Transcription-Based Prediction of Response to IFNβ Using Supervised Computational Methods

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Changes in cellular functions in response to drug therapy are mediated by specific transcriptional profiles resulting from the induction or repression in the activity of a number of genes, thereby modifying the preexisting gene activity pattern of the drug-targeted cell(s). Recombinant human interferon beta (rIFNβ) is routinely used to control exacerbations in multiple sclerosis patients with only partial success, mainly because of adverse effects and a relatively large proportion of nonresponders. We applied advanced data-mining and predictive modeling tools to a longitudinal 70-gene expression dataset generated by kinetic reverse-transcription PCR from 52 multiple sclerosis patients treated with rIFNβ to discover higher-order predictive patterns associated with treatment outcome and to define the molecular footprint that rIFNβ engraves on peripheral blood mononuclear cells. We identified nine sets of gene triplets whose expression, when tested before the initiation of therapy, can predict the response to interferon beta with up to 86% accuracy. In addition, time-series analysis revealed potential key players involved in a good or poor response to interferon beta. Statistical testing of a random outcome class and tolerance to noise was carried out to establish the robustness of the predictive models. Large-scale kinetic reverse-transcription PCR, coupled with advanced data-mining efforts, can effectively reveal preexisting and drug-induced gene expression signatures associated with therapeutic effects.

Introduction

Interferons are small, inducible proteins secreted by nucleated cells in response to viral infection and other stimuli. They act in a paracrine fashion on other cells in their immediate vicinity, triggering a state of growth arrest, so that infected cells cannot be forced to produce viral proteins, and activating the process of programmed cell death, so that infected cells can be removed [1]. Interferons are important not only in the defense against a wide range of viruses but also in the regulation of immune responses and hematopoietic cell development [2,3]. Recombinant human interferon beta (rIFNβ) is routinely used to control exacerbations in relapsing-remitting multiple sclerosis (MS) [4,5]. Although effective in reducing the number of exacerbations and brain disease activity in some patients, rIFNβ produces no benefit in almost one-half of these patients [6,7]. Furthermore, it is not at all certain how significant its long-term effects on disease progression are. Therapy has been associated with a number of adverse reactions, including flu-like symptoms, transient laboratory abnormalities, menstrual disorders, increased spasticity, and dermal reactions [8].

We generated and analyzed longitudinal patterns of gene expression from interferon beta (IFNβ)-treated patients suffering from MS with the aim of identifying preexisting and drug-induced signatures that would predict or explain the clinical response to the drug.

Results/Discussion

Fifty-two patients with relapsing-remitting MS were followed for at least 2 y after initiation of therapy with IFNβ. Clinical follow-up included a neurological examination every 3 mo and at the time of relapse. At each visit, a blood sample was obtained by venipuncture. After the 2-y endpoint, patients were classified as either good or poor responders based on strict criteria, as described in Materials and Methods. We measured the expression profile of 70 carefully selected genes from peripheral blood mononuclear cells isolated from each patient at each time point, using one-step kinetic reverse-transcription PCR (Dataset S1). This process offers remarkable sensitivity and specificity and a dynamic range of several orders of magnitude, allowing the compar-
ison of expressed transcripts from many different genes without compromising accuracy. Targets for analysis were selected on the basis of their presumed biological action and included genes coding for type I and II IFN-responsive molecules, cytokine receptors, members of the interferon (IFN) signaling and apoptosis pathways, and several transcription factors involved in immune regulation (Table S1). Altogether, more than 70,000 reactions were carried out. A common inherent prediction performance limitation of most high-throughput gene-expression profiling projects arises from the largely asymmetric expression data matrix obtained as a result of measuring far more genes than samples [9]. Such ill-conditioned data matrices inevitably lead to overfitting of predictive models (among other difficulties), some effects of which can be mitigated by judicious application of various established inverse and regularization schemes [10]. The undesirable properties (i.e., overfitting) of such massively under-determined datasets are largely avoided in this study design because the number of genes measured is commensurable with the numbers of samples.

Using linear discriminant analysis–based integrated Bayesian inference system (IBIS), we were able to detect the gene MX1 as the single best discriminating variable between samples obtained at baseline (T = 0) and at 3 mo after initiation of therapy (T = 3) with a classification accuracy of 79% (data not shown). Given that MX1 is a known marker of IFN bioavailability [11], this result validates our experimental approach as well as our sample handling and processing.

