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DISCOVERING DIAGNOSTIC GENE TARGETS FOR EARLY DIAGNOSIS OF ACUTE GVHD USING METHODS OF COMPUTATIONAL INTELLIGENCE ON GENE EXPRESSION DATA

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Abstract

This is an application paper of applying standard methods of computational intelligence to identify diagnostic gene targets and to use them for a successful diagnosis of a medical problem - acute graft-versus-host disease (aGVHD). This is the major complication after allogeneic haematopoietic stem cell transplantation (HSCT) in which functional immune cells of donor, recognize the recipient as "foreign" and mount an immunologic attack. In this paper we analyzed gene-expression profiles of 47 genes associated with allo-reactivity in 59 patients submitted to HSCT. We have applied different dimensionality reduction techniques of the variable space, combined with different classifiers to detect the aGVHD at onset of clinical signs. This is a preliminary study which utilises both computational and biological evidence for the involvement of a limited number of genes for the diagnosis of aGVHD. Directions for further studies are also outlined in this paper.

1 Introduction

With the completion of the first draft of the human genome the task is now to be able to process this vast amount of ever growing dynamic information and to create intelligent systems for detection, prediction and knowledge discoveries about human pathology and disease. When genes are in action, the dynamics of the processes, in which a single gene is involved, are very complex, as this gene interacts with many other genes and mediators, and is influenced by many environmental factors. The genes in an individual may mutate, change slightly their code, and may therefore express differently at a next time. Modeling these events, learning about them and extracting knowledge are major goals for *bioinformatics* [1, 2].

The potential applications of microarray technology are numerous and include identifying markers for classification, diagnosis, disease outcome prediction, target identification and therapeutic responsiveness [1, 2]. Microarray analysis might help to identify unique markers (e.g. a set of gene) of

clinical importance. Diagnosis and prediction of a biological state/disease is likely to be more accurate by identifying clusters of gene expression profiles (GEPs) performed by macroarray analysis. Based on a genetic profile, it is possible to set a diagnostic test, so a sample can be taken from a patient, the data related to the sample processed, and a profile related to the sample obtained [2]. This profile can be matched against existing gene profiles and based on similarity, it can be confirmed with a certain probability the presence or the risk for a disease. We apply this approach here to detect acute graft-versus-host disease (aGVHD) in allogeneic hematopoietic stem cell transplantation (HSCT), a curative therapy for several malignant and non malignant disorders [3].

Acute GVHD remains the major complication and the principal cause of mortality and morbility following HSCT [4, 5]. At present, the diagnosis of aGVHD is merely based on clinical criteria and may be confirmed by biopsy of one of the 3 target organs (skin, gastrointestinal tract, or liver) [6]. The severity of aGVHD is graded clinically from I to IV using a standardized system, with increased mortality rates associated with significant aGVHD (grades II-IV) [7]. There is no definitive diagnostic blood test for aGVHD, although a lot of blood proteins have been described as potential biomarkers in small studies [8, 9]. A recent report indicates a preliminary molecular signature of aGVHD in allogeneic HSCT patients [10].

In the current project, our primary objective was to validate a novel and not invasive method to confirm the diagnosis of aGVHD in HSCT patients at onset of clinical symptoms. For this purpose, a database has been built using pre-processed experimental measurements from patients, and features were selected to enable a good class separation without using the large amount of variables recorded features thus facing the "curse of dimensionality" problem (i.e., an excessive number of training inputs that increases the system complexity without remarkable advantages in terms of prediction performances). This problem can be considered as a typical inverse problem of pattern classification, starting from experimental database.

The proposed approach uses different dimensionality reduction techniques, such as Principal Component Analysis (PCA), Correlation-based Feature Selection (CFS) algorithm combined with an Artificial Neural Network (ANN) classifier, and also a wrapper method combined with the Naïve Bayesian classifier and with a Support Vector Machine (SVM) classifier to select the most important features (genes) for the diagnosis.

This is the first paper which discusses both computational and biological evidence to confirm the early statement of aGVHD based on selected genetic diagnostic markers. The organization of the rest of the paper is as follows: section 2 explains the data analyzed; a dimensionality reduction technique is applied in order to reduce the number of variables; section 3 describes the results obtained with our approach; section 4 discusses the results of the diagnostic method and the last section of the paper gives conclusions inferred with some possible future applications.

2 Methodology

Feature selection is the process of choosing the most appropriate features (variables) when creating a computational model [11]. Feature evaluation is the process of establishing how relevant to the problem in hand are the features used in the model. Features can be:

- Original variables: used in the first instance to specify the problem.
- Transformed variables: obtained through mapping the original variable space into a new one.

