An Approach to Identify miRNA Associated with Cancer Altered Pathways

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Abstract. MicroRNAs play an important role in the regulation of gene expression by binding mRNA targets causing their degradation or blocking their translation. Several genes has been found to be implicated as miRNA targets in different types of malignant tumors suggesting their involvement in cancer pathogenesis. Detecting direct miRNA–targets associations is not straightforward as in principle targets expressions are not altered except when they are completely repressed by the degradation complex.

In this paper we propose an approach to identify direct miRNA–targets associations hypotheses by means of indirect association measures such as mutual information. Indirect regulons of miRNA and Transcription Factors (TFs) are compared with the Fisher's exact test to identify potential co-regulations which may constitute potential miRNA–TF direct associations.

We apply the method on two cancer datasets, Colon and Lung, drawn from the Cancer Genome Atlas (TGCA) obtaining promising results.

Keywords: miRNA, reverse engineering, gene regulatory networks.

1 Introduction

MicroRNAs (miRNAs) are small noncoding, endogenous, single-stranded RNA molecules of approximately 22 nucleotides. They play an important role in the regulation of gene expression by binding mRNA targets causing mRNA degradation or blocking mRNA translation. About 5300 human genes are found to be implicated as targets of miRNAs and have function in various human diseases [1]. In particular, deregulation of miRNAs in different types of malignant tumors suggests their involvement in the pathogenesis of cancer. Similarly to oncogenes the over-expression of miRNAs may contribute to tumor formation if they bind to tumor suppressor genes, instead, they act as onco-suppressors if they bind oncogenes slowing down cancer progression [15]. Such a behavior makes the study of miRNA regulation associations crucial in cancer diagnosis, prognosis and therapy. For instance, Calin *et al.* found that two genes, coding

for *miR-15* and *miR-16*, are located within a 30 kb region of loss in chronic lymphocytic leukemia and that both genes are deleted or down-regulated in the majority of cases [6]. The repression activity modulated by miRNAs depends on the recognition of binding sites located mainly on the 3' untranslated regions of target mRNAs [1]. In order to understand the role of miRNAs in biological processes, it is important to identify their gene targets and which conditions make such binding pivotal. In addition to experimental techniques to identify miRNA-target interactions, several computational algorithms have been developed to predict putative targets [16]. The most common prediction algorithms identify miRNA targets using sequence–based approaches and rank the candidate targets by considering the sequence complementarity between miRNA seeds and genes 3'–UTRs. Such algorithms yield usually a very high number of false positives. On the other hand, these tools, tested against the available experimentally validated miRNA-target pairs, are not able to predict every known gene target them (false negatives) [3]. Alternative approaches exploit reverse engineering algorithms to reconstruct miRNA–gene regulatory networks. For instance, Zhao *et al.* analyzed the *feed-forward loop* consisting of miRNAs, transcription factors (TFs) and genes to construct a miRNA-TF regulatory network in glioblastoma and identified miRNA components involved in the Notch signaling pathway [20]. Genovese *et al.* adopted a network-modeling algorithm to compute pairwise measures of associations and identified some important miRNA, such as miR-34a proposed as a novel regulator of TGF-β signaling [8]. The algorithm evaluates the mutual information of miRNA-mRNA pairs. This analysis resulted in the identification of some putative interactions, whose only the minimal proportion (0.17%) was predicted to be direct.

In this paper we propose an approach inspired by the work of Genovese *et al.* to identify potential miRNA–TF associations in cancer altered pathways. In principle the expression of miRNA's targets is not altered except when they are completely repressed by the degradation complex. Thus expression correlation methods, such those adopted by Genovese *et al.*, are not able to identify such direct relationships. To avoid such a limitation we adopt an indirect approach. The regulons of both miRNA and TF are computed through the mutual information of their expression profiles. Such regulons, that comprises both direct and indirect associations, are then compared by means of Fisher's exact test to identify miRNA–TF co-regulations. We show on two cancer datasets that such a computed co-regulation is a valuable criterion to suggest direct miRNA–TF associations. We test our approach on two datasets of common malignant tumors, colon adenocarcinoma (COAD) and lung squamous cell carcinoma (LUSC), obtaining a relevant number of strong literature confirmed interactions, such as *miR-17/miR-20a → BCL2* [4,10,14].

The paper is organized as follows. The next Section covers material and methods detailing the proposed approach and defining the evaluation procedure adopted. Section 3 reports and discusses the results obtained by applying the approach on two datasets. Section 4 draws conclusions and outlines future directions.