To search for expression signatures associated with therapeutic outcome (good or poor responder), we conducted clustering of samples using normalized data for all 70 genes at each time point [12]. Despite applying several different similarity measures and clustering algorithms [13], we did not observe concomitant segregation of samples according to their responder status, with the exception of a few local clusters of small size (Figure 1). This result may suggest that overall differences in gene expression in the two groups of patients, as assessed by conventional similarity measures, are small or negligible. The clustering null results with respect to concomitant class segregation, however, do not rule out the possibility of discovering outcome-predictive combinatorial and nonlinear relationships. To investigate this possibility further, we used quadratic discriminant analysis–based IBIS, implemented for three-dimensional

Figure 1. Nonsupervised Two-Way Hierarchical Clustering of Samples at T = 0
A clear aggregation of samples cannot be seen by this technique. The first column indicates the type of responder to which each sample belongs (red, good; blue, poor).
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(3D) searches, in the search for highly predictive sets of three genes whose expression at \( T = 0 \) correlated with a good or poor outcome of therapy at the 2-y endpoint. This process exhaustively carried out sample classification by searching through all 54,740 possible three-gene combinations of 70 genes. Higher-order combinatorial searches beyond combinations of three genes are possible using IBIS and are currently under investigation. However, higher-order predictive variable combinations do require the support of many more samples to prevent overfitting of the model.

We implemented a stringent method for examining the statistical validity of our classification results that consisted in testing the obtained classifier in an independent set of samples not previously “seen” by the program. All of the following statistical analyses were thus performed on split datasets, namely, training (75% of the samples) and test (25%), each reflecting the same relative proportion of classes (63% good and 37% poor responders). We started by conducting 3D IBIS searches using expression data from only the training set and selected the top nine scoring triplets (on the basis of their high prediction accuracy and low mean squared error [MSE] values). For each gene triplet, and using the training data only, a committee of classifiers was built based on an internal cross-validation scheme. Subsequently, the classifiers were used to predict the outcome of an independent test set of samples. Gene triplets were ranked on the basis of the prediction accuracies of the classifiers on this independent test set. We identified nine gene triplets with a predictive accuracy of at least 80% (Table 1). We considered it essential to empirically rule out the chances of fortuitous data splits in the accuracy results obtained from the top-scoring gene triplets.

Consequently, for the nine top-scoring gene triplets and their corresponding classifiers, we generated 100 random splits and built classifiers for each new resulting training set. Next, we tested how well the classifiers predicted therapeutic outcome in the corresponding test datasets. Figure 2 illustrates the distribution in the prediction accuracies obtained for the triplet composed of Caspase 2, Caspase 10, and FLIP, which yielded a predictive accuracy of 86% in the original split. The bell-shaped distribution resulting from 100 tests for this triplet displayed a mean accuracy of 87.8% and a tenth percentile of 78.6%, meaning that if the prediction were performed multiple times, in 90% of these instances an accuracy of almost 79% or better would be obtained. This histogram only reflects the range of accuracies obtained, should the initial data split be different. Notably, the genes in the top-scoring triplet were Caspase 2, Caspase 10, and FLIP—three apoptosis-related molecules. The second-highest-scoring triplet was that of Caspase 2, Caspase 3, and IRF4 (86.8%...
mean accuracy after 100 splits). Other high-scoring triplets included IL4Ra and MAP3K1, in addition to other apoptotic molecules (Table 1). When we repeated this experiment with the top three scoring genes, using F-test, the obtained mean accuracy was 64% (tenth percentile at 50%) (Figure 3).

In Figure 4, the predictive capability of the best-scoring triplet (Caspase 2, Caspase 10, and FLIP; 3D model) was compared with those obtained with the single-gene (1D) and gene-pair (2D) models. We observed that the classification accuracy improves as more genes are added to the classifier. We next plotted the samples of a test dataset (25% of samples) on the predictive probability model shown in Figure 4G and compared the performance of the 3D IBIS model to those of the individual 2D models (Figure 5). Overall, the 2D projections of the 3D predictive model show that the Caspase 2/Caspase 10 and Caspase 10/FLIP gene pairs show significant predictive capability, but that all three genes are required to provide the highest level of model accuracy and robustness.

To validate the specificity and predictive capability of the top-scoring gene triplet (for the good and poor responding classes) and its associated classifiers, we examined the model exhibiting the best performance on a “default” expression dataset. This null dataset was built keeping the original gene expression data and randomly permuting the class labels of the outcomes 1,000 times (keeping the same counts of good and poor responding patients as were in the original dataset).

