NNF77756: Role of the glucocorticoid receptor isoforms in the pathogenesis of adrenocortical tumors

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Publications and participations on scientific meetings related to the proposal
Publications:

1. Attila Patocs, Bence Acs, Agnes Szappanos, Karolina Feldman, Judit Majnik, Peter Igaz, Miklos Toth, Karoly Racz: Polymorphisms of the glucocorticoid receptor gene associated with hormonally inactive adrenal adenoma ENDO2010 92th Annual Meeting of the Endocrine Society, ENDO10, San Diego, USA, abstract, poster presentation

2. Tamás Bence Ács, Orsolya Dóra Ács, Ágnes Szappanos, Judit Majnik, Nikolett Lendvai, Nikolette Szücs, János Horányi, Miklós Tóth, Károly Rácz, Attila Patócs: A glukokortikoid receptor gén polimorfizmusainak vizsgálata mellékvesekéreg adenomas betegekben, presentation at the 23th Annual Meeting of the Hungarian Society of Endocrinology and Metabolism, Visegrad, Hungary, abstract, oral presentation

3. **Tamás Bence Ács, Karolina Feldmann, Judit Majnik, Ágnes Szappanos, Károly Rácz, Attila Patócs:** Polymorphisms of the glucocorticoid receptor gene, as phenotype modifiers in patients with hormonally inactive adrenal adenomas, poster P1-55 in Program booklet of Congress on Steroid research 27-29 March, 2011, Chicago, USA, abstract, poster presentation

4. Boyle B, Butz H, Liko I, Zalatnai A, Toth M, Feldman K, Horanyi J, Igaz P, Racz K, Patocs A.: Expression of glucocorticoid receptor isoforms in human adrenocortical adenomas.**,** Steroids. 2010 Oct;75(10):695-700., article

5. Patócs Attila, Boyle Belema, Szappanos Ágnes, Feldman Karolina, Likó István, Rácz Károly: A glukokortikoidok hatásmechanizmusában szerepet játszó génpolimorfizmusok és fehérje izoformák szerepe komplex öröklődésmenetű betegségek patogenezisében.
Magyar Biokémia Egyesület Vándorgyűlés, 2010 (2010), oral presentation

6. Henriett Butz, István Likó, Károly Rácz, Attila Patócs: Role of nuclear receptors and cofactors in the pathogenesis of adrenal tumors Semmelweis University Conference of PhD students, 2009, Budapest, Hungary, oral presentation.

Manuscripts in preparation:

Tamás Bence Ács, Karolina Feldmann, Judit Majnik, Ágnes Szappanos, Károly Rácz, Attila Patócs: Polymorphisms of the glucocorticoid receptor gene, as phenotype modifiers in patients with hormonally inactive adrenal adenomas. Invited submission to The Journal of Steroid Biochemistry and Molecular Biology. Deadline is August 1, 2011

Henriett Butz, Istvan Liko, Karolina Feldman, Karoly Racz, Attila Patocs: Expression of nuclear receptors and cofactors in adrenal cortical tumors. Plan to submit to Steroids

Tamas Bence Acs, Karolina Feldman, Henriett Butz, Peter M Szabo, Istvan Liko, Szilvia Toth, Zsolt Tulassay, Karoly Racz, Attila Patocs: Glucocorticoid receptor beta-regulated genes involved in the pathogenesis of inflammatory bowel disease, Plan to submit to Gastroenterology

Participations on scientific meetings:

1. Attila Patocs ENDO2010; 92th Annual Meeting of the Endocrine Society, San Diego, USA between June 19-22. Abstract presentation related to NNF77756
2. Attila Patocs Congress on Steroid research 27-29 March, 2011, Chicago, USA, poster presentation

3. Attila Patocs, Magyar Biokémia Egyesület Vándorgyűlés, Budapest august 25-28 2010, invated oral presentation

Background: The process of tumorigenesis in adrenal tumors that occur sporadically is obscure. However, incidentally discovered adrenal masses are frequently observed (estimated incidence is between 0.35-4.36% in the general population) and they raise serious diagnostic and therapeutic problems. The percentage of bilateral tumors among adrenal incidentalomas is between 9-15%, and this high prevalence of bilateral tumors raise the possibility that systemic effects, such as genetic alterations, could play a role in their pathogenesis. The metabolic syndrome, hypertension, obesity and abnormalities of the hypothalamic-pituitary-adrenal (HPA) axis, such as lack of cortisol suppression after dexamethasone administration are more common in patients with incidentally discovered adrenal adenomas than in healthy individuals.