2   Material and Methods

2.1   Datasets

We test our approach on two datasets drawn from The Cancer Genome Atlas (TCGA) portal (`http://cancergenome.nih.gov/`): Colon Adenocarcinoma (COAD) and Lung Squamous cell Carcinoma (LUSC). The COAD dataset contains the expression levels of 1046 miRNAs in 187 tumor samples obtained with Illumina Genome Analyzer miRNA Sequencing and the expression level of 20531 genes in 193 tumor and 18 normal samples obtained with IlluminaHiSeq RNASeqV2. The LUSC dataset contains the expression levels of 1046 miRNAs in 213 tumor and 45 normal samples obtained with Illumina HiSeq miRNASeq and the expression level of 20531 genes in 259 tumor and 35 normal samples obtained with IlluminaHiSeq RNASeqV2.

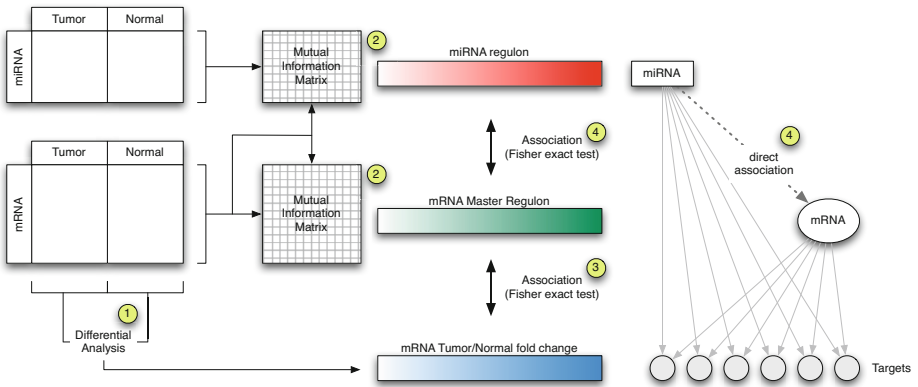### 2.2   The Proposed Approach

Fig. 1. The proposed analysis workflow

Our aim is to identify hypothesis of association between miRNAs and targets in the context of cancer altered pathways. We adopt the workflow shown in Figure 1 which consists of 5 steps detailed below:

Step1: Differential expression analysis. To determine whether a gene is differential expressed, we apply a test of hypothesis and the fold-change between two conditions, e.g. tumor and normal. In particular we adopt the quantile-adjusted conditional maximum likelihood (qCML) method for experiments with single factor, implemented in the edgeR package from Bioconductor [17]. Compared against several other estimators, qCML is the most reliable in terms of bias on a wide range of conditions and specifically performs best in the situation of many small samples with a common dispersion. The p-values generated from the

analysis sorted in ascending order, are corrected using the Benjamini-Hochberg procedure for multiple testing correction.

Step2: miRNA and TF regulon estimation. Network inference, which is the reconstruction of biological networks from high-throughput data, can provide valuable information about the regulation of gene expressions in cells. Several methods have been proposed in literature [19]. Among all we adopt mutual information implemented in the parmigene R package, as it is particular efficient for large dataset. The mutual information provides us with an index of dependence between miRNAs and genes and between TFs and genes. We estimate an appropriate threshold with a permutation test to filter out non significant dependences and thus obtaining the set of genes regulated (directly or indirectly) by miRNAs and TFs.

Step3: Transcription factor master regulon estimation. Master Regulators are those transcription factors whose targets are enriched for a particular gene signature (e.g. differentially expressed genes). The enrichment is evaluated using a statistical test, such as Fishers Exact Test. The objective is to place the signature genes within a regulatory context and identify the Master Regulators responsible for coordinating their activity, thus highlighting the regulatory apparatus driving phenotypic differentiation [7]. We adopt Fisher's Exact Test to perform an enrichment of TF-regulons obtained in step 2 respect to differentially expressed genes obtained in step 1, identifing the TFs master regulators. The known list of 1503 transcription factors in human is obtained from TRANSFAC [22]. The p-values are corrected using the Benjamini-Hochberg (BH) procedure for multiple testing correction.

Step4: miRNA–mRNA association estimation. As for Step 3 we adopt Fisher's Exact Test to perform an enrichment of miRNA-regulons obtained in Step 2 with respect to the master regulons of TFs obtained in Step 3. This analysis let us to identify the most significant direct associations between miRNAs and TFs through the indirect associations with their regulons obtained by means of mutual information.