The prediction accuracies for all the gene triplets obtained with this dataset dropped dramatically as the means ranged from 49.2% to 53.6% (data not shown), emphasizing the specificity of the classifiers. In addition, for the top-scoring gene triplet (for good and poor responder classes), we calculated the probability of achieving, under the null hypothesis, an equal or better accuracy than that obtained in the original prediction (86%), as previously described [14]. This achieved significance level was 0.009, suggesting that it is very unlikely that the prediction accuracies observed for this classifier are caused by chance.

Finally, we tested the robustness of each of these gene sets as predictors of IFNβ response by simulating experimental measurement error. To accomplish this, we first calculated the standard deviation of the expression measurements for all genes as an estimation of the overall experimental error. We then added a fixed amount of Gaussian noise corresponding to one standard deviation (taken from 20 random deviations) to each expression value and repeated the classification/prediction in 30 different splits of the data (a total of 600 tests). Notably, the mean drop in predictive accuracy after the addition of noise was less than 10%, denoting a significant tolerance to reasonable measurement errors (Table 1).

Because all the patients in this study were systematically followed up for a period of 2 y, we were able to perform a longitudinal analysis. Using a repeated-measures analysis of variance (ANOVA), we searched for genes with significantly different expression patterns based on models that tested for responder effect, time effect, and interaction effect (time × response). Significant responder effect for 20 genes (Figure 6) and significant time effect for 13 genes were detected (Figure 7). Interestingly, six of the genes that showed statistically significant differences between good and poor responders, IRF4 (p = 0.03), IL4Ra (p = 0.01), Caspase 10 (p = 0.0008), Caspase 7 (p = 0.01), IRF2 (p = 0.02), and IRF6 (p = 0.03) are among the 12 genes that best predict response at T = 0 (shown in bold in Figure 7B). A pattern consistent with increased apoptosis (five members of the Caspase family of
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proteins, TRADD, and BAX) was observed for the poor responders.

Although we successfully identified informative, combinatorial relationships, establishing the causality of the association between gene expression and outcome to therapy is beyond the scope of this work, and these genes are therefore considered surrogate markers. Moreover, although extensive in vivo and in vitro experiments have been conducted, the full mechanism of action of IFNβ in MS remains unknown.

Transcription profiling experiments have involved IFNβ in the regulation of apoptosis in both cancer and MS [15,16,17,18]. Induction of programmed cell death could lead to a reduction in the number of activated lymphocytes, macrophages, and monocyte-derived dendritic cells—all key components of the pathogenic process leading to tissue damage in MS [19,20,21]. However, increased levels of some anti-apoptotic molecules have also been observed in IFNβ-treated MS patients, possibly reflecting a compensatory mechanism [16]. Furthermore, even the inhibition of activated T cell apoptosis in response to IFNα and IFNβ has been reported [22]. Our finding of increased apoptosis in poor responders does not support the hypothesis of programmed cell death as a primary therapeutic mechanism for IFNβ. We hypothesize that a net increase in pro-apoptotic transcripts in peripheral blood mononuclear cells from poor responders could be reflecting undesired elimination of certain regulatory cell populations that are much needed to maintain a homeostatic balance.

Other differentially expressed transcripts included IRF4, a gene essential for mature T and B lymphocyte function and homeostasis, and a transcription factor with dual function (activator/repressor) that regulates transcription of IL4 through physical interaction with Nfatc2 [23]. Remarkably, IRF4 is a repressor of other IFN-induced genes [24], an observation consistent with the elevated expression of IRF4 observed in the poor responders before initiation of therapy.

As expected, the gene MX1 showed a significant time effect independent of clinical response (p = 0.01). This result is in agreement with previous findings indicating substantial MX1 upregulation in response to type I IFNs [25]. Interestingly, upregulation of MX1, which occurs minutes after IFN stimulation [26], is sustained over at least 2 y, spanning several orders of magnitude of time units. This also correlates well with our results identifying MX1 as the best single classifier for samples from patients before (T = 0) and after (T = 3) initiation of therapy. In fact, as Figure 7 illustrates, most of the significance for the time effect in MX1 comes from the difference between T = 0 and T = 3.

