The lack of correlation and wide gap between GCRs' expression in SRA show that GCR β does not inhibit GCR α in a competitive manner. On the other hand, it seems that the reduction in GCR α /GCR β expression ratio is a major notable finding, but not a causative factor, in GC resistance and further studies on its role in diagnosis or treatment of GC resistance cases may be useful. However, other causative and/or confounding factors and mechanisms (like medications, metabolic pathways, cytokines, hormones, minerals, *etc.*) most probably interfere with the complex pathogenesis of GC resistance in severe asthmatics.

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References

- (1) Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention. Global Initiative for Asthma (GINA) (2012). Available from URL: <http://www.ginasthma.org/>.
- (2) Bousquet J, Mantzouranis E, Cruz AA, Ait-Khaled N, Baena-Cagnani CE, Bleecker ER, Brightling CE, Burney P, Bush A, Busse WW, Casale TB, Chan-Yeung M, Chen R, Chowdhury B, Chung KF, Dahl R, Drazen JM, Fabbri LM, Holgate ST, Kauffmann F, Haahtela T, Khaltaev N, Kiley JP, Masjedi MR, Mohammad Y, O'Byrne P, Partridge MR, Rabe KF, Togias A, van Weel C, Wenzel S, Zhong N, and Zuberbier T. Uniform definition of asthma severity, control, and

- exacerbations: Document presented for the World Health Organization Consultation on Severe Asthma. *J. Allergy Clin. Immunol.* (2010) 126: 926-38.
- (3) To T, Stanojevic S, Moores G, Gershon AS, Bateman ED, Cruz AA and Boulet LP. Global asthma prevalence in adults: Findings from the cross-sectional world health survey. *BMC Public Health* (2012) 12: 204.
 - (4) Barnes PJ. Severe asthma: Advances in current management and future therapy. *J. Allergy Clin. Immunol.* (2012) 129: 48-59.
 - (5) Bel EH, Sousa A, Fleming L, Bush A, Chung KF, Versnel J, Wagener AH, Wagers SS, Sterk PJ, Compton CH and Unbiased Biomarkers for the Prediction of Respiratory Disease Outcome Consortium CG. Diagnosis and definition of severe refractory asthma: an international consensus statement from the Innovative Medicine Initiative (IMI). *Thorax* (2011) 66: 910-7.
 - (6) Goleva E, Jackson LP, Gleason M and Leung DY. Usefulness of PBMCs to predict clinical response to corticosteroids in asthmatic patients. *J. Allergy Clin. Immunol.* (2012) 129: 687-93.e1.
 - (7) Adcock IM and Lane SJ. Corticosteroid-insensitive asthma: Molecular mechanisms. *J. Endocrinol.* (2003) 178: 347-55.
 - (8) Bonnans C, Chanez P and Chavis C. Lipoxins in asthma: Potential therapeutic mediators on bronchial inflammation? *Allergy* (2004) 59: 1027-41.
 - (9) Marwick JA, Adcock IM and Chung KF. Overcoming reduced glucocorticoid sensitivity in airway disease: Molecular mechanisms and therapeutic approaches. *Drugs* (2010) 70: 929-48.
 - (10) De Iudicibus S, Franca R, Martellosi S, Ventura A and Decorti G. Molecular mechanism of glucocorticoid resistance in inflammatory bowel disease. *World J. Gastroenterol.* (2011) 17: 1095-108.
 - (11) Barnes PJ. Mechanisms and resistance in glucocorticoid control of inflammation. *J. Steroid Biochem. Mol. Biol.* (2010) 120: 76-85.
 - (12) Lewis-Tuffin LJ and Cidlowski JA. The physiology of human glucocorticoid receptor beta (hGRbeta) and glucocorticoid resistance. *Ann. N. Y. Acad. Sci.* (2006) 1069: 1-9.
 - (13) Wikstrom AC. Glucocorticoid action and novel mechanisms of steroid resistance: Role of glucocorticoid receptor-interacting proteins for glucocorticoid responsiveness. *J. Endocrinol.* (2003) 178: 331-7.
 - (14) Leung DY and Bloom JW. Update on glucocorticoid action and resistance. *J. Allergy Clin. Immunol.* (2003) 111: 3-22.
