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Expression of Early Growth Response Gene-2 and Regulated Cytokines Correlates with Recovery from Guillain–Barré Syndrome

Ernesto Doncel-Pérez,*1 Lourdes Mateos-Hernández,*1 Eduardo Pareja,‡ Ángel García-Forcada,* Margarita Villar,† Raquel Tobes,‡ Francisco Romero Ganauz,* Virginia Vila del Sol,* Ricardo Ramos,§ Isabel G. Fernández de Mera,† and José de la Fuente‡,*§

Guillain–Barré syndrome (GBS) is an immune-mediated peripheral neuropathy. The goal of this research was the identification of biomarkers associated with recovery from GBS. In this study, we compared the transcriptome of PBMCs from a GBS patient and her healthy twin to discover possible correlates of disease progression and recovery. The study was then extended using GBS and spinal cord injury unrelated patients with similar medications and healthy individuals. The early growth response gene-2 (EGR2) was upregulated in GBS patients during disease recovery. The results provided evidence for the implication of EGR2 in GBS and suggested a role for EGR2 in the regulation of IL-17, IL-22, IL-28A, and TNF-β cytokines in GBS patients. These results identified biomarkers associated with GBS recovery and suggested that EGR2 overexpression has a pivotal role in the downregulation of cytokines implicated in the pathophysiology of this acute neuropathy. 

Guillain–Barré syndrome (GBS) is an immune-mediated peripheral neuropathy involving both the myelin sheath and axons that affects the peripheral nervous system (1). It has been identified as the main cause of the acute neuromuscular paralysis worldwide, with an annual incidence ranging from 0.81 to 1.89 cases per 100,000 people (2). GBS is characterized by rapidly evolving ascending weakness, mild sensory loss, and hyporeflexia or areflexia, progressing to a nadir over up to 4 wk (2). Besides the classic presentation of ascending paralysis in demyelinating GBS, clinical variants are based on the types of nerve fibers involved (motor and/or sensory, cranial or autonomic), predominant mode of fiber injury (demyelinating versus axonal), and alterations in consciousness. Consequently, different subtypes of GBS such as acute inflammatory demyelinating polyneuropathy, Miller Fisher syndrome, acute motor axonal neuropathy (AMAN), acute motor sensory axonal neuropathy (AMSAN), acute panautonomic neuropathy, and Bickerstaff’s brainstem encephalitis have been described previously (1–3).

Conventional treatment strategies for patients with GBS include plasmapheresis, i.v. Ig administration, and immunosuppressive drugs (2, 4). However, these treatments are relatively inefficient, invasive, and expensive (4). Therefore, it is necessary to implement new treatments to prevent both the development of the syndrome as well as the disability persistent in GBS patients.

Although GBS is considered to be an autoimmune disease with the involvement of both cellular and humoral immune responses (2), little is known about the molecular mechanisms involved in the pathogenesis of GBS and its variants (2, 5–9). Strong evidence support a synergistic effect of these two viruses on the peripheral nerve (13). In addition, different types of viral hepatitis have been related to GBS (12). A high proportion of patients monoinfected or coinfected with the HIV and hepatitis C virus (HCV) develop GBS, pointing to an additive or synergistic effect of these two viruses on the peripheral nerve (13). Recent results showed that infection with one of these microorganisms leads to Ab production, which cross-reacts with gangliosides.
and other glycolipids leading to myelin destruction by complement activation or by Abs targeting macrophages via the FcR and leading to both demyelination and nerve conduction failure (2, 14).

The goal of the research reported in this paper was the identification of biomarkers associated with recovery from GBS. To address this objective, we first characterized differences in the transcriptome of PBMC between a GBS patient and her healthy twin to discover possible correlates of disease progression and recovery in individuals with the same genetic background. The results were then corroborated with the mRNA level and contrasted with serum cytokine levels in unrelated GBS and spinal cord injury (SCI) patients with similar medications and in healthy individuals.

Materials and Methods

Patients and controls

The use of human material, including PBMC and peripheral blood serum samples from GBS and SCI patients and healthy individuals, was approved by the Clinical Research Ethics Committee for Hospitals of Toledo City (permit number 17), and informed consent was obtained from all individuals in compliance with the Helsinki Declaration. Blood samples of patients and controls were extracted by nursing personnel to patients and controls in the Paraplegics National Hospital (Toledo, Spain). Data on patients and control individuals are described in Table I.

