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A computational study to identify TP53 and SREBF2 as regulation mediators of miR-214 in melanoma progression

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Abstract: In the complex world of post-transcriptional regulation, miR-214 is known to control in vitro tumor cell movement and survival to anoikis, as well as in vivo malignant cell extravasation from blood vessels and lung metastasis formation. miR-214 has also been found to be highly expressed in human melanomas, and to directly and indirectly regulate several genes involved in tumor progression and in the establishment of distant metastases (Penna et al., 2011). In this work, we exploit a computational pipeline integrating data from multiple online data repositories to identify the presence of transcriptional or post-transcriptional regulatory modules involving miR-214 and a set of 73 previously identified miR-214 regulated genes. We identified 27 putative regulatory modules involving miR-214, NFKB1, SREBF2, miR-33a and 9 out of the 73 miR-214 modulated genes (ALCAM, POSTN, TFAP2A, ADAM9, NCAM1, SEMA3A, PVRL2, JAG1, EGFR1). As a preliminary experimental validation we focused on 9 out of the 27 identified regulatory modules that involve two main players, miR-33a and SREBF2. The results confirm the importance of the predictions obtained with the presented computational approach.

1 INTRODUCTION

Aberrant expression of coding and non-coding genes, such as microRNAs (miRNAs), occurs in melanomas, one of the most aggressive human tumors. miRNAs are 20 to 24 nucleotides long non-coding RNAs involved in the post-transcriptional down-regulation of protein-coding genes through imperfect base pairing with their target miRNAs. miRNAs have been implicated as possible key factors in several diseases because of their capability to affect the simultaneous expression of multiple genes involved in the cell biology (Beezhold et al., 2010; Tu et al., 2009; Benso et al., 2013; Di Carlo et al., 2013; Yuan et al., 2009). Referring to melanomas, miRNAs such as let-7a/b, miR-23a/b, miR-148, miR-155, miR-182, miR-200c, miR-211, miR214 and miR-221/222 have been found to be differentially expressed in benign melanocytes versus melanoma cell lines or in benign melanocytic lesions versus melanomas in human samples. Targets of some of the above listed miRNAs are well-known melanoma-associated genes like the oncogene NRAS, the microphthalmia-associated transcription factor (MITF), the receptor tyrosine kinase c-KIT, or the AP-2 transcription factor (TFAP2). We previously showed that miR-214, the product of an intron of the Dynamin-3 gene on human chromosome 1, coordinates melanoma metastasis formation by modulating the expression of over 70 different genes, including 2 activating protein transcription factors (TFAP2A and TFAP2C) and the adhesion molecule ALCAM (Penna et al., 2011; Penna et al., 2013). In fact, alterations in the expression level of some of these genes leads to downstream effects on a number of key processes such as apoptosis, proliferation migration and invasion. In order to elucidate the regulatory networks mediated by miR-214 we designed a computational pipeline able to search for different classes of regulatory modules between miR-214 and the set of 73 modulated proteins. In this analysis we focused on the interplay between transcription factors (TFs) and
microRNAs (miRNAs) since several studies as (Zhao et al., 2013) suggested its critical role in cellular regulation during tumorigenesis. Three different classes of regulatory modules (see Figure 1) have been analyzed:

1. Type-0 (direct interactions), where miR-214 directly down-regulates one of the target proteins;
2. Type-1 (one-level indirect interactions), where miR-214 down-regulates a Transcription Factor which eventually regulates one of the targets;
3. Type-2 (two-level indirect interactions), where miR-214 targets a Transcription Factor regulating a gene which hosts another miRNA that down-regulates one of the target proteins.

Although Type-0 and Type-1 interactions may be quite straightforward to detect, Type-2 interactions are not immediately evident and require a more complex data integration process. Other types of interactions may be similarly interesting (e.g. three-level interactions like: miR-214 → TF1 → TF2 → Target Protein) but have not been considered, at this stage, because they are a lot more difficult to experimentally validate. The search process for the three classes of interactions was completely automated and based on the integration of heterogeneous data extracted from different public available repositories. The pipeline highlighted no interactions of Type-0 and Type-1, and 27 possible Type-2 interactions. An experimental validation of a subset of the identified interactions is shown in the Results section.

2 Methods

2.1 Computational analysis

Searching for the three classes of interactions involving miR-214 presented in Figure 1 requires the integration of heterogeneous data sources. This section introduces the selected public repositories used to retrieve the required information as well as the computational flow followed to integrate these sources and to search for the chosen regulatory modules.