Another important aspect of adrenal adenomas is its association with complex hormonal and metabolic profile which resembles to those observed in patients with glucocorticoid excess. Glucocorticoids produced in the adrenal cortex act by binding to a specific intracellular protein, the glucocorticoid receptor (GR), which in turn modulates transcription of genes containing a special sequence, the glucocorticoid responsive element (GRE). Two isoforms of the GR were originally described; the functionally active GRα isoform encoded by exons 2–8 and part of exon 9 of the *GR* gene consists of 777 amino acids, and the GRß isoform in which the 50 amino acids at the C-terminal encoded by part of exon 9 are replaced by 15 different amino acids encoded by part of the exon 9ß. It was earlier reported that the GRß isoform represents a potential endogenous inhibitor of glucocorticoid action in humans and its expression is increased in steroid resistant states and in patients with Cushing's syndrome.

The concentration of GR within each cell depends on several factors, including the cell type, cell cycle and stage of differentiation. In the absence of their ligands, the GR receptor remains sequestered in the cytoplasm through interactions with large multiprotein complexes containing heat shock proteins and cofactors. In our previous work we studied some aspects of the possible role of polymorphic GR gene variants in the pathophysiology of unilateral and bilateral adrenal incidentalomas. We found significantly higher carrier and allelic frequencies of the N363S variant of the *GR* in patients with bilateral incidentalomas compared to those with unilateral tumors or controls, which raised the possibility that an increased sensitivity to glucocorticoids reportedly present in N363S carriers may play a role in the development of bilateral adrenal tumors. Recently, it was reported that the GR is expressed in the human adrenal gland. In addition, nuclear cofactors are also involved in the modulation of gene expression during development, differentiation and endocrine therapies. Wang and co-workers using two different cell lines and cell-free pull-down and immunoprecipitation assays showed that nuclear receptor corepressor and silencing mediator of retinoid and thyroid hormone receptor associate with agonist and antagonist complexes of the GRs. It was suggested that the mutually antagonistic equilibrium interactions of corepressors and coactivators modulate the dose-response curve and partial agonist activity of the GR complexes.

Aims and results

1. **Genetic variants of *GR* gene can be related to the adrenal tumorigenesis (Objective 1).**

For this specific aim we evaluated 134 patients with adrenal adenoma (102 with hormonally inactive adrenal adenoma: HI; and 32 with cortisol producing: CP tumor) and 129 healthy, Hungarian controls. Written informed consent form for genetic analysis will be collected. The molecular biological examination was performed using a Taqman allele-discrimination assay,

on ABI7500 real-time PCR instrument. The carrier frequency of the N363S was higher in HI than in CP or healthy population, while the prevalence of A3669G (located in GRβ) was lower in patients with HI than in CP or controls (N363S: 7.4% vs 2,7% $p<0.05$; A3669G: 14.7% vs. 22.1% $p<0.05$). These associations were even stronger in patients with bilateral HI tumors: N363S: 10.5% vs. 2.7% $p<0.05$; A3669G: 10.5% vs. 22.1% $p<0.05$) (Table 1). Similarly to N363S, the prevalence of haplotypes containing this variant was significantly higher in patients with HI than in controls or patients with CP especial in bilateral HI group (0,25 in bilateral HI vs. 0.05 in healthy or 0.03 in CP, $p<0.05$), while the prevalence of haplotypes containing A3669G was lower in HI than in controls or patients with CP (0.15 vs. 0.34 or 0.34, $p<0.05$). The ER22/23EK was identified both in patients and controls together with A3669G. In CP group no associations between *GR* SNPs were observed.