Samples

PBMC were isolated by Ficoll gradient from blood samples and resuspended in nucleic acid preserving solution (TRizol; Invitrogen) and conserved at −80°C until used for RNA extraction. For separation of serum from the total blood, a sterile tube without anticoagulant was used. The blood from each individual (5 ml) was maintained in standing position at room temperature for clotting (20–30 min) and centrifuged at 1500 × g for 20 min at room temperature. Serum was collected and conserved at −20°C until used for cytokine protein analysis.

RNA sequencing

Samples from 0.5 to 10 × 10^6 PBMC collected simultaneously from the GBS patient and her healthy twin at three different time points during disease progression from hospitalization in the intensive care unit (T1), at intermediate care (T2), and finally at conclusion of the locomotion rehabilitation program when the patient was close to be discharged the hospital (T3) were used for RNA extraction using the RNeasy Minikit (Qiagen). RNA quality was assessed by measuring RNA integrity number in the bioanalyzer (Agilent), showing an average RNA integrity number value of 9.4 (8.8–9.8). The polyA + RNA was purified, and RNA libraries were prepared according to standard procedures using the TruSeq RNA kit (Illumina). After library preparation, samples were sequenced in the same temperature range for every sample. The Clorfl31 mRNA levels were normalized using the genNorm ΔΔct method as implemented by Bio-Rad iQ5 Standard Edition, version 2.0 (17). Normalized Ct values were compared between samples by two-tailed parametric moderated t test (Limma) using Benjamini–Hochberg false-discovery rate correction (p = 0.05; n = 1 or 3 independent samples with two replicates each). For Clorfl31, real-time RT-PCR was performed on RNA samples with primers designed on National Center for Biotechnology Information Primer-Blast Tool (MIW29C_F: 5'-ATCTTCTACAACGGCGTG-3' and MIR29C_R: 5'-TCCCCCTCATCFAACC-3') normalizing against PGK1 (H.PKG1_F: 5'-GCTGGACAACGCTGAGCTTA-3' and H.PKG1_R: 5'-TTGGCTTCAATTGTCGAACCA-3') and GAPDH (H.GAPDH_F: 5'-AAAAAGAAGATGCCGGCTGACTGT-3' and H.GAPDH_R: 5'-GCCAGATTAAAGACCGCCCT-3') using the iScript one-step RT-PCR kit with SYBR green and the iQ5 thermal cycler (Bio-Rad, Hercules, CA) following the manufacturer’s recommendations. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicons denatured consistently in the same temperature range for every sample. The Clorfl31 mRNA levels were normalized using the genNorm ΔΔct method as implemented by Bio-Rad iQ5 Standard Edition, version 2.0 (17). Normalized Ct values were compared between samples by Student t test with unequal variance (p = 0.05; n = 1 or 3 independent samples with two replicates each).

Human Th1/Th2/Th17 Ab array

Cytokine serum levels were determined in GBS (n = 8) and SCI (n = 4) unrelated patients and healthy individuals (n = 4) using the quantitative Human Th1/Th2/Th17 Ab Array (20 targets) (Abcam) following manufacturer’s protocol. Four technical replicates were included for each sample. Patient and control values were compared by Student t test with unequal variance (p = 0.05).

Results

Transcriptomics analysis identifies EGR2 as a potential correlate for GBS in blood cells

The first objective of this study was to identify in PBMC relevant correlates for GBS progression and recovery. The analysis was conducted with blood samples collected from a GBS patient and her healthy identical twin to reduce variations because of differences in genetic background. The patient was diagnosed with GBS (AMSAN variant) and suffered from infection with HCV (Fig. 1A). The patient’s clinical GBS profile was characterized by a monophasic course, during which the patient went from no weakness to rapid loss of muscular function including respiratory muscles, and then started to recover muscle strength and movement in 7 wk (Fig. 1A). Her twin sister came every day to the hospital to look after the patient and remained healthy during the duration of the study (Fig. 1A). Blood samples were collected simultaneously from the patient and control healthy twin at three different time points during disease progression from hospitalization in the intensive care unit (T1), passing to intermediate care (T2), and at conclusion of the locomotion rehabilitation program when the patient was close to be discharged from the hospital (T3) (Fig. 1A). The transcriptomics analysis was conducted to compare GBS patient and control individual gene expression profiles at different time points, T1–T3. Differential gene expression analysis resulted in significant differences between GBS patient and control healthy twin at T3 only with 4 upregulated and 52 downregulated genes (Supplemental Table II). The lack of differences in the transcriptome between GBS patient and control healthy
twin at T1 and T2 supported the selection of individuals with similar genetic backgrounds to reduce individual variations in the transcriptomics analysis. Furthermore, the identification of only 56 differentially expressed genes between GBS patient and the control individual at T3 suggested a role for these genes during recovery from GBS.