There are different groups of methods for feature selection:

- Filtering methods: features are 'filtered', selected and ranked in advance, before a model is created (e.g. a classification model). Traditional filtering methods are: correlation, t-test, and signal-to-noise ratio.
- Wrapping methods: features are selected on the basis of how well the created model performs using these features.

In this paper we consider three general approaches to feature subset selection, more specifically, wrapper and filter approaches, for gene selection and a

feature extraction technique based on the variance of data (PCA) for obtaining a new problem space of lower order.

Wrappers and filters differ in the way the feature subsets are evaluated. Filter approaches remove irrelevant features according to general characteristics of the data. Wrapper approaches, by contrast, apply machine learning algorithms to feature subsets and use cross-validation to evaluate the score of feature subsets. In theory, wrappers should provide more accurate classification results than filters (Langley, 1994) [11]. Wrappers use classifiers to estimate the usefulness of feature subsets.

The use of "tailor-made" feature subsets should provide better classification accuracy for the corresponding classifiers, since the features are selected according to their contribution to the classification accuracy of the classifiers. The disadvantage of the wrapper approach is its computational requirement when combined with sophisticated algorithms such as support vector machines.

As a filter approach, CFS was proposed by Hall [12]. The rationale behind this algorithm is "a good feature subset is one that contains features highly correlated with the class, yet uncorrelated with each other." It has been shown in Hall [12] that CFS gave comparable results to the wrapper and executes many times faster. It will be shown later in this paper that combining CFS with a suitable classifier, provides a good classification accuracy for diagnosis of aGVHD.

Another point of view is to consider PCA, for finding a representation of the problem space into another orthogonal space, having a smaller number of dimensions defined by another set of variables (eigenvectors). The new set of variables will be considered as an input for a suitable ANN.

2.1 Experimental Data

Fifty-nine HSCT patients were enrolled in our study between March 2006 and July metricconverterProductID2008 in2008 in Transplants Regional Center of Stem Cells and Cellular Therapy "A. Neri" Reggio Calabria, Italy, during a Governative Research Program: *"Project of Integrated Program: Allogeneic Hemopoietic Stem Cells Transplantation in Malignant Hemopathy and Solid Neoplasia Therapy - Predictive and prognostic value for graft vs. host disease of chimerism and gene expression"*.

Because experimental design plays a crucial role in a successful biomarker search, the first step in our design was to choose the most informative specimens and achieve adequate matching between positive cases aGVHD (YES) and negative controls aGVHD (NO) to avoid bias. This goal is best achieved through a database containing high-quality samples linked to quality controlled clinical information. Patients with clinical signs of aGVHD (YES) were selected, and in more than 95% of them aGVHD was confirmed by biopsy including those with grade I.

We used 26 samples from aGVHD (YES) patients that were taken at the time of diagnosis and we selected 33 samples from patients that did not experienced aGVHD (NO). All together YES/NO patient groups comprised a validation set. Total RNA was extracted from whole peripheral blood samples using a RNA easy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription of the purified RNA was performed using Superscript III Reverse Transcriptase (Invitrogen). A multigene expression assay to test occurrence of aGVHD were carried out with TaqMan® Low Density Array Fluidic (LDA-macroarray card) based on Applied Biosystems 7900HT comparative dd CT method, according to manufacturer's instructions. Expression of each gene was measured in triplicate and then normalized to the reference gene 18S mRNA, who was included in macroarray card. About the project of macroarray card, we selected 47 candidate genes from the published literature, genomic databases, pathway analysis. The 47 candidate genes were involved in immune network and inflammation pathogenesis.

2.2 Dimensionality Reduction Approach

2.2.1 Feature Extraction with PCA

In statistics, PCA [13] is a technique that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components (PCs). PCA can be used for dimensionality reduction in a data set while retaining those characteristics of the data set that contribute most to its variance, by keeping lower-order PCs and ignoring higher-order ones. Such low-

order components often contain the "most important" aspects of the data, considering, moreover, that data set has been preliminarily dewhitened. PCA has the distinction of being the optimal linear transformation for keeping the subspace that has the largest variance.

In this paper we have applied PCA after a suitable data normalization. It is shown that the first 9 PCs account for more than 83% of the variation among the data samples (Fig. 1). A cut-off of the PCs selected has been established in order to balance the retained information and the "curse of dimensionality" problem. In section 3 it will be analyzed an evaluation about the usefulness of this new problem space representation, using a classifier.