Table 1: Allele prevalence of GR polymorphisms among patients with adrenal incidentaloma and healthy individuals

GR polymorphisms	Unilateral	Bilateral	Unilateral and bilateral	healthy control
Bcl*I*	0.22*	0.25	0.23*	0.34
N363S	0,04	0.105**	0.07**	0.027
A3669G	0.17	0.1**	0.14**	0.22
ER22/23EK	0.03	0.07	0.03	0.08

*p=0.011; **p<0,05

Various **genotype-phenotype associations** have been observed:

- the N363S polymorphism associated with type 2 diabetes mellitus
- the polymorphism A3669G associated with higher body weight and body mass index
- the BclI polymorphisms of the GR associated with higher systolic blood pressure and higher cholesterol level (Table 2)

Table 2: Associations of GR polymorphisms with clinico-pathological and biochemical parameters in patietns with adrenal incidentalomas

	Wild Type (n=30)	BCL (n=28)	N363S (n=15)	A3669G (n=23)	BclI +A3669G (n=8)
Serum cortisol					
Morning (08.00)	11.9 ± 6.6	10.8 ± 4.7	9.6 ± 3.7	10.7 ± 5	10.8 ± 6.8
Night (24.00)	4.2± 4.4	4.4 ± 4.6	3.3 ± 1.9	4.3 ± 3.4	3.8 ± 2.6
After ACTH stimulation	60.5 ± 29.8	63.5 ± 28.9	71 ± 35	56 ± 20	65 ± 46
After LDD	1.9 ± 0.8	2.07 ± 1.1	2 ± 0.9	2.2 ± 1.6	2.3 ± 0.96
ACTH					
Morning (08.00)	27 ± 16	36 ± 63	17 ± 6	18.1 ± 15	12.2 ± 10
After metyrapon test	148 ± 132	**346 ± 317***	168 ± 119	136 ± 143	172 ± 70
DHEAS	84 ± 89	59 ± 44	82 ± 100	78.2 ± 148	140 ± 243
Tumorsize (mm)	31 ± 19	27 ± 15	23.2 ± 12.6	26.9 ± 17	26 ± 20
Blood pressure before surgery (Hgmm)					
systolic	134 ± 21	143 ± 21	144 ± 26	146 ± 23	144 ± 20
diastolic	83 ± 7	86 ± 12	88 ± 14	89 ± 10	88 ± 11
Total cholesterol	5.7 ± 0.8	6.3 ± 0.9	6.4 ± 1.7	6 ± 1.3	3.9 ± 1.2
Serum triglyceride	1.6 ± 0.9	1.9 ± 1.2	2.2 ± 1.5	1.7 ± 0.9	1.2 ± 0.4

Weight (kg)	72.1 ±14.5	**85.1 ± 23.9***	79.7 ± 13.7	**84.3 ± 15.7***	**91.8 ± 17.7***
BMI (kg/m2)	27.4 ± 5.7	**31.2 ± 5.3***	31.1 ± 6.5	**30.9 ± 4.3***	31.8 ± 3.4
Prevalence of HT	0.77	0.75	0.64	0.76	0.71
Prevalence of T2DM	0.32	0.24	**0.57***	0.38	0.42

*: p<0,05

Our results together suggest that the increased sensitivity against endogenous glucocorticoids can play a role in the pathogenesis of hormonally inactive adrenal incidentalomas especial in bilateral cases. In addition BclI, A3669G may contribute to the development of obesity in these patients, while the N363S represents susceptibility for type 2 diabetes mellitus in this patient group. The abstract containing all these data have been submitted and accepted at the 92th Annual Meeting of the Endocrine Society, ENDO10, San Diego, USA, at the 23th Annual Meeting of the Hungarian Society of Endocrinology and Metabolism, Visegrad, Hungary and at Congress on Steroid Research in Chicago, 2011[1-3]. Manuscript containing these data is in preparation, the deadline for its submission to The Journal of Steroid Biochemistry and Molecular Biology is August 1, 2011.