The downregulated genes affected biological processes relevant for GBS such as immunity, blood pressure and coagulation, nervous system, osteogenesis, stress, and metabolic processes (Fig. 1B). However, the study was focused on the upregulated genes coding for Clorfl31 microRNA, EGR1, EGR2, and GBP1 as possible correlates for GBS progression and recovery (Fig. 1C). The expression of these genes was characterized by quantitative RT-PCR in PBMC from the GBS patient and control healthy twin at T3 (Fig. 1D) and in GBS (n = 3) and SCI unrelated patients (n = 3) with similar medical treatments (E). Normalized Ct values were compared between samples by Student t test with unequal variance (p = 0.05; two replicates for each sample). The same color code was used to identify upregulated genes in (C)–(E).

Nevertheless, the results showed that only EGR2 expression was confirmed as upregulated in GBS patients when compared with SCI patients (Fig. 1E). These results suggested that the expression of the other upregulated genes identified in the GBS patient when compared with the control twin were probably affected by pharmacological treatments and provided additional support for the role of EGR2 in GBS and its possible use as a correlate for disease recovery.

The analysis of serum cytokines possibly regulated by EGR2 reveals potential markers for GBS

Previous results suggested a relationship between EGR2, IL-17 cytokine levels, and GBS (18, 19), and therefore, the second objective of this study was to identify the possible effect of EGR2 upregulation on the serum cytokine protein levels in GBS patients. For this analysis, the human Th1/Th2/Th17 Ab array targeting 20 cytokines was used to analyze serum samples collected from GBS (n = 8) and SCI (n = 4) unrelated patients with similar medications and healthy control individuals (n = 4) (Table I).

The results showed that IL-2, -5, -12, -13, -17, -17F, -21, -22, -23, and -28A; TGF-β; and TNF-α and TNF-β levels were significantly lower in GBS patients when compared with healthy individuals (Fig. 2A). Serum levels for GM-CSF, IFN-γ, IL-1β, IL-6, IL-10, and MIP-3α were not affected in GBS patients (Fig. 2A).

