miRNA based pathway analysis tool in nephroblastoma as a proof of principle for other cancer domains

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Abstract— **Wilms tumor, or nephroblastoma, is a cancer of the kidneys that typically occurs in children and rarely in adults. Around 10% of Wilms tumor patients are diagnosed having a concurrent syndrome that enhances the risk of Wilms tumor. A screening method for early detection of Wilms tumor in these patients would be beneficial, since the size or stage of a tumor is related to outcome. We introduce a miRNA pathway analysis methodology that takes into account the topology and regulation mechanisms of the gene regulatory networks and identify disrupted sub-paths in known pathways, using miRNA expressions. The methodology was applied on a miRNA-expression study and a predictive model was developed, using machine-learning (decision-tree induction) approaches. The model is able to identify putative mechanisms that underlie and govern the Wilms tumor phenotype, and discriminate between diseased and healthy subjects. Initial experimental results are promising and in line with the relevant biomedical literature.**

Keywords— miRNAs, pathway analysis, clinical predictive model, disrupted pathways, gene regulatory networks, systems biology

I. Introduction

MicroRNAs (miRNAs) are endogenous molecules containing about 22 nucleotides that can play an important regulatory role in animals and plants by targeting messenger RNAs (mRNAs) for cleavage or translational repression[1]. miRNA research has revealed multiple roles in negative regulation [2] (transcript degradation and sequestering, translational suppression) and possible involvement in positive regulation (transcriptional and translational activation). A miRNA controls gene expression post-transcriptionally either via the degradation of target mRNAs or the inhibition of protein translation. Using high-throughput profiling, dysregulation of miRNAs has been widely observed in different stages of cancer [3, 4]. The up-regulation (overexpression) of specific miRNAs could lead to the repression of tumor suppressor gene-expression, and conversely the down-regulation of specific miRNAs could result in an increase of oncogene expression; both these situations induce subsequent malignant effects on cell proliferation, differentiation, and apoptosis that lead to tumor growth and progress [5, 6]. Since their discovery, miRNAs are recognized as crucial regulators of gene-expression that regulate a range of processes [7]. Nowadays, it is evident that miRNAs regulate diverse cellular pathways and are widely believed to regulate most biological processes [8, 9, 10]. As Chen et al [5] state, miRNAs play key roles in human cancer; identifying the underlying pathways will provide a more complete understanding of their functions and regulations during cancer progression, and may have clinical applications in the future.

The activation of miRNAs can result in the post-transcriptional down-regulation or up-regulation of the expression of certain genes [11], which subsequently affect other genes downstream in pathways or biological processes. Given such knowledge, a clinical question appears: "*Can we use the disrupted by miRNAs sub-paths to train a predictive model, and utilize this knowledge in biomedical research and clinical practice*?" For a patient such knowledge is critical since, the treating physician may proactively select targeted drugs for his/her treatment. Figure 1 shows an indicative example where, the overexpressed miRNAs (in green, Figure 1, part A) target specific genes (in blue, Figure 1, part B) and disrupted the MAPK signaling path (targeted down-regulated genes in blue, Figure 1, part C).

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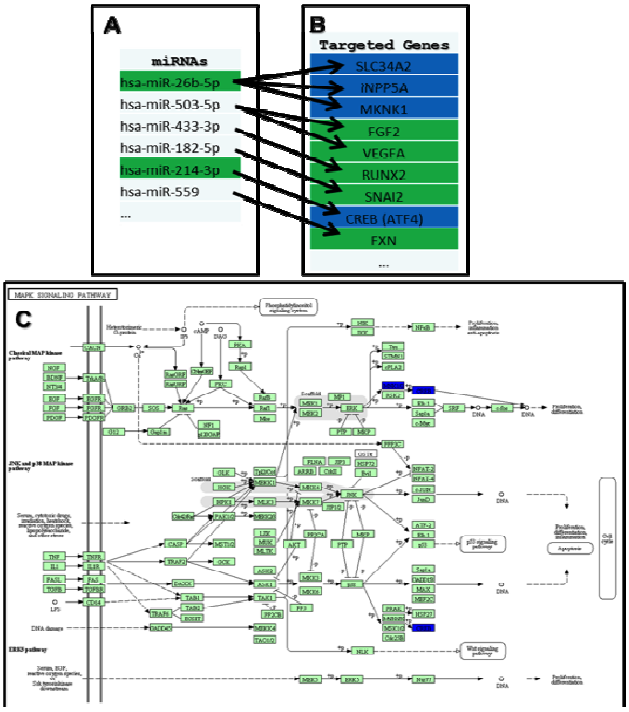