2. The second objective was evaluation of the expression of the GR isoforms at mRNA and protein levels in different adrenal adenoma tissues. The GR, using an N-terminal antibody is expressed in normal human adrenal cortex, however, no data have been reported about the GR expression in pathologic adrenocortical tissues and about expression of specific isoforms of the GR. We examined the GRα and GRβ on mRNA level by quantitative real-time PCR (qRT-PCR) in 31 adrenal tissues including 19 non-functioning adenomas (NFA), 6 cortisol-producing adenomas (CPA) and 6 normal adrenocortical tissues, and on protein level by immunohistochemistry. First, we extracted RNA from fresh frozen adrenal tumor samples using Qiagen RNA extraction kit, following the manufacturer's instruction. Reverse transcription and qRT-PCR measurements were performed using our Taqman assays. The specificity of primers and probes were demonstrated by gel electrophoresis and by direct DNA sequencing (**Figure 1., Panel A).**

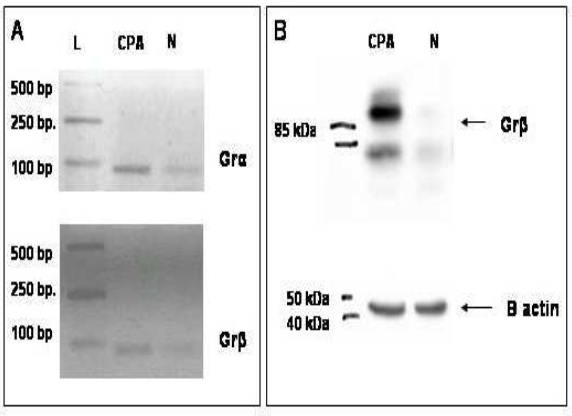
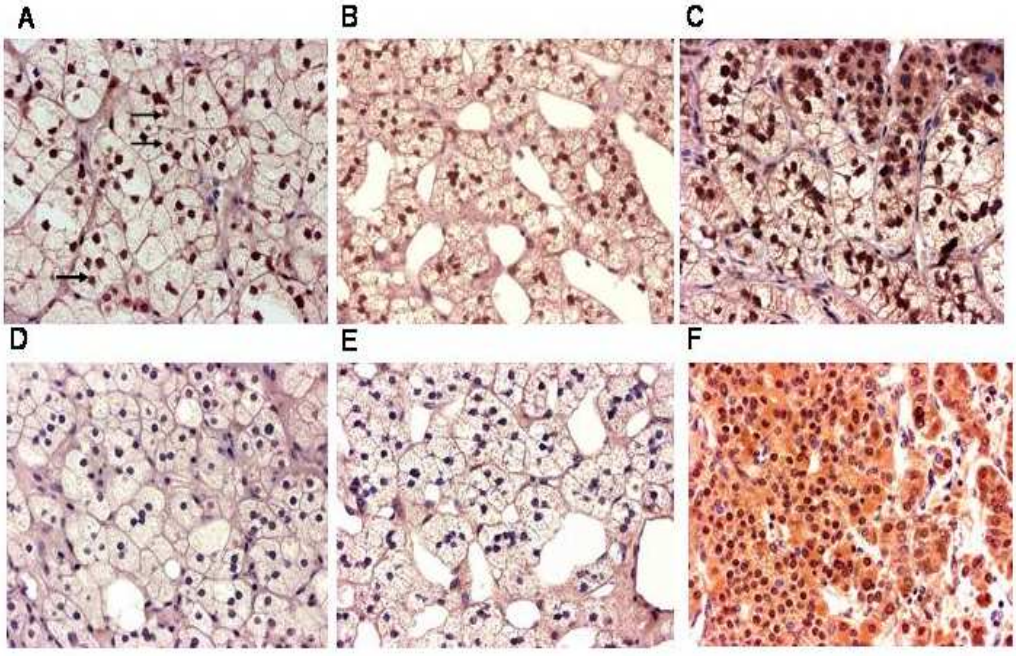


Figure 1: Panel A: Gel electrophoresis of quantitative RT-PCR amplicons of Grα and GRβ showing a single specific band for both Grα and GRβ in CPA and normal adrenal tissue (N). Panel B: Western blot analysis of GRβ and β-actin in CPA and normal adrenal tissue (N) illustrating a strong signal of 90 kDa in CPA and the absence of specific signal in normal adrenal tissue (N). L: DNA ladder.

