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Monika Ray, Sekhar Dharmarajan, Johannes Freudenberg, Weixiong Zhang, and Alexander G. Patterson

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Department of Computer Science & Engineering

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Type of Report: Other

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Running Title: Gene expression profiling in lung transplantation

Abstract

Lung transplantation is the treatment of choice for end-stage pulmonary diseases. A limited donor supply has resulted in 4000 patients on the waiting list. Currently, 10-20% of donor organs offered for transplantation are deemed suitable under the selection criteria, of which 15-25% fails due to primary graft dysfunction (PGD). This has resulted in increased efforts to search for alternative donor lungs selection criteria. In this study, we attempt to further our understanding of PGD by observing the changes in gene expression across donor lungs that developed PGD versus those that did not. Our second goal is to use a machine learning tool - support vector machine (SVM), to distinguish unsuitable donor lungs from suitable donor lungs, based on the gene expression data. From our analysis, we have obtained transcripts that were involved in signalling, apoptosis and stress-activated pathways. Results also indicate that metallothionein 3 may prevent lungs from developing PGD. Preliminary classification results for distinguishing suitable and unsuitable lungs for transplantation using a SVM were promising. This is the first such attempt to use human lungs used for transplantation and combine the identification of a molecular signature for PGD, with machine learning methods for donor lung prediction.

Introduction

Lung transplantation has gained widespread acceptance for the treatment of end-stage pulmonary diseases. However, two significant problems in clinical lung transplantation are a major shortage of donor organs and the incidence of primary graft dysfunction (PGD). PGD is a severe allograft ischemia-reperfusion (I/R) injury syndrome occurring in the hours following transplantation. It significantly affects morbidity as well as early and late mortality. Improvements in operative techniques, donor lungs management, and immunosuppressive protocols have decreased perioperative mortality to below 10% at most experienced lung transplant centres (1, 2). The one- and five-year survival rates have improved to 76% and 49%, respectively (1). These results, however, continue to lag behind those achieved for other solid organ transplants. The occurrence of PGD after lung transplantation significantly increases the duration of mechanical ventilation, hospital length of stay and short-term mortality after lung transplantation (3). Survivors of PGD have a significantly protracted recovery with impaired physical function up to one year after transplantation and an increased risk of death extending beyond the first year after transplantation (3, 4).

The current criteria used to evaluate potential donor lungs appear to be inadequate at predicting how these lungs will function post-transplantation (5, 6, 7). Donor organs are evaluated for lung transplantation on the basis of criteria that are primarily historically founded and largely arbitrary (8). Relatively crude measures of lung function such as chest radiography, arterial oxygen tension in blood gases, and bronchoscopy are currently used to assess the quality of potential donor lungs. That these tools are inadequate in evaluating organs from prospective donors is evidenced by two recent developments. First, the liberalisation of the selection criteria and the use of 'marginal' donor lungs by many centres have not had a negative impact on outcome after transplantation (9-11). A

recent study showed no significant difference in a number of indices for infection and inflammation between donor lungs that were accepted and rejected for transplantation (7). Second, the incidence of PGD or I/R injury, after transplantation remains unchanged at 15-25% despite the increased use of marginal donor lungs and improvements in all areas of lung transplantation (2, 4, 12). These issues have led investigators to search for criteria that may subject lungs to increased risk of PGD. While recent studies have identified donor age and recipient diagnosis of primary pulmonary hypertension (PPH) as risk factors for the development of PGD, the aetiology of PGD in most cases after transplantation is unknown and thought to be due to complex interactions between donor lung and recipient immune system (13, 14).

A limited donor supply has dramatically increased the waiting time for transplant recipients. Approximately 4,000 patients are currently on the transplant waiting list and this has resulted in intense pressure to search for alternative strategies. Unfortunately, up to 10-20% of these patients on the waiting list will die from their underlying lung disease before an organ becomes available. Currently, only 10-20% of cadaveric donor organs offered for transplantation are judged to be acceptable under the current selection criteria (15). More biologically meaningful donor lungs selection criteria may result in significant expansion of the number of lungs accepted from this potential donor pool (5).

The results of the above mentioned studies suggest that there may be complex, occult biological factors present in donor lungs which contribute to the development of PGD that are not detected by the current donor organ evaluation. Gene expression profiling is a powerful, high-performance tool of molecular biology that allows the analysis of the levels of expression of thousands of genes simultaneously. It has been previously used to study gene transcripts involved in I/R injury using a rat model (16). To the best of our knowledge, however, this is the first report where gene expression profiling has been used on actual human lungs used for transplantation, along with the application of machine learning techniques to attempt to distinguish unsuitable donor lungs from suitable donor lungs.

Our objective is two fold - the first is to obtain a set of genes involved in PGD and identify new gene products relevant to allograft transplantation; and the second is to use this set of genes for classification of donor lungs into PGD positive (i.e. lungs that develop PGD) or PGD negative (i.e. lungs that do not develop PGD) categories. The first objective would provide greater insights into the mechanisms of PGD as well as extend the work of Yamane et al (16). The set of genes identified as being involved in PGD can be designated as the 'molecular signature' of PGD. As many donor lungs that may be actually suitable for transplantation, are discarded by the current selection criteria, it would be useful to classify unseen donor lungs, using the molecular signature derived in the first objective coupled with machine learning techniques. Such a classification can ultimately perhaps increase the potential donor pool for lung transplantation and is the motivation behind our second objective.

Materials and Methods

Donor lung sampling

From August 2003 to January 2005, 80 transplants were performed in our programme at Washington University School of Medicine. Three of these were excluded from the study as one was a single lung transplant, another was a heart-lung transplant, and the third was a combined coronary artery bypass graft with lung transplant. This resulted in biopsies of 77 donor lungs used for bilateral sequential lung transplantation. The biopsies were obtained from the anterior right middle lobe or lingula immediately prior to cold-flushing. Of these 77, 9 patients did not give consent. Of the remaining biopsies, some samples were excluded due to technical errors or complexities during expression profiling, resulting in a net total of 50 biopsies being used for the study. Five lungs were considered *marginal* donor lungs based on them portraying one or more of the following conditions - either PaO_2 in arterial blood gas < 300 on 100% inspired oxygen, or smoking history > 20 pack-years or donor age > 55. These samples were immediately snap-frozen in liquid nitrogen and then stored in a -70° Celsius freezer until used for analysis. Specimens were sampled using standard techniques for open lung wedge biopsy. An area of lung tissue approximately 1 x 1 cm was isolated and excised using 2 staple lines from a 30 mm EndoGIA stapler (US Surgical, Norwalk, CT). This protocol was approved by the Human Studies Committee and Institutional Review Board at Washington University School of Medicine and protection of human subjects, namely recipients, was afforded by detailed informed consent before entrance into this research protocol. No complications related to sampling of the donor lungs occurred in this study. As this is a pilot study done on actual human lungs used for transplantation, we did not have enough tissue to perform RT-PCR.