Figure 1: From miRNAs to disrupted sub-paths. Part A expressed (green) miRNAs, part B targeted genes in blue and functional genes in green, part C functional sub-paths in green and disrupted sub-paths in blue

In the literature we can find pathway analysis tools for miRNAs, e.g., DIANA-miRPath [12], GeneTrail [13] or InnateDB [14]. One critical drawback of these tools comes from the way they handle the pathways. Each pathway is viewed and represented just as the set of the genes it engages, and using gene-enrichment methodologies they determine which biological pathways are significantly over-represented (i.e., more than expected by chance). Following this pathway analysis approach, these tools cannot access and do not provide information for parts (i.e., sub-paths) of the pathway. Over-representation pathway analysis approaches aim to determine whether certain pathways are over-represented (i.e., enriched) in the given miRNA expression profiles. Furthermore, such tools neglect the pathway topology and disregard the underlying regulatory interactions between genes (e.g. activation, expression, inhibition).

In the present study, we introduce a novel miRNA pathway analysis methodology that takes into account the pathway topology as well as the regulatory interactions between the involved genes. The key feature of the methodology is that it tries to access the differential (between phenotypes) power of pathway sub-paths and not just genes. We applied this methodology on known pathways from the KEGG repository in order to identify pathways disrupted from miRNAs and utilize that knowledge in order to discriminate between Wilms tumor (nephroblatoma) and healthy subjects. The work reported here is conducted in the context of the p-medicine project (www.p-medicine.eu). The following section outlines the methodology of our approach, the publicly available datasets and the evaluation of the devised prediction models. In section III we present and assess the experimental results, accompanied by their biological validation. Finally, in the last section we summarize and conclude with an outlook on the potential of the approach in the clinical setting.

II. Methodology

A. *Outline of the Methodology*

The reference cohort for the creation of the model is based on the hsa (human) KEGG pathways (223 in total). The proposed methodology for the identification of disrupted pathways by miRNAs, and the induction of classification models to predict Wilms Tumor (WT) diseased vs. healthy subjects, unfolds into two steps:

Step-1 – Identification of disrupted pathways. The task is to identify sub-paths in pathways being disrupted by miRNAs utilizing the gene-expression profiles of patients and healthy subjects from a reference cohort (selected by expert clinicians). Pathway analysis and identification of disrupted pathways is based on the MinePath (www.minepath.org) tool [15]. MinePath is able to identify differentially expressed pathway sub-paths, i.e., sub-paths that are functional for one class (phenotype) and not functional for another. MinePath takes in consideration the underlying pathway regulatory functions (i.e., the type of gene-gene interactions). The tool has been extended and appropriately customized in order to support miRNA expression data. The output of the first step is a *matrix of disrupted pathway sub-paths profiles* for the reference cohort subjects. The rows of the matrix refer to the identified sub-paths and the columns to samples, with the matrix cells to be binary-valued: '1' for the sub-path being disrupted for the respective sample, and '0' otherwise. For the identification of genes targeted by miRNAs we utilize the MirTarBase database (http://mirtarbase.mbc.nctu. edu.tw/).

Step-2. In the second step, the disrupted sub-paths matrix feeds an induction process (e.g., support-vector machine, decision-tree induction etc) in order to induce a classification model able to predict if a person candidate for a WT test is actually diseased or not. The prediction is based on the person's miRNA expression profile, the reference cohort (selected by the expert physician), and the respective list of miRNA-targeted genes.

The whole methodology is realized by a harmonized bunch of components (from data-extraction to induction of prediction models), and it is encapsulated in a clinico-genomic decision-making tool to be operated by expert clinicians. The tool is wrapped as a service that rests in the p-medicine's workbench of tools. The whole methodology, its constituent components and interactions, as well as the underlying data-flow are summarized and illustrated in Figure 2.