<table>
<thead>
<tr>
<th>Gene (ID)</th>
<th>Description</th>
<th>Patient/Control Log2-fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clorfl31 (MIR29C)</td>
<td>Chromosome 1 open reading frame 1</td>
<td>8.001</td>
</tr>
<tr>
<td>EGR1 (X52541)</td>
<td>Early growth response 1 protein</td>
<td>3.347</td>
</tr>
<tr>
<td>EGR2 (AF139463)</td>
<td>Early growth response 2 protein</td>
<td>2.685</td>
</tr>
<tr>
<td>GBP1 (BT006847)</td>
<td>Guanylate binding protein 1, interferon-inducible, 67kDa</td>
<td>1.848</td>
</tr>
</tbody>
</table>
ever, cytokine levels were also lower in SCI patients when compared with controls (Fig. 2A), suggesting a possible effect of the pharmacological treatment on serum cytokine levels. An analysis was then conducted to compare cytokine levels between GBS and SCI patients, resulting in six proteins, IL-1β, IL-17, IL-22, IL-28A, MIP-3α, and TNF-β, with significant differences between groups, all showing higher levels in GBS patients (Fig. 2B). Of them, IL-17, IL-22, IL-28A, and TNF-β levels were also different between GBS patients and healthy individuals (Fig. 2A, 2B). Using serum samples from the GBS patient and her healthy twin included in the transcriptomics analysis, IL-17, IL-22, IL-28A, and TNF-β cytokine levels were always higher at T1 during disease acute phase than at T3 during recovery from GBS (Fig. 2C). These results suggested a connection between EGR2 and IL-17, IL-22, IL-28A, and TNF-β cytokine levels and implicated these molecules in GBS and particularly in recovery from disease.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Clinical Diagnosis</th>
<th>Age (y)</th>
<th>Gender</th>
<th>Functional Status and Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBS patient twin</td>
<td>GBS, AMSAN variant</td>
<td>23</td>
<td>F</td>
<td>Able to stand and walk with help Drugs: gabapentin, TD fentanyl, clorazepate dipotassium, venlafaxine, pantoprazole, laxatives, furosemide</td>
</tr>
<tr>
<td>GBS patient I</td>
<td>GBS, AMAN variant</td>
<td>31</td>
<td>M</td>
<td>Generalized muscle atrophy of 4 limbs, able to stand, wheelchair-bound for displacements, dysphagia, dysarthria Drugs: laxatives</td>
</tr>
<tr>
<td>GBS patient II</td>
<td>GBS, undetermined variant</td>
<td>84</td>
<td>F</td>
<td>Wheelchair-bound, lack of voluntary movements on 4 limbs Drugs: amoxicillin-clavulanate, gabapentin, furosemide, fluoxetine, zolpidem, alprazolam, atorvastatin, laxatives, LMWH, ipratropium bromide, salbutamol, esomeprazole</td>
</tr>
<tr>
<td>GBS patient III</td>
<td>GBS, AMAN variant</td>
<td>68</td>
<td>M</td>
<td>Able to stand and walk short distances with help, wheelchair for long displacements Drugs: atenolol, lorazepam, laxatives, mirtazapine, aspirin, clodigodrel, pantoprazole, escitalopram, gramine insulin, solifenacin</td>
</tr>
<tr>
<td>GBS patient IV</td>
<td>GBS, AMAN variant</td>
<td>70</td>
<td>M</td>
<td>Able to perform daily basic activities with help, stands and walks with GaitAid walker device Drugs: salbutamol, ipratropium, aspirin (acetylsalicylic acid), laxatives, pantoprazole, venlafaxine, gabapentin, paracetamol (acetaminophen), lorzepam</td>
</tr>
<tr>
<td>GBS patient V</td>
<td>GBS, AMAN variant</td>
<td>54</td>
<td>M</td>
<td>Wheelchair-bound for displacements, able to stand short periods with help Drugs: laxatives, gabapentin, amitriptyline, zolpidem, atenolol, duloxetine, omeprazole</td>
</tr>
<tr>
<td>GBS patient VI</td>
<td>GBS, AMAN variant</td>
<td>71</td>
<td>F</td>
<td>Mild tetraparesia: able to stand and walk short distances with help, able to perform basic daily activities with help Drugs: gabapentin, potassium, metformin, amitriptyline, zolpidem, pantoprazole, dimethicone, hydroxychloroquine sulfate</td>
</tr>
<tr>
<td>GBS patient VII</td>
<td>GBS, undetermined variant</td>
<td>55</td>
<td>F</td>
<td>Able to perform basic daily activities, stands and walks with GaitAid walker device Drugs: laxatives, metimazoline, lorazepam, alprazolam</td>
</tr>
<tr>
<td>SCI patient I</td>
<td>SCI at cervical level 7</td>
<td>64</td>
<td>M</td>
<td>Complete paraplegia, wheel chair for any displacement Drugs: furosemide, folic acid, salbutamol, ipratropium, budesonide, levothyroxine, midodrine, clorazepate dipotassium, ibuprofen, pregabalin, lorazepam, fluoxetine, laxatives, pantoprazole</td>
</tr>
<tr>
<td>SCI patient II</td>
<td>SCI at cervical level 4</td>
<td>56</td>
<td>M</td>
<td>Tetraplegic, wheelchair-bound Drugs: mupirocin, laxatives, diazepam, lorazepam, fluoxetine, clonidine, oxycodone, esomeprazole, LMWH, gabapentin, ipratropium bromide, salbutamol, baclofen, amitriptyline</td>
</tr>
<tr>
<td>SCI patient III</td>
<td>SCI at cervical level 4</td>
<td>44</td>
<td>M</td>
<td>Tetraplegic, wheelchair-bound Drugs: amitriptyline, oxycodone, gabapentin, laxatives, pantoprazole, baclofen, levomepromazine</td>
</tr>
<tr>
<td>SCI patient IV</td>
<td>SCI at cervical level 5</td>
<td>51</td>
<td>F</td>
<td>Incomplete tetraplegia, able to stand with help, wheelchair-bound for displacements Drugs: Fe, solifenacine, gabapentin, baclofen, alprazolam, amitriptyline, fluoxetine, lorazepam, esomeprazole, pantoprazole, LMWH</td>
</tr>
<tr>
<td>Control healthy twin</td>
<td>Healthy</td>
<td>23</td>
<td>F</td>
<td>Healthy Drugs: none</td>
</tr>
<tr>
<td>Control healthy individual I</td>
<td>Healthy</td>
<td>30</td>
<td>F</td>
<td>Healthy Drugs: none</td>
</tr>
<tr>
<td>Control healthy individual II</td>
<td>Healthy</td>
<td>41</td>
<td>F</td>
<td>Healthy Drugs: none</td>
</tr>
<tr>
<td>Control healthy individual III</td>
<td>Healthy</td>
<td>55</td>
<td>M</td>
<td>Healthy Drugs: none</td>
</tr>
</tbody>
</table>
The EGR2 or Krox-20 protein is a transcription factor involved in several processes such as immune response, early smooth muscle-like cell differentiation, pathogenesis of fibrosis, and myelination in the peripheral nervous system with implications for the inherited peripheral neuropathies (5–9, 18–20). Furthermore, recent results have shown that EGR2 is induced by IL-6 and TGF-β and negatively regulates the expression of IL-17 but not IL-2 or IFN-γ in effector T cells (20). The IL-6 and IL-17 are produced by activated Th17 cells, which are significantly increased in GBS patients (5–8), and IL-17 and IL-22 levels have been implicated in the acute stage of GBS (7, 8).