RNA Isolation

Single isolates of donor lung samples were homogenised in the presence of RNAzolB and finally dissolved in RNase-free H2O. 25 g of total RNA was treated with DNase using the Qiagen RNase-free DNase kit and samples were further purified using RNeasy spin columns (Qiagen, Valencia, CA). Total RNA treated with DNase was dissolved in RNase-free H2O to a final concentration of 0.2 g/l. RNA quality was assessed by 1% agarose gel electrophoresis in the presence of ethidium bromide. Samples that did not reveal intact and approximately equal 18S and 28S ribosomal bands were excluded from further study.

cDNA Synthesis and Gene Expression Profiling

This study used commercially available high-density microarrays (Affymetrix, Santa Clara, CA) that produce gene expression levels on 22,278 probe sets (Affymetrix Human Genome U133Av2.0 Array). Each donor lung biopsy was analysed on a different GeneChip. Preparation of cDNA, hybridisation, and scanning of the arrays were

performed according to the manufacturer's instructions. The arrays were scanned using the Affymetrix GeneArray scanner. Image analysis was performed with the Affymetrix GeneChip software. We also performed a quality control test on the dataset using the R package 'affyQCReport' (17) and the results were favourable.

Data

The data from all 50 gene chips was normalised using the GCRMA method developed by (18). The 50 donor lung samples were divided into two groups - those that developed PGD after transplantation (PGD positive) and those that did not (PGD negative). PGD was defined as T0 Grade III dysfunction according to International Society for Heart and Lung Transplantation criteria, that is, a ratio (referred to as the P/F ratio) of partial pressure of arterial oxygen (PaO$_2$) to fraction of inspired oxygen (FiO$_2$) less than 200 in the first arterial blood gas in the intensive care unit after transplantation (generally 4-6 hours after actual reperfusion) (19). Although definitions of PGD at later time points may more accurately reflect outcomes after transplantation, they may also be potentially affected by other postoperative factors such as overall fluid balance or presence of infection. Sixteen samples were classified as PGD positive according to this definition and the remaining thirty-four were PGD negative.

Transcripts Selection

We then proceeded to the next step in our study - the identification of differentially expressed (DE) transcripts. The objective was to find a set of DE transcripts/probes that could be used as a molecular signature for the condition. DE transcript extraction falls into two broad categories - wrapper methods and filter methods. In wrapper transcript selection methods, the DE transcript identification phase is integrated with the classification phase. In filter methods, the DE transcript extraction phase is independent of the classification phase. In this study, we used two packages for the identification of DE transcripts - RankGene (20), and significance analysis of microarrays (SAM) (21).

RankGene is a programme for analysing gene expression data, feature selection and ranking genes based on the predictive power of each gene/transcript to classify samples into functional or disease categories. It supports eight different measures for quantifying a gene's ability to distinguish between classes. For our analysis, we used the t-statistics measure of predictability. The t-statistic value is a score for each gene's ability to discriminate between the 2 classes. RankGene ranks genes according to the decreasing order of the absolute value of the t-statistic for each gene. The group of top genes from this ranked list is considered to be the most informative for distinguishing between the classes. SAM is open-source software which identifies DE genes based on the change in gene expression relative to the standard deviation of repeated measurements (21). It uses the false discovery rate (FDR) and q-value method presented in (22) to select genes. As microarrays result in the measurement of several thousand probes, the individual p-values are not a good measure of significance. The q-value is used to adjust for multiple testing. It is analogous to the p-value and is corrected, through a permutation process, for the variability of the expression data. The q-value of a transcript is the FDR for the transcript

list that includes that transcript and all transcripts that are more significant. SAM also provides the tail strength (TS) value which measures the deviation of each p-value from its expected value. Therefore, large positive TS values indicate evidence against the null hypothesis, i.e., there are more small p-values than one would expect by chance (23).

We first ran RankGene on the complete set of probes. Since we were interested in the most highly DE transcripts, we chose to take the top 100 transcripts from the ranked list for further analysis. On this list of 100 DE transcripts, we applied SAM. SAM displayed 81 differentially expressed transcripts based on a FDR of 0% and a TS of 92.7%. After averaging the values of and removing multiple probes mapping to the same gene name, 23 upregulated and 42 downregulated transcripts were obtained. These sets of up and down regulated transcripts were used for further analysis in Ingenuity Pathway Analysis software.

Pathway analysis

Ingenuity Pathway Analysis (IPA) (www.ingenuity.com) was used to perform pathway analysis on the two sets of DE transcripts - upregulated and downregulated, to identify networks of genes that are known to interact functionally. IPA uses the Ingenuity Pathways Knowledge Base (IPKB) which contains large amounts of individually modelled relationships between objects (e.g., genes, proteins and mRNAs) to dynamically generate significant biological/gene expression networks and pathways. The identified DE transcripts from our analysis that are mapped onto the IPKB are called 'focus genes'. These are used as starting points for building the networks. IPA consists of genes that have functions assigned to them and are in published literature. First, IPA queries the IPKB for interactions between the focus genes and all other genes stored in IPKB and then generates a set of networks/pathways with a maximum of 35 genes. A p value for each network is calculated according to the user's list of DE genes. This is accomplished by comparing the number of focus genes that are present in a given pathway, relative to the total number of occurrences of those genes in all pathways stored in IPKB. The score of the network is shown as the negative logarithm of the p value, indicating the likelihood of the focus genes in a network being found together by random chance. In our study, we further analysed networks that had a network score of 10 or higher. If genes do not have any known functions assigned to them, they do not become focus genes in IPA although they have a gene name. This network analysis is an exploratory *in silico* approach and does not necessarily indicate that the pathway or network actually exists.