 - (15) Pujols L, Mullol J, Perez M, Roca-Ferrer J, Juan M, Xaubet A, Cidlowski JA and Picado C. Expression of the human glucocorticoid receptor alpha and beta isoforms in human respiratory epithelial cells and their regulation by dexamethasone. *Am. J. Respir. Cell Mol. Biol.* (2001) 24: 49-57.
 - (16) Pujols L, Mullol J, Roca-Ferrer J, Torrego A, Xaubet A, Cidlowski JA and Picado C. Expression of glucocorticoid receptor alpha- and beta-isoforms in human cells and tissues. *Am. J. Physiol. Cell Physiol.* (2002) 283: C1324-31.
 - (17) Online Mendelian Inheritance in Man, OMIM®. Nuclear receptor subfamily 3, group C, member 1; NR3C1. *McKusick-Nathans Institute of Genetic Medicine*, Johns Hopkins University, Baltimore, MD. OMIM number 138040 [Last Edit 09/02/2016]. Available from: URL: <https://www.omim.org/entry/138040>.
 - (18) Vazquez-Tello A, Halwani R, Hamid Q and Al-Muhsen S. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J. Clin. Immunol.* (2013) 33: 466-78.
 - (19) Hamid QA, Wenzel SE, Hauk PJ, Tsicopoulos A, Wallaert B, Lafitte JJ, Chrousos GP, Szefer SJ and Leung DY. Increased glucocorticoid receptor beta in airway cells of glucocorticoid-insensitive asthma. *Am. J. Respir. Crit. Care Med.* (1999) 159: 1600-4.
 - (20) Christodoulouopoulos P, Leung DY, Elliott MW, Hogg JC, Muro S, Toda M, Laberge S and Hamid QA. Increased number of glucocorticoid receptor-beta-expressing cells in the airways in fatal asthma. *J. Allergy Clin. Immunol.* (2000) 106: 479-84.
 - (21) Bergeron C, Fukakusa M, Olivenstein R, Lemiere C, Shannon J, Ernst P, Martin JG and Hamid Q. Increased glucocorticoid receptor-beta expression, but not decreased histone deacetylase 2, in severe asthma. *J. Allergy Clin. Immunol.* (2006) 117: 703-5.
 - (22) Panek M, Pietras T, Antczak A, Gorski P, Kuna P and Szemraj J. The role of functional single nucleotide polymorphisms of the human glucocorticoid receptor gene NR3C1 in Polish patients with bronchial asthma. *Mol. Biol. Rep.* (2012) 39: 4749-57.
 - (23) Pietras T, Panek M, Tworek D, Oszajca K, Wujcik R, Gorski P, Kuna P and Szemraj J. The Bcl I single nucleotide polymorphism of the human glucocorticoid receptor gene h-GR/NR3C1 promoter in patients with bronchial asthma: pilot study. *Mol. Biol. Rep.* (2011) 38: 3953-8.
 - (24) Rivers C, Levy A, Hancock J, Lightman S and Norman M. Insertion of an amino acid in the DNA-binding domain of the glucocorticoid receptor as a result of alternative splicing. *J. Clin. Endocrinol. Metab.* (1999) 84: 4283-6.
 - (25) Turner JD, Schote AB, Keipes M and Muller CP. A new transcript splice variant of the human glucocorticoid receptor: Identification and tissue distribution of hGR Delta 313-338, an alternative exon 2 transactivation domain isoform. *Ann. N. Y. Acad. Sci.* (2007) 1095: 334-41.
 - (26) Kurokawa K, Tanahashi T, Murata A, Akaike Y, Katsuura S, Nishida K, Masuda K, Kuwano Y, Kawai T and Rokutan K. Effects of chronic academic stress on mental state and expression of glucocorticoid receptor alpha and beta isoforms in healthy Japanese medical students. *Stress* (2011) 14: 431-8.
 - (27) Homo sapiens nuclear receptor subfamily 3, group

- C, member 1 (glucocorticoid receptor) (NR3C1), transcript variant 7, mRNA. 2014 [Date published May 18, 2014], [date accessed August 4, 2014]. National Center for Biotechnology Information, U.S. National Library of Medicine: Bethesda MD. Available from URL: http://www.ncbi.nlm.nih.gov/nuccore/NM_001024094.1.