2.1.1 Data sources

The following public repositories represent the main sources of information in our computational process:

- **microRNA.org** database (Betel et al., 2008) is used to search for miRNA target genes. microRNA.org uses the miRanda algorithm (John et al., 2004) for target predictions. The algorithm computes optimal sequence complementarity between a miRNA and an mRNA using a weighted dynamic programming algorithm. The overall database consists of 16,228,619 predicted miRNA target sites in 34,911 distinct 3'UTR from isoforms of 19,898 human genes. Predictions are associated to a mirSVR score, a machine learning method for ranking miRNA target sites by a down-regulation score (Betel et al., 2010). The mirSVR score is a real number that indicates the prediction confidence (lower negative scores correspond to better predictions). Data from microRNA.org are available for download in 4 different zipped packages: (1) Good mirSVR score, Conserved miRNA, (2) Good mirSVR score, Non-conserved miRNA, (3) Non-good mirSVR score, Conserved miRNA, (4) Non-good mirSVR score, Non-conserved miRNA. They are differentiated in terms of mirSVR score (high or low) and conservation (highly, low conserved). The four archives have been unified in a single database, keeping the information of the source archive in a specific field as well as the related mirSVR score, in order to be able to filter the retrieved targets and to work with the most reliable predictions. It is necessary to point out that the identification of any regulatory module involving miRNA targets is always affected by the type-I and type-II errors embedded in the miRNA target prediction algorithms, and therefore an experimental validation, at least of the most promising results, is unavoidable.

- **Transcription Factor Encyclopedia** (Wasserman Lab, 2012) and **Targetmine** (The Mizuguchi Laboratory, 2013; Chen et al., 2011) have been used to identify genes Transcription Factors (TF). TFE provides details of transcription factor binding sites in close collaboration with Pazar, a public database of transcription factors and regulatory sequence information. Targetmine contains only Upstream Transcription Factors. For each gene, the database retrieves all upstream regulatory genes from the AMADEUS and ORegAnno compiled TF-Target gene sets. Amadeus (Linhart et al., 2013; Linhart et al., 2008) contains TF and miRNA target sets in human, mouse, D. Melanogaster, and C. Elegans, collected from the literature. For each TF it is reported its set of targets, given as a list of Ensembl gene ids.

- **Eutils programming utilities** (NCBI, 2013) and **Mirbase.org** (mirbase.org, 2013; Griffiths-Jones et al., 2006) are used for retrieving coordinates of precursor miRNAs and genes. miRBase is a searchable database of published miRNA se-
sequences and annotations. About 94.5% of the available mature miRNA sequences considered in this database have experimental evidence, thus representing a reliable source of information. Each miRNA entry in miRBase is correlated with the related information on the location that is exploited to identify the host genes.

2.1.2 Computational flow

Figure 2 highlights the computational flow implemented to search for miR-214 mediated interactions. The full pipeline has been developed in PHP language and coupled with a MySQL database, which mirrors an optimized subset of data coming from multiple online repositories. As previously discussed, we focused our analysis on the set of 73 protein-coding genes reported in Table 1. These proteins, denoted as Target Proteins in Figure 2, have been found to be modulated in a direct or indirect manner by miR-214 in previous microarray experiments presented in (Penna et al., 2011).

The computational flow is organized into four main data integrations steps that, starting from miR-214, search for Type-0, Type-1 and Type-2 interactions.

**Step 1 - detection of Type-0 interactions**

Type-0 interactions require searching for target genes that are directly regulated by miR-214.

We queried microRNA.org database to search for miR-214 direct targets. Due to the computational approach used by microRNA.org to predict miRNA targets, false positives are in general present in the query results. To limit these errors we restricted the query to the "Good mirSVR score, Conserved miRNA" and to the "Good mirSVR score, Non-conserved miRNA", which represent the most reliable subsets of computed targets. Moreover, miRNA targets have been further filtered according to their mirSVR score. Such score is considered meaningful with a cutoff of at most -0.1, based on the empirical distribution of the extent of target down-regulation (measured as log-fold change) that is expected given a mirSVR score (Betel et al., 2010). For scores closer to zero the probability of meaningful down-regulation drops while the number of predictions sharply rises (MicroRNA.org, 2013). In order to work with high reliable predictions we selected only those targets with mirSVR < -0.3.
Then, in order to identify Type-0 interactions, the full list of obtained miR-214 targets have been intersected with the set of 73 Target Proteins.

**Step 2 - detection of Type-1 interactions** Starting from the full list of miR-214 targets computed during Step 1, the identification of Type-1 interactions requires filtering out those targets that have not been identified as Transcription Factors (TF) for other genes. Each miR-214 Target is searched both in Transcription Factor Encyclopedia and in TargetMine to check whether it represents a TF. For each identified TF the related target gene is then extracted. This step allows us to build a list of TF Targets that can be intersected with the list of 73 Target proteins to identify Type-1 interactions.