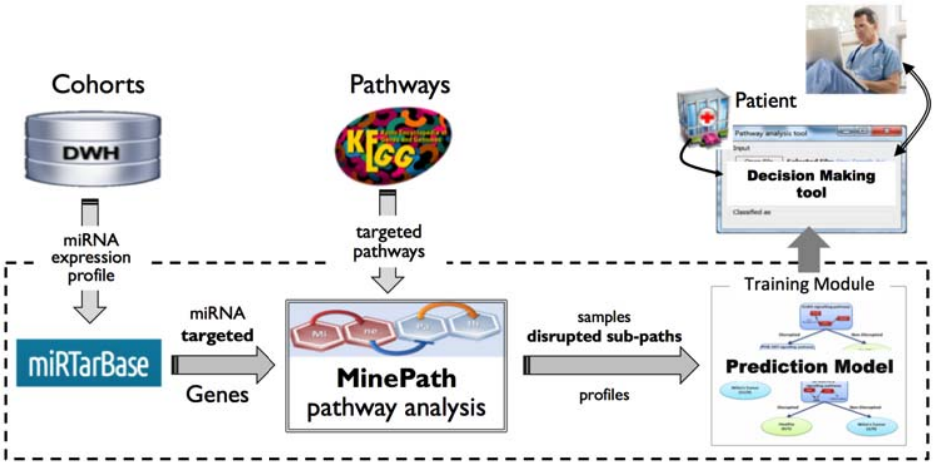

Figure 2: The identification of disrupted by miRNAs pathways methodology: components, tools and data-flow

B. Data

The reference cohort for the creation of the model is based on the KEGG human pathways (223 in total) and the GSE38419 public (form Gene Expression Omnibus – GEO; www.ncbi.nlm.nih.gov/geo) miRNA dataset [16]. The utilized expression profiling platform, Geniom Biochip Homo sapiens v12, febit biomed GmbH, contains 7 replicates of 848 miRNAs as annotated in the Sanger miRBase [17] v12.0. The dataset contains 23 WT patient samples (prior to chemotherapy) and 19 healthy controls. The mean age of the treated patients was 3.3±2.2 and the mean age of healthy controls was 37.8±14.2.

C. Data pre-processing

Data pre-processing is an important step in the mining of genomic data, and in our case this step concerns the discretization of gene-expression data (MinePath operates on such data). Discretization of expression values means that each value is assigned to an interval of numbers that represents the expression-level of the gene for a specific sample. A variable set of such intervals may be utilized and assigned to naturally interpretable values e.g., 'low', 'high'. The discretization process is applied to each miRNA separately. Initially the expression levels of each miRNA across all samples are sorted in descending order. Then all the midpoints between consecutive values are calculated and the samples are dichotomized into two respective groups. For each midpoint, an information-gain formula is applied, which computes the entropy of the system with respect to its division into subgroups. Finally, the midpoint that exhibits results the highest information-gain is selected as the optimal discretization point. Details regarding the discretization process can be found in [18].

D. miRNA targets

Many miRNA-related database systems have been developed to provide further insight into miRNAs and their target genes. For the identification of the targeted genes we used the miRTarBase [19], a comprehensive collection of experimentally validated miRNA–target interactions (MTI). The biological features of miRNA-target duplex are observed based on the largest collection of human MTIs currently available. miRTarBase has accumulated more than fifty thousand miRNA target interactions, being collected by both manual and text-mining operations. The collected MTIs are validated by reporter assay, western blot, microarray and next-generation sequencing experiments. miRTarBase provides the most updated collection compared with other similar databases. We used the current release (release 4.5) of the database that refers to: 2636 number of articles, 18 species, 17520 gene targets, 1232 miRNAs and 51460 miRNA target interactions. For the GSE38419 miRNA expression dataset that contains 848 miRNAs, we identified 7067 validated miRNA target interactions.