- (28) Homo sapiens nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) (NR3C1), transcript variant 1, mRNA. 2014. [Date published May 18, 2014], [date accessed August 4, 2014]. National Center for Biotechnology Information, U.S. National Library of Medicine: Bethesda MD. Available from URL: http://www.ncbi.nlm.nih.gov/nuccore/NM_000176.2.
- (29) Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-Delta Delta C(T)} Method. *Methods* (2001) 25: 402-8.
- (30) Webster JC, Oakley RH, Jewell CM and Cidlowski JA. Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative β isoform: A mechanism for the generation of glucocorticoid resistance. *Proc. Natl. Acad. Sci.* (2001) 98: 6865-70.
- (31) Hauk PJ, Goleva E, Strickland I, Vottero A, Chrousos GP, Kisich KO and Leung DY. Increased glucocorticoid receptor Beta expression converts mouse hybridoma cells to a corticosteroid-insensitive phenotype. *Am. J. Respir. Cell Mol. Biol.* (2002) 27: 361-7.
- (32) Barnes PJ. Inhaled Corticosteroids. *Pharmaceuticals* (2010) 3: 514.
- (33) Pujols L, Mullol J and Picado C. Alpha and beta glucocorticoid receptors: Relevance in airway diseases. *Curr. Allergy Asthma Rep.* (2007) 7: 93-9.
- (34) Gagliardo R, Chanez P, Vignola AM, Bousquet J, Vachier I, Godard P, Bonsignore G, Demoly P and Mathieu M. Glucocorticoid receptor alpha and beta in glucocorticoid dependent asthma. *Am. J. Respir. Crit. Care Med.* (2000) 162: 7-13.
- (35) Barnes PJ and Adcock IM. Glucocorticoid resistance in inflammatory diseases. *Lancet* (2009) 373: 1905-17.
- (36) Jakiela B, Bochenek G and Sanak M. Glucocorticoid receptor isoforms in steroid-dependent asthma. *Pol. Arch. Med. Wewn.* (2010) 120: 214-22.
- (37) Chang PJ, Michaeloudes C, Zhu J, Shaikh N, Baker J, Chung KF and Bhavsar PK. Impaired nuclear translocation of the glucocorticoid receptor in corticosteroid-insensitive airway smooth muscle in severe asthma. *Am. J. Respir. Crit. Care Med.* (2015) 191: 54-62.
- (38) Li LB, Leung DY, Martin RJ and Goleva E. Inhibition of histone deacetylase 2 expression by elevated glucocorticoid receptor beta in steroid-resistant asthma. *Am. J. Respir. Crit. Care Med.* (2010) 182: 877-83.
- (39) Butler CA, McQuaid S, Taggart CC, Weldon S, Carter R, Skibinski G, Warke TJ, Choy DF, McGarvey LP, Bradding P, Arron JR and Heaney LG. Glucocorticoid receptor beta and histone deacetylase 1 and 2 expression in the airways of severe asthma. *Thorax* (2012) 67: 392-8.
- (40) Kino T, Manoli I, Kelkar S, Wang Y, Su YA and Chrousos GP. Glucocorticoid receptor (GR) beta has intrinsic, GRalpha-independent transcriptional activity. *Biochem. Biophys. Res. Commun.* (2009) 381: 671-5.
- (41) Kalra N and Ishmael F. Cross-talk between vitamin D, estrogen and corticosteroids in glucocorticoid resistant asthma. *OA inflammation* (2014) 2: 2.
- (42) Barnes PJ. Glucocorticosteroids: Current and future directions. *Br. J. Pharmacol.* (2011) 163: 29-43.
- (43) Lo CY, Michaeloudes C, Bhavsar PK, Huang CD, Wang CH, Kuo HP and Chung KF. Increased phenotypic differentiation and reduced corticosteroid sensitivity of fibrocytes in severe asthma. *J. Allergy Clin. Immunol.* (2015) 135: 1186-95.e1-6.
- (44) Sousa AR, Lane SJ, Cidlowski JA, Staynov DZ and Lee TH. Glucocorticoid resistance in asthma is associated with elevated *in-vivo* expression of the glucocorticoid receptor beta-isoform. *J. Allergy Clin. Immunol.* (2000) 105: 943-50.