**Steps 3 and 4 - detection of Type-2 interactions** The last two steps of the proposed computational flow are used to identify Type-2 interactions that represent the most complex considered mechanism.

For each TF Target identified during Step 2 we
searched for its candidate intragenic miRNAs (Step 3). Intragenic miRNAs represent around 50% of the mammalian miRNAs. Most of these intragenic miRNAs are located within introns of protein coding genes (miRNA host genes) and are referred to as intronic miRNAs, whereas the remaining miRNAs are overlapping with exons of their host genes and are thus called exonic miRNAs. Moreover the majority of intragenic miRNAs are sense strand located, while only a very small portion is anti-sense strand located. Our analysis considers intronic and exonic miRNAs both sense and anti-sense strand located. Intragenic miRNAs are retrieved from the miRBase database. To identify intragenic miRNAs of a given host gene we first searched for the genomic coordinates of the gene using e-Utils; with the gene coordinates we searched in the miRBase database for all miRNAs with coordinates embraced in the ones of the gene.

Similarly to Step 1, for each detected Intragenic miRNA we then searched microRNA.org for the related Intragenic miRNA Targets (Step 4), and finally we filtered out those targets that do not correspond to any of the 73 target proteins. Each resulting target protein then corresponds to a Type-2 interaction. It is important to point out here that the computational analysis cannot predict the sign of the resulting differential expression (up or down regulation). In fact, following the Type-2 regulatory chain, if \textit{miR-214} is silenced the expression of the target protein is very likely inhibited. If, instead, \textit{miR-214} is over expressed, the regulatory module “removes” the inhibition and allows the target gene expression to possibly change. The only realistic way to experimentally verify the presence of the Type-2 regulatory module is to correlate the over expression of \textit{miR-214} with the under expression of the cascade TF \rightarrow gene \rightarrow miRNA that follows \textit{miR-214} (see Figure 1). This is obviously true unless the transcription factor acts as a repressor of its own target, which is statistically unlikely to happen. As for now, since public repositories do not provide this information we can only assume the TF to be an enhancer of its target.

### 2.2 Biological methods

Computational predictions have been validated against the following biological setup.

#### 2.2.1 Cell culture

MA-2 cells were provided by R.O. Hynes (Xu et al., 2008) and maintained as described in Penna et al., 2011.

#### 2.2.2 Transient transfections of pre-miRs

To obtain transient \textit{miR-214} over expression, cells were plated in 6-well plates at 30-50% confluency and transfected 24h later using RNAiFect (QIAGEN, Stanford, CA) reagent, according to manufacturers instructions, with 75 nM Pre-miR™ miRNA Precursor Molecules-Negative Control (a non-specific sequence) or Pre-miR-214.

#### 2.2.3 RNA isolation and qRT-PCR for miRNA or mRNA detection

Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA). qRT-PCRs for miR detection were performed with TaqMan® MicroRNA Assays hsa-miR-33a assay ID 002306, U6 snRNA assay ID001973 (all from Applied Biosystems, Foster City, CA) on 10 ng total RNA according to the manufacturer's instructions. For mRNA detection, 1 ug of DNAse-treated RNA (DNA-free™ kit, Ambion, Austin, TX) was retrotranscribed with RETROscript™ reagents (Ambion, Austin, TX) and qRT-PCRs were carried out using SREBP2 gene-specific primers (FW:gcctggaagtgacagagag, RV: tgctttcactggagtga) and the Probe #21 of the Universal Probe Library (Roche, Mannheim, GmbH) using a 7900HT Fast Real Time PCR System. Quantitative normalization was performed on the expression of the U6 small nucleolar RNA or of 18S, for miR or mRNA detection, respectively. The relative expression levels between samples were calculated using the comparative delta CT (threshold cycle number) method (2-DDCT) with a control sample as the reference point (Bookout and Mangelsdorf, 2003).

### 3 Results and discussion

The computational pipeline presented in Section 2.1 leaded to the identification of zero Type-0, zero Type-1, and 27 Type-2 interactions. The fact that no Type-0 and Type-1 interactions were found does not mean that they do not exist, but that in the available databases there is no evidence of their presence.
The 27 Type-2 interactions target 22 out of the 73 considered miR-214 potential interacting proteins, which have been marked in green in Table 3. The full list of the 27 identified regulatory modules is shown in Table 3.