E. Pathway analysis - MinePath

MinePath is a pathway analysis web-based platform that implements a methodology for the identification of differentially expressed functional paths or sub-paths within Gene Regulatory Networks (GRNs) using gene-expression data. We use the term 'GRN' for the signaling/cellular pathways. The analysis takes advantage of the topology and the types of interactions between GRN genes (e.g. activation/expression, inhibition). The methodology initially locates all functional paths encoded in (selected and targeted) GRNs and tries to assess which of them are compatible with the gene-expression values of samples that belong to different clinical phenotypes (e.g., disease vs. healthy). The differential power of the selected paths is computed and their biological relevance is assessed.

Currently MinePath copes with GRNs from the KEGG database [20]. Since its first introduction in 1995, KEGG pathways have been widely used as a reference knowledge base for understanding biological pathways and the function of cellular processes. Human related GRNs could be downloaded in KGML format (the KEGG Markup Language). The GRN is described through standard graph annotation. Nodes are used to represent either genes, groups

of genes, compounds or other networks. Edges represent known biological gene relations, e.g., activation, inhibition, expression, phosphorylation, association, dissociation etc. Each gene relation possesses different semantics, according to the precise biology phenomenon that take place during the regulation of the specific network. MinePath relies on a novel processing of GRNs that takes into account all possible functional interactions of the network. The different interactions correspond to the different functional sub-paths that can be followed during the regulation of a target gene. The network is decomposed to all possible functional sub-paths, as exemplified in Figure 3.

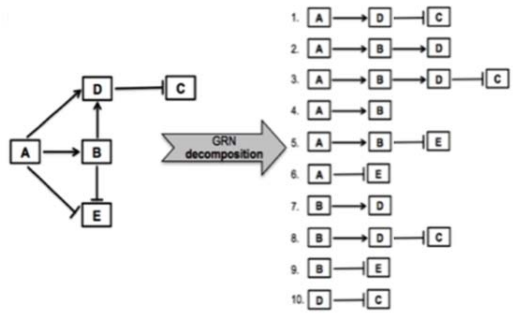

Figure 3: Decomposition of GRNs in MinePath (artificial example)

Each GRN sub-path is interpreted according to Kauffman's principles and semantics [21]: (i) the network is a directed graph with genes (inputs and outputs) being the graph nodes and the edges between them representing the causal links between them, i.e., the regulatory reactions; (ii) each node can be in one of the two states, 'ON', the gene is expressed or up-regulated (i.e., the respective substance being present) or, 'OFF', the gene is not-expressed or targeted from a specific miRNA; and (iii) time is viewed as proceeding in discrete steps - at each step the new state of a node is a Boolean function of the prior states of the nodes with arrows pointing towards it.

Initially, the samples of the discretized miRNA-targets expression matrix are matched against functional sub-paths of selected GRNs. In the next step, MinePath identifies the sub-paths that exhibit high matching scores for one phenotypic class and low matching scores for the other.

In order to cope with and reveal disrupted regulatory mechanisms, MinePath impose over the formed sub-paths the following requirement: for a sub-path to be considered as disrupted it should be 'non-active' (not functional) during the GRN regulation process. In other words, we assume that all genes in a sub-path are targeted by overexpressed miRNAs. At the final step, the sub-paths are ranked according to a ranking formula that computes the differential power of the sub-path to discriminate between the phenotypes.

III. Experimental Results

For the purposes of our scenario the core methodology of MinePath has been extended and adopted to handle targeted (down-regulated) genes from miRNA expression values.

After the decomposition of each of the selected pathways in its functional and disrupted components, each sub-path has been matched against the (selected) cohort's samples gene-expression profiles. The result is a matrix of sub-paths with binary values ('1' for disrupted, '0' not disrupted) for every sample in the cohort. The ranking formula identified 980 sub-paths of high differential power. Utilizing the Weka [22] machine-learning library we devised and trained two different classification models able to predict the phenotypic state of the samples, i.e., 'Wilm's Tumor' vs. 'Healthy'.

A. *Support Vector Machines*

Support Vector Machines [23] (SVM; 'SMO' Weka package) are supervised learning models that analyze data and recognize patterns used for classification and regression analysis. The SVM/SMO linear kernel classifier created a model using 780 sub-paths (out of the 980 most discriminant ones). Assessment of the results was performed using both 10-fold and leave-one-out (LOOCV) cross-validation modes. The performance of the SVM/SMO linear kernel model was 100% accurate for both validation modes.