In order to detect the GRβ isoform on protein level we needed to examine the specificity of the anti-GRβ antibody. We performed Western blot analysis of total protein extracts of adrenal samples. GRβ protein was detected by immunoblotting with GRβ antibody (GRß, Abcam 3581, dilution: 1:1000, Abcam plc, Cambridge, UK) followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (sc-2004; dilution 1:7000) (Santa Cruz Biotechnology, Santa Cruz, USA) (**Figure 1. Panel B**). Compared to normal adrenocortical tissues, both GRα and GRβ mRNAs were significantly increased in CPA

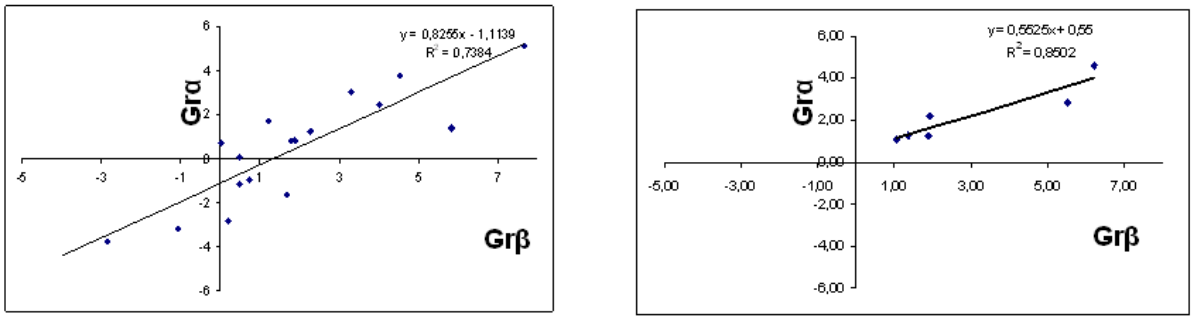
whereas GRα, but not GRβ mRNA expression was moderately increased in NFA. Using anti-GRα antibody a strong nuclear staining was observed in NFA and CPA, and a less remarkable immunoreactivity was detected in some nuclei of normal adrenocortical cells (Figure 2). GRβ immunostaining was absent in normal adrenal tissues and NFA, while a strong cytoplasmic and nuclear immunoreaction was found in CPA. These data together may indicate that the altered expression of GRα and GRβ in CPA and that of GRα in NFA raises their possible role in the pathophysiology of these adrenal tumors [3].

Figure 2. Immunohistochemical detection of GRα (A-C) and GRβ (D-F) in human adrenocortical tissues (magnification 400x). A and D, normal adrenocortical tissues; B and E, non-functioning adrenocortical adenomas; C and F, cortisol-producing adrenocortical adenomas. Arrows show nuclear staining for Grα in some normal adrenocortical cells (Boyle et al, Steroids. 2010 Oct;75(10):695-700)



In addition a strong correlation was observed between the expression of GRα and GRβ in hormonally inactive and cortisol-producing adrenal adenomas (**Figure 3**), which may suggest that the expression of the GR isoforms are regulated by a common promoter.

Figure 3: Correlation between mRNA expression of GRα and GRβ in hormonally inactive adrenal adenomas (left panel) and in cortisol producing tumors (right panel). (Boyle et al, Steroids. 2010 Oct;75(10):695-700)



Objective 3. Evaluation of the expression of nuclear cofactors at mRNA level in different adrenal adenoma tissues (3a); and in *in vitro* models of glucocorticoid action (3b).

Many aspects of the GR related gene regulation machinery complex are poorly understood. Our proposal aims to evaluate systematically the role of the GR and other nuclear cofactors and corepressors in the pathogenesis of different adrenal tumors. Using custom designed gene expression array (TLDA: Taqman Low-density Array) we analyzed the expression level of 94 different genes in normal and pathological adrenal tissues. To date, we have collected a total of 48 adrenal tissue specimens (10 normal-N, 12 CPA, 23 HI and 3 adrenocortical carcinomas). RNA extraction and gene expression analysis were performed in 12 samples (5 CPA, 4 HI and 3N). Statistically significant differences were observed between CPA and N samples, and these genes were also upregulated after treatment of H295R human adrenocortical cells with dexamethasone (100nm), suggesting that these alterations are related to the increased concentration of glucocorticoids in the adrenal gland (Objective 3b)[6].