Support Vector Machines

Originally developed by Vapnik (24), the support vector machine (SVM) is a statistical learning tool which has been extensively used for binary classification with great success. Ranging from classification of cancer (25) to determination of haemodialysis dosage (26), SVMs have proven to be an effective tool in a wide-range of applications.

SVM was used for the classification of patient samples into PGD positive or PGD negative categories. The dataset consisted of 50 patient samples and 100 transcripts (ranked transcripts from RankGene). Following is the manner in which SVM was used. The dataset is divided into training and test (unseen by the classifier) sets. The test set is also the validation set because although the user knows the classes of the samples in the test set, the classifier does not see the samples in the test set while it is training. The SVM is trained on the training set. The classifier performance is measured by the prediction accuracy on the test set. It is quite well known that the set of significant genes (SG) from a particular set of training data is very often very different from one chosen from a different set of training data. Obtaining a SG set from the complete dataset (i.e. from all 50 patient samples), leads to a selection bias. In order to avoid selection bias, an external cross-validation (CV) was performed i.e. the classifier performance was measured using only the set of genes (i.e. a subset of the 100 transcripts) obtained from the training set and not from the complete dataset of 50 patients. Ten fold CV was carried out rather than leave-one-out (LOO) CV, as the variability in the list of SG is much lower with 10 fold CV and this is what is preferred. Results were averaged over 20 runs.

Results and Discussion

The definition of PGD used in this study was T0 Grade III PGD as described by the International Society for Heart and Lung Transplantation consensus statement on the definition of primary graft dysfunction (19). In this statement, any time between immediately post-transplant (T0, ideally defined as arrival in the ICU, within 6 hours post-reperfusion) and 72 hours after transplantation (T72) can be used to measure blood gases and define PGD. Although definitions of PGD at later time points may more accurately reflect outcomes after transplantation, they may also be potentially affected by other postoperative factors such as overall fluid balance or presence of infection. Our objective in this study was to identify biologic risk factors in donor lungs that may contribute to PGD and therefore we felt that this was most purely measured at T0, where lung function may most clearly reflect the status of the donor lung at the time of harvest. Furthermore, data from our institution suggests that PGD as early as T0 is associated with the development of bronchiolitis obliterans syndrome (chronic rejection) (27).

The characteristics of the donor lungs are depicted in Table 1. The operative factors and the outcome of patients with PGD versus those without are shown in Table 2 and Table 3, respectively. Despite other studies correlating donor age and recipient diagnosis of PPH with PGD, we have not seen a significant correlation in our samples. Although, cardiopulmonary bypass (CPB) seemed to be significant, it could also occur *as a result of* PGD rather than be a causative factor of PGD. SAM analysis resulted in 81 differentially expressed (DE) transcripts which resolved into 65 unique genes using DAVID (http://niaid.abcc.ncifcrf.gov/tools.jsp) at the time of writing this paper. This list along with the fold change is presented in Table 4. A flowchart depicting the sequence of analysis is shown in Figure 1.

Pathways and gene products involved in PGD:

The upregulated transcripts were analysed using the Ingenuity Pathway Analysis (IPA) software. There were 23 upregulated transcripts, of which 13 were focus genes. Focus genes are the genes that map onto the Ingenuity Pathways Knowledge Base (IPKB). The network generated from these genes is shown in Figure 2.

Network 1 primarily centres on tumour protein p53 (TP53). The focus genes are shown in shaded/solid shapes and more details on these nodes are given in the supplementary material (Table 1). Figure 3 shows the location of the different gene products and the canonical pathways present in Network 1. The legend for the network is shown in Figure 7. It is natural to expect many pathways related to apoptosis and cell signalling as over 50% of the donor lungs (PGD positive and PGD negative) were involved in some kind of trauma. Interestingly, a few transcripts identified are also cancer related genes. There is growing evidence of genetic parallels between lung development and several types of cancer (28, 29). The authors of (30) have shown that Wnt signalling, cell cycle, and apoptosis pathways play important roles in lung development. We also have noticed an increased presence of genes in these pathways in our study (Figure 3).

Next, we analysed the 42 downregulated transcripts using IPA, and obtained 11 focus genes. The network created from these 11 genes is shown in Figure 4. Network 2 shows a lot of activity around beta-5 integrin (ITGB5) and GRB2-associated binding protein 2 (GAB2). The focus genes are shown in shaded/solid shapes and further description of these nodes are given in the supplementary material (Table 2). Figure 5 shows the location of the different gene products and the canonical pathways present in Network 2. The legend for the network is shown in Figure 7. We observe similar pathways, as the ones present in Network 1, in Network 2. This is not unexpected since a pathway can consist of up and downregulated genes.

Both the networks show the presence of nuclear factorKB (NFKB), stress-activated protein kinases NH_2-terminal Jun kinase (SAPK/JNK) and p38 mitogen-activated protein kinase (MAPK) signalling pathways. NFKB plays a vital role in mediating immune and inflammatory responses, and apoptosis. It regulates the expression of a large number of genes. Many of the gene products regulated by NFKB in turn activate NFKB, such as vascular endothelial growth factor (VEGF), and receptor for advanced glycation end product (RAGE). Activation of NFKB involves the phosphorylation-induced, proteasome-mediated degradation of the inhibitory subunit - inhibitory protein KB. This protein is phosphorylated by an upstream serine kinase, which, in turn is phosphorylated and activated by additional upstream serine kinases. SAPK/JNK are members of the superfamily of MAP serine/threonine protein kinases. This family also includes p38 MAP kinases (p38 MAPK) and extracellular signal-related kinases (ERK) (31). JNK/SAPK and p38 MAPK are known as stress-activated kinases, and are responsive to numerous exogenous and endogenous stress-inducing stimuli, such as reactive oxygen species (ROS), oxidative stress, osmotic stress, proinflammatory cytokines, heat shock,

and ultraviolet irradiation. Oxidative stress is defined as a persistent imbalance between the production of highly reactive molecular species (primarily oxygen and nitrogen) and antioxidant defences, finally resulting in tissue damage. There is evidence in literature that NF-KB, SAPK/JNK and p38 MAPK signalling pathways are stress-sensitive intracellular signalling systems, activation of which results in the increased expression of numerous gene products that cause cellular damage (32).