Step5: post-filtering. To further enrich the set of associations obtained with the previous steps we propose two post-filtering steps:

1. *Directional interaction.* The association of a miRNA with a TF could determine or not the degradation of the TF mRNA. Consequently the TF expression could be down-regulated, in the case of degradation, or at least unchanged, when mRNA degradation does not occur, allowing mRNA stability and accumulation. To this aim we filter out up-regulated TFs as they are non representative of the canonical functioning of miRNA regulation.

2. *Tool prediction.* The association of a miRNA with a TF should be supported by the complementarity of miRNA seed sequence with the 3' UTR mRNA. To support such a condition we adopt a representative set of tools provided by miRWalk [5], such as: DIANA-mT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR4, PICTAR5, PITA, RNA22, and TargetScan. We consider associations predicted by at least 3 tools in at least 5 TF.

2.3   Evaluation against the Literature

To evaluate miRNA–mRNA association hypotheses we conduct a survey to test to which extend such hypotheses are confirmed in literature. To this aim we adopt the miRWalk database [5] (`http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/`) and IPA®, (Ingenuity Pathways Analysis, `http://www.ingenuity.com`). The miRWalk database provides experimentally verified miRNA interaction information obtained from literature. The database, last updated on 15th March 2011, is build semi-automatically through text mining approaches and may provides false positives when for instance the names of miRNAs and TFs co-appears in the article text but are in fact not directly correlated. We assess manually the set of miRWalk validated associations by performing a manual inspection of the referred article and annotate the associations with: *text co-occurrence* for terms co-occurrence in the published article; *expression pattern evidence* for less strong validations by high-throughput gene expression experiments, such as microarrays, which not necessarily demonstrate if the miRNA causes the changed expression patterns directly or not; and *direct interaction evidence* for strong experimental evidence by western blot, qPCR or luciferase reporter assay which accurately validate miRNA target genes. IPA®, is a tool able to identify biological and molecular networks by mapping gene expression data into relevant pathways based on their functional annotation and known molecular interactions. Annotation information comes from published, peer-reviewed scientific publications and is continuously updated. For our purpose we adopt the following IPA enrichment analyses: *Biological Functions*, that allows for the identification of the biological functions that are most significantly enriched for a given set of genes; *Canonical Pathways*, that identifies molecular pathways from the IPA library that are significantly associated to a given set of genes; and *Network enrichment*, that generates regulatory networks of a given set of genes based on their literature known connectivity and constructs networks that optimize for both inter-connectivity and number of focus genes under the constraint of maximal network size.

3   Results and Discussions

3.1   COAD

Starting from the expression values of 20531 genes and applying specific filters, we found 1944 deregulated genes between cancer and normal colon tissues. Among the 1046 miRNAs in COAD dataset, we selected the 105 top expressed miRNAs in cancer condition. The expression data of both genes and miRNAs were then analyzed to calculate their mutual information and estimate the gene regulon for each miRNA. Similarly, we computed the mutual information between the expression profiles of genes and TFs identifying 482 TF master regulators (p-value < 0.01), *i.e.* those that are enriched with differentially expressed genes. By comparing miRNA and master regulons we obtained 61738 significantly associated miRNA-TF pairs (BH corrected p-value $< 1.0 \times 10^{-2}$). Those