3a: Using custom designed gene expression array (TLDA; Taqman low density array) we analyzed the expression level of 94 different genes including 47 nuclear receptors, 12 nuclear receptor coactivators and 4 endogenous controls in normal and pathological adrenal tissues.
Adrenocortical tumor tissues were obtained from 17 patients undergoing adrenalectomy, including 11 patients with hormonally inactive adrenal adenoma (HI; 10 females, one male, median age at the time of diagnosis: 41.8±10.5 years) and 6 patients with Cushing's syndrome due to cortisol-producing adrenal adenoma (CPA; 5 females and one male, median age at the time of diagnosis: 49±9.3 years). In addition 6 normal adrenal were also examined. We found 2 genes showing significant decreased expression in CPA compared to normal tissue, one gene (RARB) showing significantly decreased expression in HI compared to normal tissue and another one (NR0B1) showing overexpression in CPA compared to normal adrenal gland **(Table 3).**

Genes showing significant alteration	**CPA vs. normal adrenal tissue**		**HI vs. healthy adrenal tissue**	
	fold change	**p**	**fold change**	**p**
HSP90B1	0.478	0.033	0.64	NS
NR2F1	0.440	0.044	0.55	NS
RARB	0.363	NS	0.362	0.047
NR0B1	2.71	NS	1,01	NS

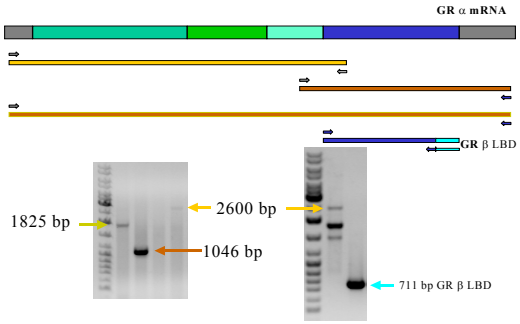
3b: Using the same experimental TLDA platform we examined the expression of nuclear cofactors in H295R human adrenocortical cell line before and after dexamethasone treatment in order to mimic conditions observed in CPA. We identified 17 nuclear cofactors and corepressors significantly altered by Dex treatment. Of particularly interesting genes HSP90B1, RARB and NR0B1 significantly changed after Dex treatment and correlated with cortisol level secreted by these cells into the maintaining media. However, expression of the NR2F1 showed just a tendency suggesting that this cofactor may not be related directly to the glucocorticoid receptor (GR) mediated effect.
From these preliminary data we may conclude that nuclear coactivators and corepressors may be involved in the pathogenesis of adrenal adenomas, mainly in hormone-secreting tumors, but further studies warranted for clarification of their role. One particularly interested factor, NR2F1 (COUP-TFI: transcription factor coup I, EAR3: ERBA related 3) has been identified as a potential GR-independent factor which downregulation may account for adenomatogenesis of CPA. NR2F1 is an orphan receptor, contains a DNA-binding and a

putative ligand-binding domain. In the mouse, COUP-TFI and COUP-TFII (Nr2f1 and Nr2f2) are essential for early neural development and organogenesis, several genes has been validated as COUP-TFI targets: Fabp7, Crabp1, Sod1 Casq1-, Foxo3a. Its potential role in adrenal tumorigenesis needs further studies. These results were presented at two scientific meetings and the manuscript containing our preliminary data is in preparation[5,6].

Objective 4: Identification of specific genes differentially regulated by the two isoforms of the GR.