Gene products associated with stress-activated pathways emerged from both our study as well as the study in the rat model for ischemia-reperfusion injury (16). As the experimental protocol, and animal model are different, one would not expect too much of an overlap.   As suggested by the recent articles in *Nature Biotechnology* by the MicroArray Quality Control (MAQC) project (33), it is better to focus on pathways and broad functional relationships, rather than on individual genes. They state that "even under the best circumstances, gene lists will still differ somewhat from person to person and place to place". In our work, we have observed a good deal of overlap in the functional categories/pathways of the identified transcripts. As not all animal model studies translate well into human analysis, our investigation takes the study performed by (16) a step further by performing the analysis on human samples and showing consensus.

An exciting observation was that the metallothionein family of gene products was identified as being upregulated in the lungs that did not develop PGD. In the work by Yamane et al (16), metallothionein levels of expression are much lower in the microarray when compared to most of the other genes considered significant. However, RT-PCR confirms that it does have an increased expression. Hence, the rat study as well as ours does confirm the elevated expression of metallothionein.

Metallothioneins (MT) are ubiquitous, low molecular weight, intracellular zinc-binding proteins with antioxidant properties. MT consists of 3 isoforms – MT1, MT2 and MT3. We extracted the metallothionein 3 (MT3) pathway from Network 1 (see Figure 6). Although the exact mechanism by which MT3 operates is not well known, there are a few studies that have explained the possible roles of metallothionein, especially MT1 and MT2. A recent study has shown that metallothioneins have positive effects during the early phase of islet transplantation (34). Another study has shown that the metallothionein gene is upregulated in wound margins particularly in regions of high mitotic activity (35). These observations reflect its role in promoting cell proliferation and re-epitheliation. Furthermore, selected growth factors may modulate metallothionein gene expression and hence, the ability of cells to proliferate (35). As can be seen from Figure 6, MT3 is connected to NF-KB1. In human fibroblasts, NFKB protein consisting of p50 [NFKB1] and of p65 v-rel reticuloendotheliosis viral oncogene homolog A (RELA) increases expression of human MT3 mRNA. There is also an indirect relationship between MT3 and epidermal growth factor (EGF). EGF is involved in EGF signalling, ephrin receptor signalling, neuregulin signalling, and NFKB signalling. EGF's role in the cell is proliferation, migration, mitogenesis, apoptosis, growth, chemotaxis, transformation, stimulation, S phase, and differentiation. Several other papers have also shown that metallothionein positively regulates the cellular level and activity of NF-KB (36, 37). Recent work by St. Croix et al (38), has also shown the protective role of

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metallothionein in acute lung injury. Cells deficient in MT1 and MT2 have shown increased sensitivity to apoptosis (39). Other work suggests that under inflammatory conditions, MT supports beneficial movement of leukocytes to the inflammation site (40). In vitro experiments have shown that modest increase in MT levels still provides protection against oxidative stress (41). All this research on MT suggests that it is a valuable gene and should be analysed in extensive detail in the context of PGD. Furthermore, whether the MT3 isoform has the same properties as MT1 and MT2 needs to be determined. The overexpression of metallothionein 3 may protect the lung graft from PGD. We feel that this is one of the most important insights into the mechanism of PGD.

Classification of donor lungs using SVM:

The set of 100 ranked transcripts, obtained using RankGene, was used for the classification of donor lungs into PGD positive and PGD negative classes by SVM. The classification accuracy of SVM in differentiating the two classes was 70%. This indicates that this set of transcripts has enough information to distinguish unsuitable and suitable donor lungs.

The SVM did better at identifying the suitable lungs (i.e. low false negative). Considering that the motivation behind using machine learning for the selection of suitable donor lungs was to detect those that otherwise would have been discarded, this observation is promising. The unsuitable donor lungs were more often misclassified and this can be attributed to the fact that there were very few unsuitable donor lungs in the dataset (16 unsuitable lungs versus 34 suitable lungs) and subsequently, an even smaller number in the training set. Furthermore, our dataset had been pre-selected by physicians based on clinical criteria. Hence, the dataset did not have *truly* unsuitable donor lungs, i.e., lungs considered unsuitable by clinical criteria. Obviously, certain lungs that passed the selection criteria developed PGD. In essence, these were lungs that *seemed* to be good by the current clinical criteria. Hence, the gene expression patterns of the unsuitable donor lungs are very similar to the patterns of suitable lungs. In fact, when the gene expression values of the DE transcripts were compared between PGD positive and PGD negative lungs, the difference was marginal. These observations are not surprising as both sets of lungs were considered suitable by clinical criteria, and therefore the difference between them would be very subtle.

The SVM had difficulty in recognising some unsuitable donor lungs as it was not being trained on the gene expression pattern of a large number of unsuitable donor lungs, or, for that matter, on a large number of *truly* unsuitable donor lungs. Given the fact that we had only 50 samples, in which we did not have truly unsuitable lungs, the classification performance is good. Increasing the sample size in both categories would lead to a more accurate and possibly larger set of DE transcript involved in PGD, as well as improved classification results.

As the differences at the macroscopic level between PGD positive and PGD negative donor lungs are minimised after employing the clinical selection criteria, gene expression

profiling would help in amplifying whatever small differences there may be. SVMs are capable of using these marginal differences to identify suitable and unsuitable donor lungs. This is where machine learning plays a valuable role - *assisting* physicians and not necessarily overruling them. Hence, machine learning methods, such as SVMs, can be used in conjunction with clinical criteria to identify unsuitable donor lungs, thereby further decreasing the chances of using donor lungs that would develop PGD. Due to the limited dataset, it would be advantageous to have a larger dataset for further validation.

The study design affects the kind of questions that can be posed as well as the quality of answers. As this was a pilot study to test the feasibility of the approaches, we restricted it to only a few samples. Furthermore, we did not have enough tissue material to perform RT-PCR to validate the microarray results. We hope that this research would motivate and warrant the need for a larger study with more sophisticated statistical methods as well as microarray validation tools. Moreover, an animal model would allow for more samples to be taken at different time points which would further strengthen the study. An interesting strategy would be to perform a random sampling of different regions of the lung and subject it to microarray analysis. This was not done in this study due to three reasons – 1) taking multiple samples from a donor lung when the primary objective is transplantation is difficult to justify; 2) taking samples from different regions of the lung necessitates the need for a larger sample set in order to reduce variance and increase statistical power and finally 3) it was convenient to take a biopsy from the lingula or anterior right middle lobe as opposed to other regions, without compromising the amount of time the lung is kept without cold flushing. Due to the limited number of samples, we could not perform an external validation and resorted to a 10-fold cross validation, which is normal in many cases where SVM has been used for classification in a clinical study. Furthermore, in order to improve the classification accuracy, it would be necessary to include lungs in the training set that have been rejected by the clinical criteria. Although we obtained 23 upregulated and 42 downregulated transcripts, only 13 of the 23 and 11 of the 42 transcripts became focus genes in IPA. This indicates that the majority of the transcripts do not have any specific function assigned to them as yet. Further research into the functions of these transcripts will also provide some insight into their role in PGD.