B. *Decision Tree learning*

A decision tree induction algorithm (C4.5 [24]; 'J48' Weka package) was also applied. The C4.5/J48 induction process builds a decision tree from the top; first the most informative feature, the sub-path PLCβ→PKC→MEKK (from the GnRH signaling pathway), is selected. Then, the algorithm searches for the next best informative variable; the sub-path PDK1→AKT→CREB (from the PI3K-AKT pathway) is selected. The third and final node of the decision tree is the P50→COX2 sub-path (from the NF-KAPPA B pathway). Figure 4 shows a graphical illustration of the induced decision-tree. The assessed predictive performance of the decision-tree model was: 80% for 10-fold cross-validation, and 78% for LOOCV.

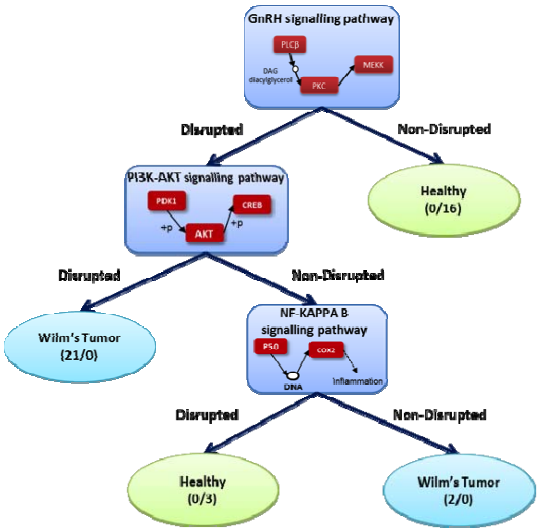

Figure 4: Decision tree for Wilms tumor prediction model

Even though the decision tree model did not achieve the LOOCV accuracy figure of the SVM/SMO model it is interesting to observe (due to the transparent nature of decision-trees) that the decision tree uses only three sub-paths. Examining further the three selected sub-paths we can see (Figure 5, Figure 6 and Figure 7 in red) that these sub-paths have a central role, in terms of topology and number of connections, in their respective pathways.

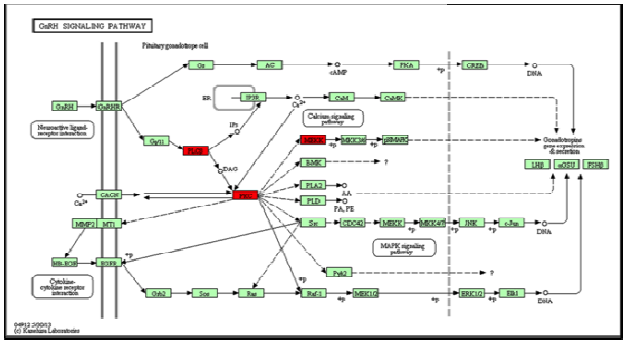

Figure 5: The PLCβ→PKC→MEKK disrupted sub-path (red) in the GnRH signaling pathway.

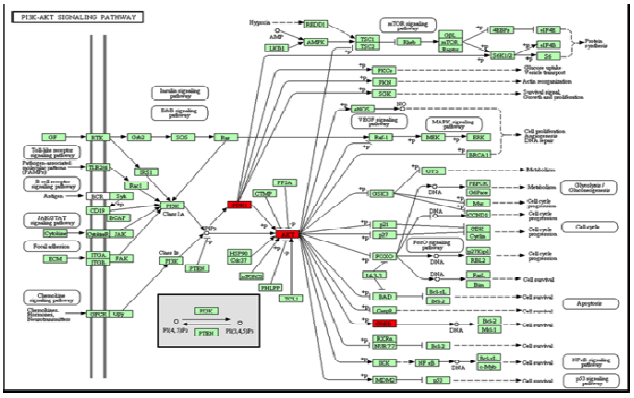

Figure 6: The PDK1→AKT→CREB disrupted sub-path (red) in the PI3K-AKT signaling pathway.