Table 1. miRWalk confirmed associations on COAD dataset

miRNA	TF	FDR	No. of tools	PubMed	Notes
hsa-mir-15b	CD36	$1.06\ 10^{-26}$	1	17379065	text co-occurence
hsa-mir-181a-1	CD36	$8.35\ 10^{-4}$	0	17379065	text co-occurence
hsa-mir-22	CD36	$5.72\ 10^{-25}$	0	17379065	text co-occurence
hsa-mir-28	CD36	$1.95\ 10^{-3}$	1	17379065	text co-occurence
hsa-let-7c	KIT	$1.54\ 10^{-10}$	3	18068232	text co-occurence
hsa-mir-17	KIT	$1.49\ 10^{-24}$	3	18068232	text co-occurence
hsa-let-7c	MEIS1	$3.48\ 10^{-70}$	1	18308931	text co-occurence
hsa-let-7i	MEIS1	$1.33\ 10^{-9}$	0	18308931	text co-occurence
hsa-mir-29a	MEIS1	$8.35\ 10^{-3}$	0	18308931	text co-occurence
hsa-mir-141	FOXF1	$1.05\ 10^{-61}$	0	18698484	text co-occurence
hsa-mir-200a	FOXF1	$1.22\ 10^{-103}$	0	18698484	text co-occurence
hsa-mir-200c	FOXF1	$1.16\ 10^{-90}$	6	18698484	text co-occurence
hsa-mir-429	FOXF1	$2.82\ 10^{-36}$	6	18698484	text co-occurence
hsa-mir-20a	BCL2	$3.21491\ 10^{-7}$	5	18941111	direct interaction evidence
hsa-mir-17	MEIS1	$3.23\ 10^{-187}$	0	19155294	text co-occurence
hsa-mir-17	BCL2	$5.48908\ 10^{-7}$	5	19666108	direct interaction evidence
hsa-let-7c	MEF2C	$4.28\ 10^{-34}$	2	19897480	text co-occurence
hsa-mir-143	KLF4	$7.82\ 10^{-3}$	1	20089806	text co-occurence
hsa-mir-27a	MEF2C	$3.64\ 10^{-3}$	6	20736237	expression pattern evidence
hsa-mir-27b	MEF2C	$3.54\ 10^{-3}$	7	20736237	expression pattern evidence
hsa-mir-183	HMOX1	$7.68\ 10^{-14}$	0	21147878	text co-occurence
hsa-mir-19b-2	BCL2	$5.7447\ 10^{-8}$	1	21883694	text co-occurence
hsa-let-7c	CLU	$3.57\ 10^{-20}$	0	22749186	text co-occurence
hsa-let-7i	CLU	$6.53\ 10^{-4}$	0	22749186	text co-occurence
hsa-mir-143	CLU	$6.04\ 10^{-114}$	0	22749186	text co-occurence
hsa-mir-200b	FOXF1	$8.4858\ 10^{-117}$	6	23022474	text co-occurence

represents the potential relationships between miRNAs and TFs that could explain the molecular alterations observed in colon cancer. We examined the 672 most interesting miRNA–TF associations by applying the filters explained in Section 2.2. These associations contains 32 unique miRNAs associated in average with 20 TFs. Among the selected miRNA-TF pairs, 27 out of 672 associations corresponded to miRWalk validated miRNA-targets (Table 1).

One of the most interesting result is observed for *miR-17* and *miR-20a*, which share the same group of TFs, thus belonging to the same family. In literature they are widely reported to be up-regulated in colon cancer tissues [4,10,14], promoting cell proliferation, tumour growth and cell cycle progression [14]. In our study, *miR-17* resulted significantly associated to 31 TFs, 9 of which are predicted by at least 3 tools among DIANAmt, miRanda, miRWalk, and TargetScan. One of such associations (*miR-17/miR-20a → BCL2*) has been biologically demonstrated by Beveridge *et al.* [2]. The fact that such an association is predicted by 5 sequence prediction tools let us suppose for a strong direct target binding association.

We investigated the enrichment in Biological Functions and Canonical Pathways for *miR-17* associated TFs with IPA. Cellular proliferation and differentiation, gene expression and cancer are the most significant biological function categories (BH corrected p-value $< 1.0 \times 10^{-6}$). Interestingly, in the cancer biological function the first annotations are "metastatic colorectal cancer" and "colorectal tumor", suggesting that the central role of *miR-17* in colon tumorigenesis could be mediated by its associated TFs. Moreover, "glucocorticoid receptor signaling" is the only significantly affected canonical pathway suggesting cortisol synthesis in a non-endocrine tumor as recently reported by Sidler *et al.* [18]. To show the putative regulatory network associated with *miR-17/miR-20a* we adopted the IPA network analysis tool that allows for reconstructing the literature known connections among a group of molecules. Figure 2A shows the most significant minimal connected network based on Ingenuity curated annotations and constructed from the set of 31 TFs significantly associated with *miR-17/miR-20a*. The network reports a total of 35 connected molecules 9 of which are comprised in the initial 31 set and whose principal functions are cell death and survival, cancer, and cell-mediated immune response.

Fig. 2. Enriched biological networks obtained with IPA tool: A) COAD miR-17/miR-20a; B) LUSC miR-663

Other interesting associations have been found for *miR-200c*, *miR-221*, and *miR-429* [9,21,11] but not described due to space limitation.