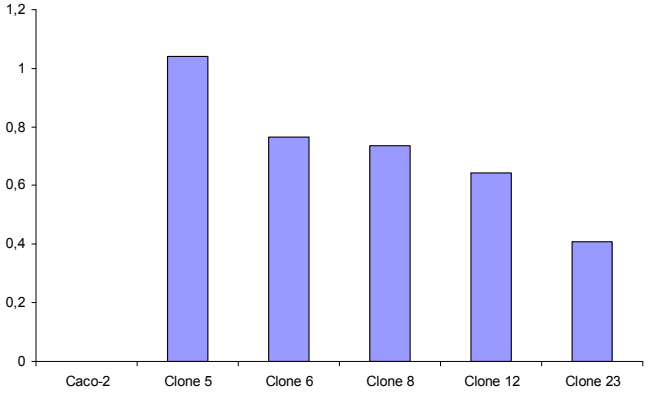
For this specific aim we need a model which contains this isoform. Therefore, we started to make a cell line which stably expresses the GRß isoform**.** GR receptor isoforms were cloned from HeLa cells by two-steps RT-PCR. The first product corresponded to common 5' α and β fragment ending at the ligand binding domain (LBD). The second fragment was the LBD domain of the GRα isoform. The fragments generated in the first two RT-PCRs were used as template for the second PCR amplification that resulted in the full length (2600bp) PCR product of the GRα. To generate the DNA fragment corresponding to the β isoform PCR reaction was performed, using the α isoform as a template. The sense primer corresponded to the common GRα/GRβ starting point of the LBD domain, and a 67 bp-long antisense primer containing a 19 bp-long sequence, which is the last common sequence in the α and β forms, and a 48 bp-long β specific sequence, which contains the full length of the β isoform specific sequence. The reaction resulted in a 711 bp-long PCR product (Fig.5).

Figure 5. PCR products of α and β isoforms of the human glucocorticoid receptor



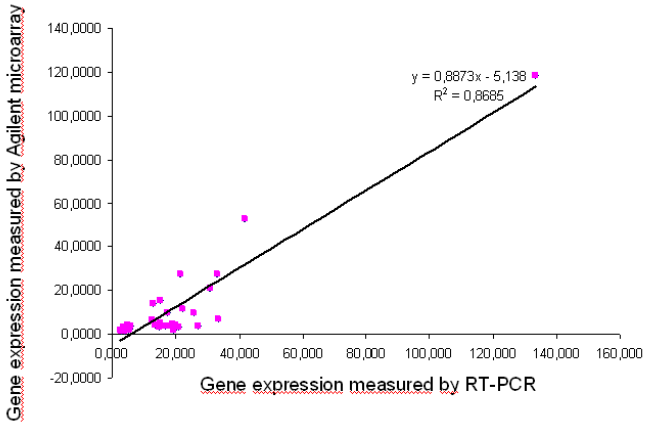
The PCR fragments coding the different GR isoforms were cloned into the eukaryotic expression vector pcDNA3.1/V5-His-TOPO. The completed constructions were verified by direct DNA sequencing. Transfection of Caco cells was performed using Lipofectamine2000 transfection reagent. The clonal selection was performed using neomycin antibiotic (500 µmol/ml). The expression of the GRβ on mRNA level was detected by RT-PCR, with the same primers as were used for qRT-PCR experiments performed in adrenal tissue. The GRß content of Caco-2-GRß cells were quantified by quantitative real-time PCR using Taqman assays. In our Caco-2GR model the GRα/GRβ ratio varied between 1:1-0,4 (clone5-clone23) which all were significantly higher of that measured in wild type Caco-2 where this ratio is: 1:0,001 **(Figure 6).**

Figure 6: Ratio between the expression levels of the Grα and GRβ isoforms in basic Caco-2 and in Caco-2-Grβ clones.



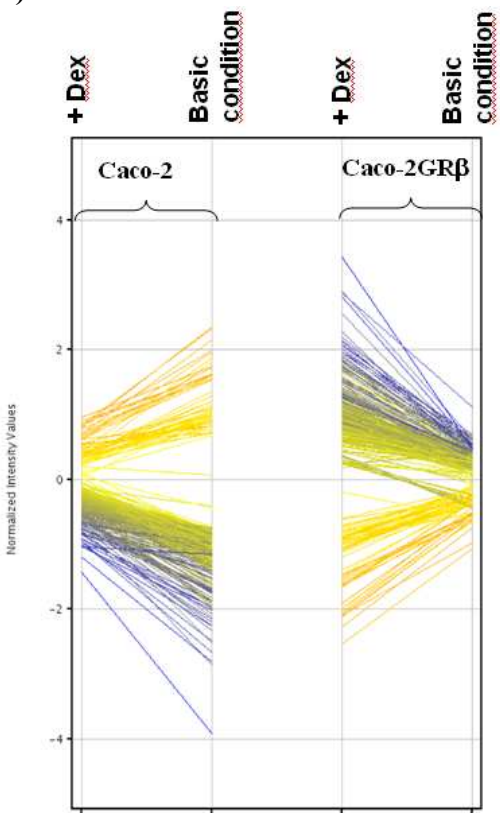
Both the basic Caco-2 and Caco-2-GRß cell lines were treated with Dexamethasone (Dex, 100 nmol) for 8 h. After Dex treatment cells were harvested and total RNA was isolated. A whole genome gene expression array was performed by Agilent 44K microarray. Microarray data analysis revealed that 1156 transcripts (978 genes) were differentially expressed in Caco-2-GRß cells compared to the basic Caco-2 cell line. Of these, 228 were underexpressed and 750 overexpressed. We validated these microarray data with individual Taqman gene expression assays. Totally, 61 genes were included into validation experiment. Of these, 51 showed the same expression pattern as was detected by microarray, demonstrating that the specificity of the microarray gene expression analysis is 82% (**Figure 7**).