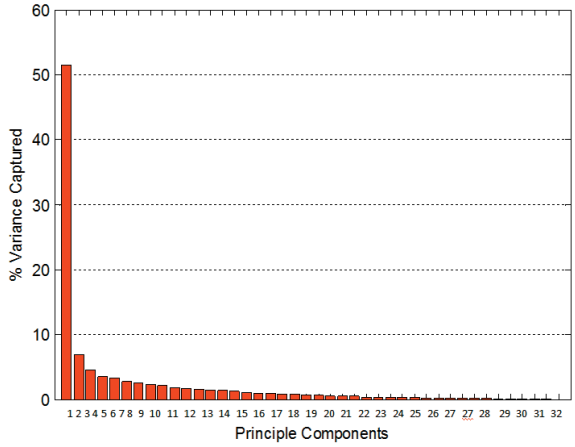

Figure 1. PCA transformation of the GvHD data, and the principal components neat for variance.

2.2.2 Gene Feature Selection

Feature Selection is a technique used in machine learning of selecting a subset of relevant features to build robust learning models. The assumption here is that not all genes measured by a macroarray method are related to aGVHD classification. Some genes are irrelevant and some are redundant from the machine learning point of view [12, 21]. It is well-known that the inclusion of irrelevant and redundant information may harm performance of some machine learning algorithms. Feature subset selection can be seen as a search through the space of feature subsets. CFS evaluates a subset of features by considering the individual detector ability of each feature along with the degree of redundancy between them

$$CFSs = \frac{k\bar{r}_{cf}}{\sqrt{k+k(k-1)\bar{r}_{ff}}} \quad (1)$$

Where

- $CFSs$ is the score of a feature subset S containing k features,
- $\bar{r}_{cf}$ is the average feature to class correlation ($f \in S$),
- $\bar{r}_{ff}$ is the average feature to feature correlation.

The distinction between normal filter algorithms and CFS is that while normal filters provide scores for each feature independently, CFS presents a heuristic "merit" of a feature subset and reports the best subset it finds. To select the genes with CFS, we have to:

a) Choose a search algorithm,

b) Perform the search, keeping track of the best subset encountered according to $CFSs$,

c) Output the best subset encountered.

The search algorithm we used was the best-first with forward selection, which starts with the empty set of genes. The search for the best subset is based on the training data only. Once the best subset has been determined, and a classifier has been built from the training data (reduced to the best features found), the performance of that classifier is evaluated on the test data. The 13 genes selected by CFS are reported in Table 1.

A leave-one-out cross validation procedure was performed to investigate the robustness of the feature selection procedures. In 29 runs, the subset of 13 genes was selected 28 times (96%) by CFS. Now it is possible to use a classifier to estimate the usefulness of feature subsets.

2.2.3 Wrapper Method

While CFS assigns a score to subset of features, Wrapper approaches take biases of machine learning algorithms into account when selecting features. The wrapper method applies a machine learning algorithm for a feature subset selection and uses cross-validation to compute a score for them.

Table 1. The 13 genes selected from CFS, with their names and meaning.

Gene Name	Official full name	Immune function
BCL2A1	BCL2-related protein A1	Anti- and pro-apoptotic regulator.
CASP1	Caspase 1, apoptosis-related cysteine peptidase	Central role in the execution-phase of cell apoptosis.
CCL7	chemokine (C-C motif) ligand 7	Substrate of matrix metalloproteinase 2
CD83	CD83 molecule	Dendritic cells regulation.
CXCL10	chemokine (C-X-C motif) ligand 10	Pleiotropic effects, including stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression.
EGR2	Early growth response 2	transcription factor with three tandem C2H2-type zinc fingers.
FAS	TNF receptor superfamily, member 6)	Central role in the physiological regulation of programmed cell death.
ICOS	Inducible T-cell co-stimulator	Plays an important role in cell-cell signaling, immune responses, and regulation of cell proliferation.
IL4	Interleukin 4	Immune regulation.
IL10	Interleukin 10	Immune regulation.
SELP	selectin P	Correlation with endothelial cells.
SLPI	Stomatin (EPB72)-like 1	Elemental activities such as catalysis.
STAT6	transducer and activator of transcription 6, interleukin-4 induced	Regulation of IL4- mediated biological responses.

Table 2. The 7 genes selected (marked with °) through the wrapper- naïve Bayes method with their names and meaning. The 5 genes selected with SVM are marked with *.