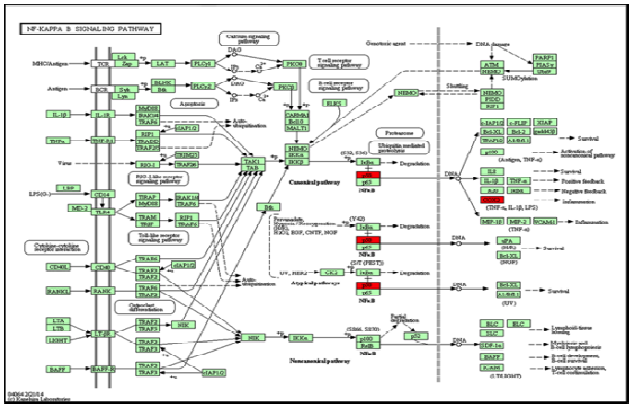

Figure 7: The P50→COX2 disrupted sub-path (red) in the NF-KAPPA B signaling pathway.

The results are in agreement and justifies an already known finding about the regulatory role of miRNAs: miRNAs preferentially regulate hub nodes, i.e., top 5% of the highly connected nodes in the network, and the network cut points which are the bottle-necks of metabolic flows, however, avoid regulating intermediate nodes which are the nodes between the hub nodes, cut points, upstream nodes and the output nodes [10].

Furthermore, the protein kinase C (PKC), which has been identified by the decision tree as the most discriminant (the first) disrupted sub-path, is implicated in the regulation of neuroblastoma (pediatric kidney tumor) cell growth and proliferation [25]. Zeidman et al [26] proved that PKCε through its regulatory domain can induce immature neurite-like processes via a mechanism that appears to be of importance for neurite outgrowth during neuronal differentiation in neuroblastoma cells.

The second sub-path of our model comes from the PI3K-AKT signaling pathway. In many types of tumor PI3K-AKT pathway inhibition can lead to a wide spectrum of direct effects including cell-cycle arrest, induction of autophagy, inhibition of metastasis as well as cell differentiation and death [27]. Recently, Santo et al [28] identified the forkhead transcription factor FOXO3a as a key target of the PI3K/AKT pathway in neuroblastoma and concluded that the inactivation of FOXO3a by AKT was essential for neuroblastoma cell survival.

Similarly, Brown et al [29] using morphoproteomic analysis revealed the activation of the NF-kappaB pathway in high risk neuroblastoma cases. Preclinical studies such as the Brignole et al [30] and Michealis et al [31] using the proteosome inhibitor bortezomib, proved that NF-kappaB pathway regulates the proliferation of human neuroblastoma cells in vitro.

IV. Conclusions

Pathway analysis using miRNA expression data could aid researchers to determine the biological relevance of the identified miRNAs. We assessed the potential biological functions of miRNAs by identifying putative miRNA targets from mirTarBase and applied pathway mining methodologies utilizing the core functionality of the MinePath web-based pathway analysis system. Using a public miRNA expression dataset (GSE38419) and the KEGG human pathways as proof of concept, we identified miRNA-disrupted pathway sub-paths, and induced a highly enough decision-tree prediction model. The decision tree provide evidence for three key sub-paths (involving central and highly connected genes) in the GnRH, the PI3K-AKT and the NF-KAPPA B KEGG signaling pathways, able to discriminate between Wilms-tumor and healthy subjects.

Around 10% of Wilms tumor patients are diagnosed having a concurrent syndrome that enhances the risk of Wilms tumor. But not all of these patients will develop such a tumor [32]. A screening method for early detection of Wilms tumor in these patients would be beneficial as the size or stage of a tumor is related with outcome [33]. In addition the detection of tumor specific disrupted pathways

might help to find targeted therapies for individual patients. In one child with relapse of a bilateral nephroblastomatosis and disrupted retinoic acid pathway the treatment with retinoic acid did cure the child without tumor surgery [34]. If it can be shown that this pathway analysis tool is beneficial for Wilms tumor it can serve as a proof of principle for usage in other cancer. From a technological point of view a translation in other cancer domains is easy as it is only necessary to link the tool with the corresponding database of patient specific miRNAs in other cancer domains.

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