Figure 7: Correlation between gene expression measured by microarray and individual Taqmn assays.



The global gene expression profile indicated that the Caco-2GRβ cell line behaves like the Dex treated Caco-2 cell line, the global gene expression profile of both under and overexpressed genes has been shifted, hence suggesting that the GRβ isoform contribute to the glucocorticoid resistance in this cell line **(Figure 8)**.

Figure 8: Gene expression change after dexamethasone treatment in Caco-2 and Caco-2-GRβ cell lines. Expression profile of dex-treated Caco-2 cell line was similar to those observed in basic condition of Caco-2-GRβ clone. In addition similar genes were altered in both cell lines, just a shift in expression profile was observed in Caco-2-GRβ clone compared to Caco-2.



Of the significantly overexpressed genes we identified genes which are involved in regulation of apoptosis (Bcl2, Casp1), metabolism (CPE, NNMT, Large, PAH, SLC26A9), immune and inflammatory response (IL1RAP, SAMD9, DEFB1), signal transduction (RHOBTB1, RICH2, TGFB2), gene transcription and/or RNA processing regulators (RBMS3, SATB1) and genes encoding matrix proteins (VIM, CDH6, SPP1, SPARC, COL4A6).

Among genes with reduced expression in Caco-2-GRß cells compared to the basic Caco-2 cell line genes involved in immune response (CXCL1, CXCL2, CXCL3), in gene transcription (NFIA, RPL39L), metabolism (PDE4A), cell signaling (SAMD1, S100P, SSTR1) have been identified. Gene set enrichment analysis (GSEA) with highly significant prediction showed that in Caco-2GRβ an inhibition of genes containing consensus site for E2F1 transcription factor, and an overexpression of genes containing consensus site for androgen receptor (AR) and GR, respectively. These results together may suggest that the GRβ isoform may forms a heterodimer not only with GRα but also with E2F1 and/or AR. In addition in basic Caco-2 cell line the GSEA analysis confirmed the previous findings that the dex treatment contributes to downregulation of genes containing consensus site for AP1 and CREB binding sites.

Caco-2 cell line is an ideal model for studying the pathomechanism of inflammatory bowel disease including diseases: Crohn disease and colitis ulcerosa. We evaluated using bioinformatical approaches the expression profiles of samples obtained from patients suffering from colitis ulcerosa and Crohn disease submitted to gene expression omnibus in order to correlate our experimental data to those obtained from patients. Our analysis revealed that expression of 90 genes were both included in Caco-2-GRβ versus Caco-2 and Crohn disease versus normal mucosa comparisons and 100 genes were common in Caco-2-GRβ versus Caco-2 and Colitis ulcerosa versus normal mucosa comparisons. Further analysis of patient samples we found that according to the treatment status in Crohn disease group we found 10 genes which were differentially expressed in responders to treatment compared to non-responders and also these 10 were found as regulated by GRβ. Similarly, in colitis group we identified 5 such genes.

These result need to be confirmed, therefore we plan to further study the relevance of our findings, but these results present starting point for identification of novel biomarkers for prediction of treatment success in patients with inflammatory bowel disease, and also potential novel therapeutic targets in this disease.