3.2 LUSC

The differential expression analysis of LUSC data resulted in 3380 and 274 deregulated genes and miRNAs respectively. Then, we calculated their mutual information to define the gene regulons for miRNAs and we identified 558 TF master regulators (p-value < 0.01). miRNA and TF regulons were compared by Fisher exact test and 11125 miRNA–TF associations resulted significant (BH corrected

Table 2. miRWalk confirmed associations on LUSC dataset

miRNA	TF	FDR	No. of tools	PubMed	Notes
hsa-mir-200c	ZEB1	$2.85\ 10^{-10}$	2	18376396	direct interaction evidence
hsa-mir-196b	TLR4	$2.32\ 10^{-4}$	0	18291670	text co-occorrence
hsa-mir-205	ZEB1	$2.12\ 10^{-3}$	2	18376396	direct interaction evidence
hsa-mir-429	ZEB1	$5.28\ 10^{-28}$	0	18381893	direct interaction evidence
hsa-mir-141	ZEB1	$2.62\ 10^{-21}$	0	18483486	direct interaction evidence
hsa-mir-429	TBX2	$3.39\ 10^{-7}$	6	18698484	text co-occorence
hsa-mir-141	TBX2	$1.37\ 10^{-4}$	3	18698484	text co-occorence
hsa-mir-205	TBX2	$2.42\ 10^{-6}$	6	18698484	text co-occorrence
hsa-mir-96	ZEB1	$7.47\ 10^{-13}$	0	19167416	text co-occorence
hsa-mir-9-1	TLR4	$1.71\ 10^{-4}$	5	19289835	text co-occorence
hsa-mir-9-1	TLR2	$1.52\ 10^{-3}$	2	19289835	text co-occorrence
hsa-mir-9-2	TLR4	$1.09\ 10^{-4}$	1	19289835	text co-occorrence
hsa-mir-9-2	TLR2	$1.15\ 10^{-3}$	6	19289835	text co-occorrence
hsa-mir-142	TLR4	$8.39\ 10^{-7}$	2	21037424	text co-occorrence
hsa-mir-369	ZEB1	$1.62\ 10^{-4}$	1	21125666	text co-occorrence
hsa-mir-429	WNT2	$1.30\ 10^{-10}$	2	21130073	text co-occorence
hsa-mir-200a	ZEB1	$7.95\ 10^{-10}$	2	18376396	direct interaction evidence
hsa-mir-200b	ZEB1	$5.02\ 10^{-5}$	6	22261924	direct interaction evidence
hsa-mir-7-1	TLR4	$2.95\ 10^{-4}$	0	23135998	text co-occorrence

p-value $< 1.0 \times 10^{-2}$). The identified miRNA–TF associations were further an-
alyzed applying the filters described in Section 2.2. We focused on 1136 associ-
ations composed by 38 miRNAs that were associated in average with 30 TFs.
Comparing the results with literature, 17 out of 1136 miRNA-TF associations
were found already described in several studies (Table 2).

Very interesting miRNA-TF associations, for elucidating the role of miRNAs
in lung tumorigenesis, were found for *miR-663*. The overexpression of *miR-663*
in lung cancer was already reported in a recent study where the authors demon-
strated it regulates cancer cell proliferation and apoptosis through targeting
TGFβ [13]. In our study, we confirmed *miR-663* is highly up-regulated in LUSC,
and it resulted significantly associated to 34 TFs, 9 of which are predicted by at
least 3 tools (such as miRanda, miRWalk, TargetScan, PICTAR5). IPA analysis
was performed to evaluate the enrichment in Biological Functions for *miR-663*
associated TFs. The most significant Biological Function (BH corrected p-value
$= 6.0 \times 10^{-9}$) is differentiation of cells which was predicted significantly decreased
(z-score $= -2.844$) by IPA algorithm. This biological annotation represents also
the top function of a proposed regulatory network built through IPA network
analysis tool (Figure 2B). Starting from *miR-663* associated TFs, this regulatory
network shows the known relationships between 35 connected molecules, 17 of
which are included in the initial 34 set.

Other remarkable result is observed for *miR-141* and *miR-200c*, which share
37/44 associated TFs and, as expected, belong to the same miRNA family. In-
terestingly, they were recently reported as novel serum biomarkers associated to

poor prognosis in LUSC [12]. Their associated TFs resulted enriched for cellular differentiation, cell death and immune cell trafficking (BH corrected p-value $< 1.0 \times 10^{-6}$) through IPA Biological Functions analysis.

4   Conclusions and Future Work

We proposed a method to identify potential associations between miRNAs and Transcription Factors playing a central role in altered cancer pathways. The algorithm is able to identify potential direct relationships where other methods, based on pure correlation measures, may fail. It is still affected by false positives thus heuristics able to filter out not relevant associations are necessary. We proposed two filtering heuristics, one based on interaction directionality and another that exploits sequence bases prediction tools.

We test the method on two cancer datasets, Colon Adenocarcinoma (COAD) and Lung Squamous cell Carcinoma (LUSC), showing important literature confirmed associations. Result obtained with this study consolidate the potential involvement of miRNAs in colon and lung cancerogenesis but further studies are necessary to examine all suggested interactions and to elucidate their role in cancer.

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