Conclusion

The incorporation of biological information into donor lung evaluation, based on studies such as this one, may deem many of the excluded organs as suitable for transplantation, directly impacting the mortality of patients on the lung transplant waiting list. Studies show that 15-25% of patients develop clinically significant primary graft dysfunction (PGD) after lung transplantation. PGD is the single most significant factor in determining perioperative morbidity and mortality and has a devastating impact on outcome following lung transplantation. It is the primary factor determining duration of mechanical ventilatory support and length of ICU and hospital stay following lung transplantation.

Perioperative mortality rates for those with clinically significant PGD are as high as 40-60%. One year survival rates fall from 69% to 40% and 2-year rates from 66% to 27% in those who suffer significant PGD. Furthermore, those that survive complications of PGD endure lengthy hospitalisation periods and a protracted and often compromised recovery, evidenced by inferior exercise tolerance and pulmonary function testing and the inability to achieve independent lifestyles. Moreover, PGD is now being identified as a risk factor for acute and chronic rejection.

In this study, gene expression profiling of donor lung samples was used to determine gene products that are associated with the development of PGD after transplantation. It also resulted in analysing possibly relevant pathways involved in PGD. When biological markers were used to differentiate between PGD positive and PGD negative lungs, good classification accuracy was achieved. The incorporation of biological markers into donor organ evaluation will have a significant impact on outcomes after lung transplantation, by potentially expanding the donor pool of organs selected for transplantation and by identifying lungs at risk for the development of PGD post-transplant, which would allow pre-treatment of these high risk organs or matching of these organs to relatively lower risk recipients. Further identification and elucidation of genetic markers in donor lungs associated with PGD could have a significant impact on lowering the incidence and preventing the morbidity and mortality of PGD after lung transplantation. Our results indicate that we have successfully achieved both our objectives.

Acknowledgements

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Supplementary material:

Table 1: Description of the upregulated transcripts from our DE list present in Network 1.

Table 2: Description of the downregulated transcripts from our DE list present in Network 2.

TABLES

TABLE 1: Clinical Donor Characteristics

Characteristics	PGD (n=16)	No PGD (n=34)	*p* value
Age (years)	26.6 ± 8.9	24.0 ± 9.8	0.53
PaO$_2$	406.7 ± 80.5	449.7 ± 80.0	0.17
Smoking history (years)	1.5 ± 2.07	2.9 ± 6.32	0.59
Gender	71% M, 29% F	83% M, 17% F	0.78
Cause of death	57% Trauma, 43% non-Trauma	75% Trauma, 25% non-Trauma	0.66
Marginal donors	1	4	0.99

TABLE 2: Operative factors

Factors	PGD (n=16)	No PGD (n=34)	*p* value
Recipient diagnosis	32% COPD, 25% CF, 43% other	35% COPD, 32.5% CF, 32.5% other	0.98
Recipients with PPH	2	1	0.24
1st lung ischemic time (min)	208.0 ± 44.0	240.0 ± 51.0	0.18
2nd lung ischemic time (min)	330.0 ± 72.0	321.0 ± 51.0	0.69
Cardiopulmonary bypass (CPB)	72%	17%	0.02

TABLE 3: Outcomes of patients with and without PGD

Outcome	PGD (n=16)	No PGD (n=34)	*p* value
Days on ventilator	9.7 ± 11.7	2.0 ± 3.7	0.01
ICU stay (days)	11.3 ± 12.6	2.9 ± 3.6	0.006
Total length of stay (days)	20.3 ± 13.0	13.4 ± 8.1	0.09
Perioperative mortality	28.5%	0%	0.02

TABLE 4: List of 81 differentially expressed transcripts output by SAM. There are 23 upregulated and 42 downregulated genes.

UP-regulated in PGD negative lungs

REFSEQ_MRNA	Gene Name	Fold Change
NM_005633	SON OF SEVENLESS HOMOLOG 1 (DROSOPHILA)	2.2183831
NM_000492	CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR, ATP-BINDING CASSETTE (SUB-FAMILY C, MEMBER 7)	1.7822446
NM_003645	FATTY-ACID-COENZYME A LIGASE, VERY LONG-CHAIN 1	1.7679424
NM_005573	LAMIN B1	1.6928441
NM_005502	ATP-BINDING CASSETTE, SUB-FAMILY A (ABC1), MEMBER 1	1.6240539
NM_017613	DOWNSTREAM NEIGHBOR OF SON	1.5424873
NM_019841	TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL, SUBFAMILY V, MEMBER 5	1.5196761
NM_017760	LEUCINE ZIPPER PROTEIN 5	1.5172142
NM_018365	MEIOSIS-SPECIFIC NUCLEAR STRUCTURAL 1	1.5161006
NM_183419, NM_015435	RING FINGER PROTEIN 19	1.4642686
NM_015024	EXPORTIN 7	1.4545849
XM_938545	SIMILAR TO FORMIN-BINDING PROTEIN 3 (FORMIN-BINDING PROTEIN 11) (FBP 11)	1.3552737
NM_017654	STERILE ALPHA MOTIF DOMAIN CONTAINING 9	1.3251204
NM_016265	ZINC FINGER PROTEIN 12	1.2960459
NM_005531	INTERFERON, GAMMA-INDUCIBLE PROTEIN 16	1.2918867
NM_007358	METAL RESPONSE ELEMENT BINDING TRANSCRIPTION FACTOR 2	1.2824896
NM_005954	METALLOTHIONEIN 3 (GROWTH INHIBITORY FACTOR (NEUROTROPHIC))	1.2808244
NM_015205, NM_032189	ATPASE, CLASS VI, TYPE 11A	1.2417599
NM_007118	TRIPLE FUNCTIONAL DOMAIN (PTPRF INTERACTING)	1.1514956
NM_001004420, NM_001004419, NM_013269	C-TYPE LECTIN DOMAIN FAMILY 2, MEMBER D	1.1469583
NM_181657	LEUKOTRIENE B4 RECEPTOR	1.1067929
NM_014229	SOLUTE CARRIER FAMILY 6 (NEUROTRANSMITTER TRANSPORTER, GABA), MEMBER 11	1.0890867
NM_201279, NM_003872, NM_201266	NEUROPILIN 2	1.0625556