Also of interest, a significant time effect (but not responder effect) was observed for IFNAR1 and STAT2 (Figure 7B). Because IFNAR1 is a subunit of the heterodimeric type I IFN receptor and STAT2 is a critical component of the DNA binding complex ISGF3a (which regulates the expression of IFN-responsive genes), their upregulation on administration of rIFNβ is likely related to mechanistic aspects of IFN signaling. Our results suggest that poor response is associated with downstream signaling events rather than deficient recognition or metabolism of the drug. Our previous finding that IFN receptor polymorphisms do not affect therapeutic response in this same set of patients partially supports this hypothesis [27].

Two genes with significant time effects, Caspase 10 (p = 0.01) and MAP3K1 (p = 0.01) were part of any predictor set (Figure 7B). In addition, MAP3K1 also showed a significant interaction effect (p = 0.05; data not shown). These results highlight the involvement of these genes in the response to IFN both at T = 0 and once therapy has started.

Here we combined large-scale, function-oriented gene expression with advanced data mining to identify a set of markers that accurately and robustly predict the response to rIFNβ therapy. Although larger, prospective studies are needed to confirm these findings, our results suggest that the underlying gene activity profile of an individual at the verge of therapy harvests sufficient information to allow investigators to estimate the chances of experiencing satisfactory therapeutic effects. As analytical tools to predict clinical outcomes based on molecular evidence evolve, these types of studies are likely to become a substantial aid to the physician, taking the paradigm of personalized medicine one step further.

Materials and Methods

Patients and samples. All studies were approved by the respective Committees of Human Research at Hospital Vall d’Hebron, Barcelona, Spain, and the University of California, San Francisco, United States. Informed consent was obtained for all study participants. All patients were examined by a trained neurologist at the CNI Unit, Vall d’Hebron Hospital. Inclusion criteria for this study were clinically definite MS (Poser’s criteria), disease in relapsing-remitting phase, age between 18 and 65 y, recorded history of at least two clearly identified relapses within the preceding 24 mo, and expanded disability status scale between zero and 5.5 (inclusive). Detailed information about clinical aspects of these patients has been recently reported elsewhere [6].

Patients were categorized as good responders (n = 33) if they experienced a total suppression of relapses and no increase in the expanded disability status scale after a 2-y follow-up period. Poor
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The same probability model generated from the training dataset (see Figure 4G) provides the background shading of volumes predictive of good response (red) and poor response (blue). Three samples are identified with arrows and followed along different graphical representations.

The fundamental question we are aiming to answer using data-mining methods is to determine the probability of a patient being a good responder if the observed gene expression level of a gene for that patient is known. Let us call this probability P(good_responder | observed expression level). According to this formula:

\[ P(\text{good responder} | g) = \frac{P(g | \text{good responder}) P(\text{good responder})}{P(g | \text{good responder}) P(\text{good responder}) + P(g | \text{poor responder}) P(\text{poor responder})}, \]

where P(g | good_responder) and P(g | poor_responder) are the distribution functions fitted to the observed gene expression levels in good responding and poor responding patients, respectively. Our fitted distribution D(g), denotes the probability of a patient having an expression level of g, given that this patient is a good responder. The fundamental question to answer using data-mining methods (here using IBIS particularly) is as follows: what is the probability of a patient being a good responder given the observed expression level of a gene for that patient? Taking advantage of the fitted distributions, a classifier applies Bayes’ formula to answer the fundamental question. According to this formula:

\[ P(\text{good responder} | g) = \frac{P(g | \text{good responder}) P(\text{good responder})}{P(g | \text{good responder}) P(\text{good responder}) + P(g | \text{poor responder}) P(\text{poor responder})}. \]

Several measures were used to assess how well the calculated probabilities matched the true patient responses to therapy. The top-performing gene triplets were selected on the basis of a mixed threshold for low MSE levels and high accuracy rates.

The algorithm. IBIS identifies genes (or gene pairs or groups of genes) that are highly predictive of the outcome based on probability distributions of those genes in different outcome classes. For example, for a given gene g, two Gaussian functions are fitted to the distributions of the observed expression levels in good responding and poor responding patients (let us call these fitted distributions Dg and Dp for good and poor responding patients, respectively). Our fitted distribution Dg(x), denotes the probability of a patient having an expression level of g, given that this patient is a good responder. The fundamental question to answer using data-mining methods (here using IBIS particularly) is as follows: what is the probability of a patient being a good responder given the observed expression level of a gene for that patient? Taking advantage of the fitted distributions, a classifier applies Bayes’ formula to answer the fundamental question. According to this formula:

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averaged over all patients. For a given patient, the clinical response determined by the end of the 2-y monitoring period is denoted by response\textsubscript{observed} and response\textsubscript{expected} and represents the probability of that patient being a good responder to rIFN\(\beta\) therapy, using the Bayes’ formula above. Classification accuracy simply expresses the percentage of patients that were correctly predicted as being good or poor responders.