Gene Name	Official full name	Immune function
CASP1°*	Caspase 1, apoptosis-related cysteine peptidase	Central role in the execution-phase of cell apoptosis.
EGR2°	Early growth response 2	transcription factor with three tandem C2H2-type zinc fingers.
CD52°*	CD52 antigen	B-cell activation.
SLPI°	Stomatin (EPB72)-like 1	Elemental activities such as catalysis.
ICOS°*	Inducible T-cell co-stimulator	Plays an important role in cell-cell signaling, immune responses, and regulation of cell proliferation.
IL10°*	Interleukin 10	Immune regulation. Foxp-3 * forkhead box P3 Regulatory T cells play important roles in the maintenance control of transplantation tolerance.
CXCL10°	chemokine (C-X-C motif) ligand 10	Pleiotropic effects, including stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression.

In general, filters are much faster than wrappers. However, as far as the final classification accuracy is concerned, *wrappers* normally provide better results. The general argument is that the classifier that will be built from the feature subset should provide a better estimate of accuracy than other methods.

The main disadvantage of *wrapper* approaches is that during the feature selection process, the classifier must be repeatedly called to evaluate a subset. The main disadvantage of wrapper approaches is that during the feature selection process, the classifier must be repeatedly called to evaluate a subset. To select the genes using a wrapper method, we have to:

a) Choose a machine learning algorithm to evaluate the score of a feature subset.

b) Choose a search algorithm.

c) Perform the search, keeping track of the best subset encountered.

d) Output the best subset encountered.

As a machine learning algorithm, here we used s simple Bayesian classifier naïve Bayes and a SVM. The naïve Bayes classifier assumes that features are independent given the class. Its performance on data sets with redundant features can be improved by removing such features. A forward search strategy is normally used with naïve Bayes as it should immediately detect dependencies when harmful redundant features are added.

SVMs use a kernel function to implicitly map data to a high dimensional space. Then, they construct the maximum margin hyperplane by solving an optimization problem on the training data. Sequential minimal optimization (SMO) [16] is used in this paper to train a SVM with a Linear Kernel. SVMs have been shown to work well for high dimensional microarray data sets [17]. However, due to the high computational cost it is not very practical to use the wrapper method to select genes for SVMs.

Also here the search algorithm was the best-first with forward selection, starting with the empty set of genes. We report here the accuracy of classifiers built from the best feature subset found during the search. The search for the best subset is based on the training data only. Once the best subset has been determined, and a classifier has been built from the training data (reduced to the best features found), the performance of that classifier is evaluated on the test data. The 5 Genes selected using the wrapper method are shown in table 2.

Most of the genes selected are also similar to those of the 13 genes selected using the CFS method and the only two genes that are different are actually correlated to other genes from the set of 13 genes.

A leave-one-out cross validation procedure was performed to investigate the robustness of the method over the training set: in 29 runs, the subset of 7 genes was selected 26 times (90%) by the nave Bayes wrapper and the group of 5 genes, 29 times (100%) by the SMO. Section 4 has shown the performance of this technique estimated on the testing data.

3 Neural Network Model for Early Diagnosis Using the Selected Gene Diagnostic Markers

Artificial neural networks (ANNs) are commonly known as biologically inspired, highly sophisticated analytical techniques, capable of modeling extremely complex non-linear functions. Some ANN models are inspired by learning processes in cognitive system and by neurological functions of the brain. ANN are capable of predicting new observations (on specific variables) from other observations (on the same or other variables) after executing a process of learning from existing data [14]. Here we aim at a comparison between two models built with the use of two different feature selection methods. We have used a popular ANN architecture called MLP with back-propagation learning algorithm. The MLP is known to be a robust function approximator for prediction/classification problems. A suitable subset of samples for biological peculiarities has been chosen as training data set. The training data set had 29 patient samples (13 aGVHD(Yes) and 16 aGVHD(No)). The test data set consisted of 30 patient samples (13 aGVHD(Yes) and 17 aGVHD(No)). The ANN's outputs were:

– 0, if aGVHD diagnosis was Yes;

– 1, if aGVHD diagnosis was No.

The ANN based system was trained with adaptive rate of learning during a period of 400 epochs. We have developed one MLP model with 13 genes as input variables, and another - with the 9 PCs as input variables. The ANN, according to a consequence of the Kolmogorov's theorem [15], has a hidden layer with 27 neurons (for the 13 gene MLP model) and 19 neurons (for the 9 PCs MLP model). As an activation function for the hidden layer the tan-sigmoid function is used. A pure linear function was used for the output layer (Figure 2). After the training phase, the ANN was tested - final results shown in section 4.