To our knowledge, the results obtained in this study provided the first evidence for the implication of EGR2 in GBS and, as previously shown for IL-17 (19), suggested a role for EGR2 in the regulation of IL-22, IL-28A, and TNF-β cytokine levels implicated in GBS pathogenesis. These findings provide new targets for GBS treatment and markers associated with disease recovery.

All patients included in the study were hospitalized, which helped to reduce the impact on the study of factors such as the sleep–wake cycle, socialization environment, diet, rehabilitation program, and pharmacological treatments. However, despite trying to match the criteria used to select patients and controls included in the study to reduce the confounders, the sample size may be too small to account for differences because of factors such as genetic heterogeneity, sex, age, medication history, and exercise, which influence serum protein levels (21).

Animal models of autoimmune diseases using rats, mice, and rabbits have improved our understanding of the pathogenesis of GBS and other autoimmune neuropathies and have facilitated testing the potential for therapies based on the manipulation of the immune system (22–25). However, none of the animal models mimic all the features of GBS but rather reflect specific facets of the syndrome (26). Nevertheless, immune-based approaches including induction of peripheral tolerance, immunotoxin targeting of activated T cells, and cytokine manipulations have been developed for the suppression of experimental autoimmune neuritis, a model for the GBS (27). Therefore, considering the limitations of these animal models, the experimental autoimmune neuritis
model based on rodents immunized with peripheral nerve myelin in adjuvant (27) could be used in future experiments to model the influence of EGR2 on recovery from GBS to provide additional support for the results presented in this paper. In these experiments, mice with pathological conditions similar to GBS developed after immunization with peripheral nerve myelin in adjuvant could be treated with EGR2 inducers such as Cannabidiol (28) to evaluate predicted recovery from GBS. If proven positive, these results will also allow the evaluation of EGR2 inducers as potential therapies for GBS.

In conclusion, the results presented in this paper showed that the gene coding for EGR2 was upregulated in GBS patients during recovery from clinical symptoms when compared with SCI patients and healthy controls, thus providing a candidate biomarker to study the physiopathology and disease progression in patients with GBS. The results suggested that EGR2 overexpression has a pivotal role in the downregulation of IL-17, IL-22, IL-28A, and TNF-β cytokines implicated in the pathophysiology of this immune-mediated peripheral neuropathy. Therefore, serum IL-17, IL-22, IL-28A, and TNF-β cytokine levels could be used to monitor disease progression from acute to recovery phase in GBS patients. In addition, if proven in experiments using animal models, EGR2 inducers could be considered as potential therapies for GBS.

Acknowledgments
We thank all the clinical staff and donors at the Paraplegics National Hospital without whom this study would not have been possible.

Disclosures
E.P. and R.T. work at Era7 Bioinformatics, which provides bioinformatics services. This does not alter their adherence to all policies on sharing data and materials. In this research group, everything that is done is open source and AGPLv3 licensed. Era7 has an open-source philosophy, and hence, it completely agrees with journal open policies. The other authors have no financial conflicts of interest.

References
Supplemental Materials

Supplemental Table I. RNA sequencing data and analysis. Includes raw data for all pair comparisons, pipeline used for statistical analysis and RNA sequencing statistics. Also available at [https://genome7.com/?abb=genome7&file_public=5286ed6787c99640af369ef277f02c14](https://genome7.com/?abb=genome7&file_public=5286ed6787c99640af369ef277f02c14)

Supplemental Table II. Differentially expressed genes in the GBS patient when compared to the healthy twin control at sampling time T3.