Down-regulated in PGD negative lungs

REFSEQ_MRNA	Gene Name	Fold Change
NM_024917	CHROMOSOME X OPEN READING FRAME 34	2.3873841
NM_024508	ZINC FINGER, BED-TYPE CONTAINING 2	2.0313459
NM_022460	HS1-BINDING PROTEIN 3	1.6525671
NM_178312, NM_178311, NM_080920	GAMMA-GLUTAMYLTRANSFERASE-LIKE ACTIVITY 4	1.5240059
NM_198544	CORTISTATIN	1.5027289
NM_014241	PROTEIN TYROSINE PHOSPHATASE-LIKE (PROLINE INSTEAD OF CATALYTIC ARGININE), MEMBER A	1.4121977
NM_079837, NM_017869	BTG3 ASSOCIATED NUCLEAR PROTEIN	1.3943394
NM_025124	HYPOTHETICAL PROTEIN FLJ21749	1.3593494
NM_001017962, NM_000917	PROCOLLAGEN-PROLINE, 2-OXOGLUTARATE 4-DIOXYGENASE (PROLINE 4-HYDROXYLASE), ALPHA POLYPEPTIDE I	1.3539779
NM_022337	RAB38, MEMBER RAS ONCOGENE FAMILY	1.3356873
NM_002035	FOLLICULAR LYMPHOMA VARIANT TRANSLOCATION 1	1.3315413
NM_024956	TRANSMEMBRANE PROTEIN 62	1.3201902
NM_003562	SOLUTE CARRIER FAMILY 25 (MITOCHONDRIAL CARRIER; OXOGLUTARATE CARRIER), MEMBER 11	1.3193721
NM_007001	SOLUTE CARRIER FAMILY 35, MEMBER D2	1.3140864
XM_929985	SIMILAR TO LARGE SUBUNIT RIBOSOMAL PROTEIN L36A	1.3078617
NM_021029	RIBOSOMAL PROTEIN L36A	1.3078617

NM_019040	ELONGATION PROTEIN 4 HOMOLOG (S. CEREVISIAE)	1.2951361
NM_015654	N-ACETYLTRANSFERASE 9	1.2935561
NM_007069	HRAS-LIKE SUPPRESSOR 3	1.2878168
XM_937648	SIMILAR TO ALVEOLAR SOFT PART SARCOMA CHROMOSOME REGION, CANDIDATE 1	1.2864441
NM_153741, NM_018973	DOLICHYL-PHOSPHATE MANNOSYLTRANSFERASE POLYPEPTIDE 3	1.2849801
NM_014320	HEME BINDING PROTEIN 2	1.2791263
NM_006476	ATP SYNTHASE, H+ TRANSPORTING, MITOCHONDRIAL F0 COMPLEX, SUBUNIT G	1.2710706
NM_002513	NON-METASTATIC CELLS 3, PROTEIN EXPRESSED IN	1.2675264
NM_018158	SOLUTE CARRIER FAMILY 4 (ANION EXCHANGER), MEMBER 1, ADAPTOR PROTEIN	1.2606948
NM_002213	INTEGRIN, BETA 5	1.2579474
NM_005865	PROTEASE, SERINE, 16 (THYMUS)	1.2495258
NM_020385	REX4, RNA EXONUCLEASE 4 HOMOLOG (S. CEREVISIAE)	1.2404477
NM_015958	DPH5 HOMOLOG (S. CEREVISIAE)	1.2402215
NM_021824	NIF3 NGG1 INTERACTING FACTOR 3-LIKE 1 (S. POMBE)	1.2392898
NM_014173, NM_001033549 NM_213622, NM_201647,	HSPC142 PROTEIN	1.2375188
NM_006463	STAM BINDING PROTEIN	1.2360276
NM_207356	CHROMOSOME 1 OPEN READING FRAME 174	1.2286265
NM_016142	HYDROXYSTEROID (17-BETA) DEHYDROGENASE 12	1.2277373
NM_016080	CHROMOSOME 17 OPEN READING FRAME 25	1.2263789
NM_080491, NM_012296	GRB2-ASSOCIATED BINDING PROTEIN 2	1.2246286
NM_032900	RHO GTPASE ACTIVATING PROTEIN 19	1.2229376
NM_004699	FAMILY WITH SEQUENCE SIMILARITY 50, MEMBER A	1.2086274
NM_003060	SOLUTE CARRIER FAMILY 22 (ORGANIC CATION TRANSPORTER), MEMBER 5	1.1931567
NM_014300	SEC11-LIKE 1 (S. CEREVISIAE)	1.1914145
NM_024766	CHROMOSOME 2 OPEN READING FRAME 34	1.1822266
NM_001004	RIBOSOMAL PROTEIN, LARGE, P2	1.1529057

FIGURE LEGENDS

FIGURE 1: Sequence of analysis undertaken in this study. Gene expression profiling was conducted on 50 lung samples. The transcripts were ranked using RankGene in descending order of their t-statistic and the top 100 were selected for further analysis. This set of 100 transcripts was used for classification by SVM and resulted in an accuracy of 70%. The set of 100 transcripts was also analysed using SAM to determine up and down regulated transcripts. SAM output 81 differentially expressed transcripts. After averaging the values of and removing multiple probes mapping to the same gene name, 23 upregulated and 42 downregulated transcripts were obtained. Of the 23 upregulated transcripts, 13 became focus genes in IPA and of the 42 downregulated, 11 were focus genes in IPA.

FIGURE 2: Network 1 - upregulated genes in PGD. This network primarily centres on tumour protein p53 (TP53). The focus genes are shown in shaded/solid shapes. Further details on the focus genes are provided in Table 4. The legend for this figure is Figure 7.