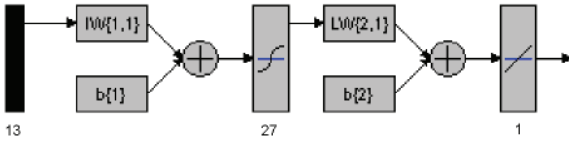

Figure 2. Structure of the implemented ANN.

In this study both the ANN and the SVM classifiers obtain similar results. The results confirm that it is possible to diagnose the aGVHD using a selected number of variables. Only one case escaped all our classification models, which achieved 97% accuracy in a leave one-out cross-validation on the testing data set. Experimental results are shown in Table 3 and Figure 3.

Table 3. Experimental results of a CFS with ANN classifier and a wrapper method combined with the naïve Bayesian classifier and with SVM. The starting set has been divided in training set and test set, a leave one-out cross-validation has been calculated for the two subsets.

Method	Training set	Test set
CFS-ANN	28(29)	29(30)
PCA-ANN	-	29(30)
Wrapper-Naïve Bayes	26(29)	29(30)
Wrapper-SVM	29(29)	29(30)

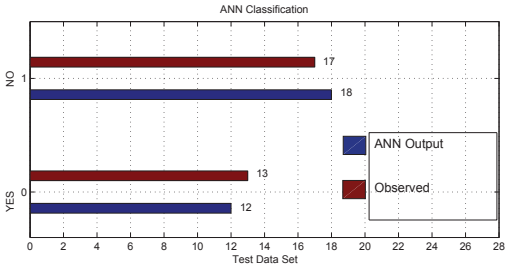

Figure 3. Test results for the ANN model.

Along with the good classification results that suggest a possible use for a clinical diagnostics, the following discoveries about related important genes were made:

a) In patients with aGVHD (YES), the following immune genes were over-expressed when compared with the reference normal values (it is assumed to be = 1): BCL2A1, CASP1, CCL7, CD83. For these genes it is very important to establish the cut-off expression value correlated with the YES event.

b) In contrast, the genes: CXCL10, EGR2, FAS, ICOS, IL-4, IL-10, SELP, SLP1, STAT6 were under-expressed during aGvHD and before of pharmacological treatment.

4 Biomedical Conclusions and Future Work

We examined the immune transcripts to study the applicability of gene expression profiling (macroarray) as a single assay in early diagnosis of aGVHD. Our interest was to select fewer number of molecular biomarkers from an initial gene panel and exploiting this to develop a fast, easy and non-invasive diagnostic test. The proposed method provides a good overall accuracy to confirm aGVHD development in HSCT setting.

From a biological point of view, the results are reliable. Others have reasoned that Th2 cell therapy could rapidly ameliorate severe aGVHD via IL-4 and IL-10 mediated mechanisms [19]. It is noteworthy that in our study a set of genes, indicated by computational analysis, included same mediators of Th2 response such as IL10, and signal transducer and activator of transcription 6, interleukin-4 induced (STAT6). All these were strongly down-regulated in aGVHD (YES) setting, suggesting ab-

sence of control mediated by Th2 cells. Therefore, we highlight the fact that defective expression of ICOS impaired the immune protective effectors during clinical aGVHD. This evidence is supported by a previous report about ICOS as regulatory molecule for T cell responses during aGVHD. It has been showed that ICOS signal inhibits aGVHD development mediated by CD8 positive effector cells in HSCT [20]. According to previous reports, mediators of apoptosis cells and dendritic cell activators were involved.

Altogether our results strongly outlined the importance and utility of non-invasive tool for aGVHD diagnosis based on GEP. We believe that to achieve an advantage from GEP performance, it is very important to know:

a) the transcript levels of immune effector cells in early time post-engraftment in order to better understand polarization of Th2 cells;

b) the CD8 positive cell action.

As a clinical trial, tissue biopsies were performed to confirm the above diagnostic results. In conclusion, our models may prevent the need for an invasive procedure.

This study demonstrated, for the first time, that the proposed here methodology for the selection of gene diagnostic targets and their use for early diagnosis of aGVHD results in a satisfactory 97% accuracy over independent test data set of HSCT population.

We plan to extend the system as a personalized model to capture peculiarity of patients through an optimization method [21-24]. A further approach to feature selection and model creation is the so called integrated approach [25], where features and model parameters are optimised together for a better accuracy of the model, which is an extension of the wrapper approach. As a classifier, a spiking neural network can be explored [25,26]. The authors are engaged in this direction.

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