From our predictions, miR-214 influences two transcription factors: NFκB1 and TP53 (average mirSVR = -0.4). Seven of the genes regulated by these two TFs were identified as host genes for miRNAs targeting at least one of the 73 miR-214 modulated proteins: APOLD1, BBC3, C11orf10, GDF15, NFATC2, SREBF2, and SVIL. The hosted miRNAs are: hsa-mir-33a, hsa-mir-604, hsa-mir-611, hsa-mir-613, hsa-mir-3189, hsa-mir-3191, and hsa-mir-3194. The average mirSVR score is significantly low (average mirSVR < -0.71). The high significance of the mirSVR scores, resulting from interactions between the intragenic miRNAs and their target proteins, is particularly evident for TFAP2A, which outperforms the others with a mirSVR score of -1.3043.

In this work, as a preliminary experimental validation, we focused our attention on the first 9 identified regulatory modules involving miR-214, NFκB1, SREBF2, miR-33a and 9 of the 73 considered proteins (ALCAM, POSTN, TFAP2A, ADAM9, NCAM1, SEMA3A, PVRL2, JAG1 and EGFR1). We evaluated miR-33a and SREBP2F expression levels following miR-214 over expression in MA-2 melanoma cells and we observed a decrease in miR-33a and SREBP2F expression as shown in Figure 3A.

This regulatory module resulted to be very interesting also because SREBP2F and miR-33a act in concert to control cholesterol homeostasis (Najafi-Shoushtari et al., 2010). In fact, SREBP2F acts by controlling the expression of many cholesterologenic and lipogenic genes, such as low-density lipoprotein (LDL) receptor, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and fatty acid synthase. Instead, miR-33a targets the adenosine triphosphate-binding cassette A1 (ABCA1) cholesterol transporter, a key mediator of intracellular cholesterol efflux from liver to apolipoprotein A-I (apoA-I) to obtain high-density lipoprotein (HDL). Considering that the lipogenic pathway is a metabolic hallmark of cancer cells, these preliminary data suggest a potential role of miR-214 in this aspect of cancer formation and progression. Our hypothesis is further supported by experimental results (not shown here), obtained from microarray analysis in a context of miR-214 over expression.

To look for molecular and cellular functions associations within the almost 500 differentially expressed genes detected by microarray analysis comparing cells over expressing miR-214 versus controls, we applied an Ingenuity Functional Analysis. The Ingenuity Pathways Knowledge Base (http://www.ingenuity.com/) is currently the world largest database of knowledge on biological networks, with annotations performed by experts. The significance value obtained with the Functional Analysis for a dataset is a measure of the likelihood that the association between a set of Functional Analysis molecules in our experiment and a given process or pathway is due to random chance. The p-value is calculated using the right-tailed Fisher Exact Test and it considers both the number of functional analysis molecules that participate in that function and the total number of molecules that are known to be associated with that function in the Ingenuity Knowledge Base. In our case, the most significant functions associated to our dataset resulted to be Cellular Assembly and Organization (7.08E-04 ÷ 3.95E-02, 25 molecules) and Lipid Metabolism (9.54E-04 ÷ 4.32E-02, 18 molecules).

The observed co-regulation of miR-33a and SREBP2F is in agreement with literature data published in Najafi-Shoushtari et al., 2010, thus supporting our computational predictions. The down-regulation of miR-33a following miR-214 over expression could contribute to miR-214-mediated cell invasion, in fact it has been demonstrated that an enforced expression of miR-33a inhibits the motility of lung cancer cells (Rice et al., 2013).
Table 2: The 27 Type-2 regulatory modules related to miR-214 as obtained by the pipeline after data scraping. The set of final targets (surface protein in the table) is limited to the 73 genes listed in Table 1. The first 9 modules have been experimentally validated.

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4 Conclusions

In this paper we presented the results of a computational pipeline created for investigating possible regulatory pathways between miR-214 and a set of 73 proteins previously identified as co-regulated with the miRNA in melanomas. Thanks to this computational flow, a set of 27 putative regulatory pathways has been identified; a preliminary experimental validation performed on 9 out of the 27 pathways provided interesting insights about the regulatory mechanisms involving miR-214 in the considered disease. The analysis suggests the involvement of miR-214 in metabolic pathways that could control metastatization. Moreover, the study highlights the relevance of specific miR-214 modulated genes, such as ALCAM, HBEGF, JAG1, NCAM1, and PVRL2, that correspond to surface proteins redundantly regulated by multiple pathways. Further laboratory experiments are under way to complete the validations of the full set of identified regulatory modules. Nevertheless, the preliminary results presented in this work already represent a significant achievement that seems to confirm the quality of the predictions obtained with the proposed computational approach.

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