FIGURE 3: Network 1 with canonical pathways overlaid. The focus genes are shown in shaded/solid shapes. The location of the gene products is also indicated. Further details on the focus genes are provided in Table 4. The legend for the figure is Figure 7.

FIGURE 4: Network 2 - downregulated genes in PGD. This network shows a lot of activity around beta-5 integrin (ITGB5) and GRB2-associated binding protein 2 (GAB2). The focus genes are shown in shaded/solid shapes. Further details on the focus genes are provided in Table 5. The legend for this figure is Figure 7.

FIGURE 5: Network 2 with the canonical pathways overlaid. The focus genes are shown in shaded/solid shapes. The location of the different gene products is also depicted. Further details on the focus genes are provided in Table 5. The legend for this figure is Figure 7.

FIGURE 6: Network 3 - Metallothionein pathway. In human fibroblasts, NFKB protein consisting of p50 [NFKB1] and of p65 v-rel reticuloendotheliosis viral oncogene homolog A (RELA) increases expression of human MT3 mRNA. The overexpression of metallothionein may protect the lung graft from PGD. The legend for this figure is Figure 7.

FIGURE 7: Network Legend (a) Key for nodes in the network, (b) Key for edges in the network, (c) Key for edge labels in the network
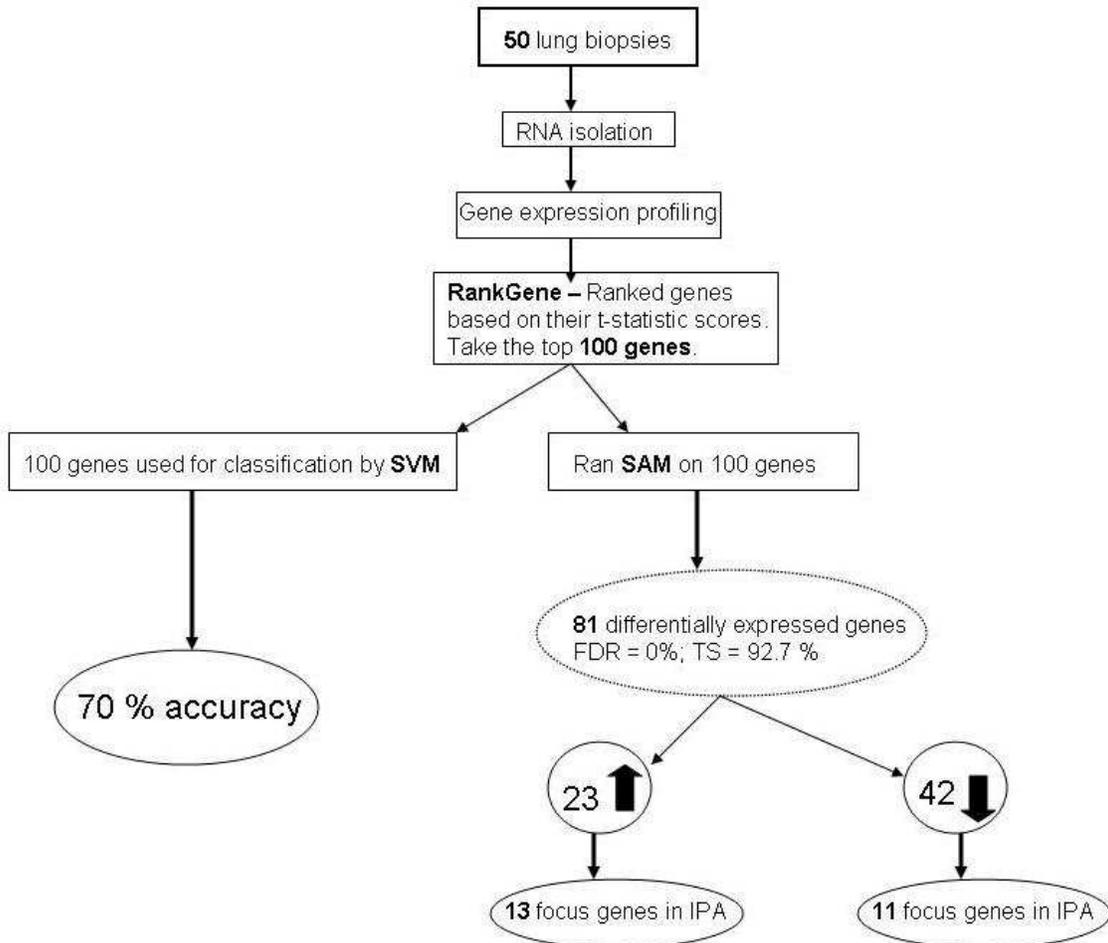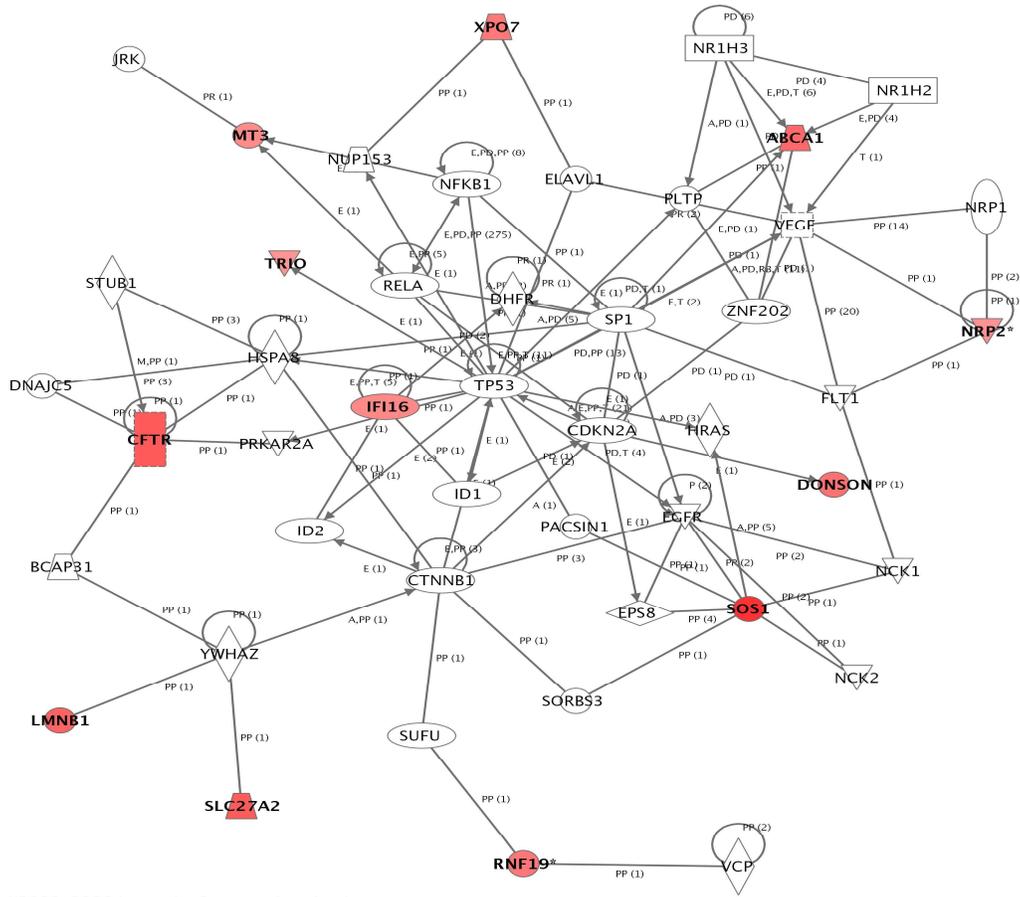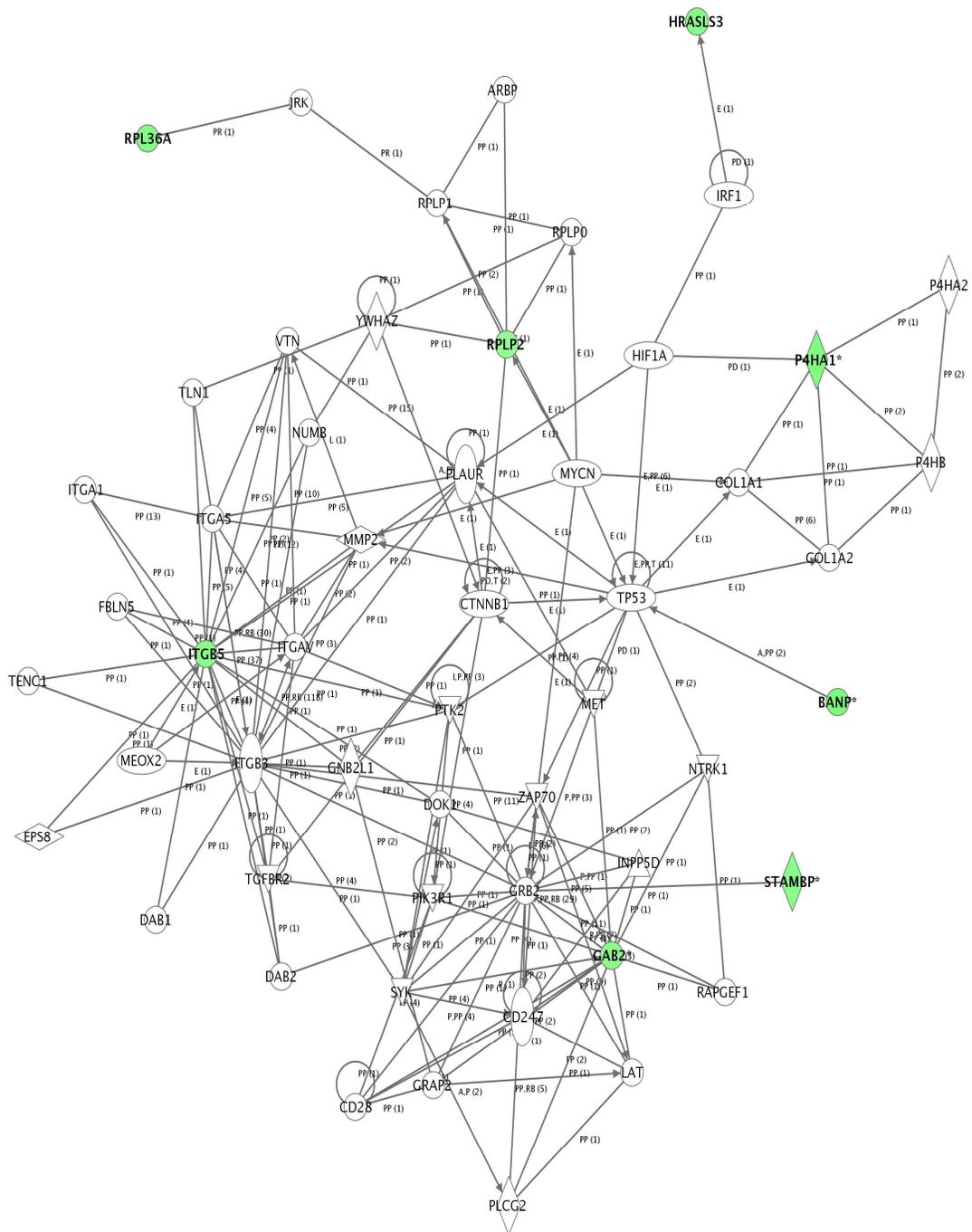
FIGURES

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

23

FIGURE 5

FIGURE 6

FIGURE 7

Nodes

☐ — Cytokine

◇ — Enzyme

☐ — Growth Factor

▯ — G-Protein Coupled Receptor

▯ — Ion Channel

▽ — Kinase

▭ — Nuclear Receptor

○ — Other

◇ — Peptidase

△ — Phosphatase

⬭ — Transcription Regulator

⬡ — Translation Regulator

⬭ — Transmembrane Receptor

△ — Transporter

A   Activation / Deactivation
RB   Regulation of Binding
PR   Protein-MRNA binding
PP   Protein-Protein binding
PD   Protein-DNA binding
B   Binding (only appears prior to IPA 3.0)
E   Expression
I   Inhibition
L   ProteoLysis
M   Biochemical Modification
O   Other (only appears prior to IPA 3.0)
P   Phosphorylation / Desphosphorylation
T   Transcription
LO   Localization

Edges

(A)———(B)
binding only

(A)———|(B)
inhibits

(A)———▶(B)
acts on

(A)———▶|(B)
inhibits AND acts on

Note: "Acts on" and "Inhibits" edges may also include a binding event.

——————
direct interaction

· · · · · · · · · ·
indirect interaction