**Classification and prediction procedure.** The initial dataset of patients was divided into two parts; namely, a training set with 75% of the samples and a test set with 25% of the samples, each reflecting the same proportion of classes (63% good and 37% poor responders). Only the training set was used for identifying the best predictive gene triplets with the IBIS method, as well as for building the classifier. A committee of classifiers was then generated using a 10-fold cross-

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**Figure 6.** Characteristic Gene Expression Profiles of Good and Poor Responders to IFN\(\beta\) over Time

(A) An unsupervised hierarchical clustering representation of the weighted difference between the average expression of good and poor responders. For each gene, the obtained differences were log normalized and multiplied by the F-statistic from an ANOVA (responder effect) run previously (shown in [B]). The “heat” colored bar represents the absolute value of this difference. With the exception of MX1 (indicated by an arrow), all genes showing a significant difference in expression between the two groups of patients were automatically arranged in only two clusters (framed in blue).

(B) List of all genes showing a significant responder effect along with their F-statistic and \(p\)-values. Genes that were part of any triplet showing more than 80% prediction accuracy at \(T = 0\) are shown in bold.

(C) A continuous representation of the longitudinal average expression of two representative genes for good (○) and poor (●) responders. TRADD shows two widely parallel curves, indicative of a significant difference in the expression averages, correlating with its profile (*) observed in the clustering shown in (A). In contrast, GATA3 displays two almost overlapping curves, consistent with its shading (*) in the clustering in (A). DOI: 10.1371/journal.pbio.0030002.g006
validation scheme during training. The training data themselves were divided into ten parts, and each time, a classifier was built using only nine parts of the data. That classifier’s predictive capability was determined by its accuracy over the one-tenth of the data withheld. A committee of ten classifiers was assembled from the results of this training stage; this committee was then applied to the test data (which have thus far been hidden from the classifiers). For a patient sample in the test data, each classifier in the committee made a prediction. A majority voting scheme then decided as to which class the sample would be assigned.

Given the initial data split into training and test sets, it was important to rule out the role of fortuitous idiosyncrasies in this split and the resulting accuracy rates. To address this point, we created 100 random splits of the data into training and test subgroups. A committee of classifiers was trained on the training set for each data split, and the accuracies were calculated over the blind test set. A histogram of the test set accuracies was then built, representing the expected ranges of accuracies had the initial data split been different. This histogram is not representative of the estimated or idealized distribution of the accuracies for a gene triplet in a machine learning sense. Rather, it is a coarse approximation of the possible range of gene triplet outcome—prediction accuracies that could be expected.

Controlling for false discoveries. To assess the significance and specificity of the top-scoring gene triplets and their corresponding trained committee of classifiers, a null dataset was created by keeping the same expression levels of genes in the dataset and randomly permuting the class labels of the patients 1,000 times (the total count of poor and good responding patients was unchanged). Classifiers were built using the training null data, and accuracies were calculated on the corresponding test sets. The mean of these accuracies for all the top-performing gene triplets was around 50%. The achieved significance level, which represents the probability of achieving
accuracy levels better than or equal to that of the nonpermuted classification was calculated to be 0.009. This value can be considered a significant level, or p-value, and indicates the number of times in 1,000 trials for which accuracies of 86% or higher can be achieved under the "no predictive capability" null hypothesis.

Time-series analysis was performed using SAS version 8.0 (SAS Institute, Cary, North Carolina, United States). Permutation analysis and histogram graphic outputs were produced with Matlab (The Mathworks, Natick, Massachusetts, United States).

Supporting Information

Dataset S1. Raw Expression Dataset
Gene expression values for all samples at all time points. This is the raw file from which all analyses were performed.

Found at DOI: 10.1371/journal.pbio.0030002.sd001 (160 KB DOC).

Table S1. Target Information
Gene names, symbols, and LocusLink and GenBank accession numbers, as well as primer sequences, are listed for all targets.

Found at DOI: 10.1371/journal.pbio.0030002.s001 (491 KB XLS).

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