A review of computational approaches detecting microRNAs involved in cancer

A review of computational approaches for the detection of micrornas involved in cancer

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1. ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs playing an essential role in gene expression regulation. Multiple studies have demonstrated that miRNAs are dysregulated in cancer initiation and progression, pointing out their potential as biomarkers for diagnosis, prognosis and response to treatment. With the introduction of high-throughput technologies several computational approaches have been proposed to identify cancer-associated miRNAs. Here, we present a systematic and comprehensive overview of the current knowledge concerning the computational detection of miRNAs involved in tumor onset and subtyping, with possible theranostic employment. An overview of the state of art in this field is thus proposed with the aim of supporting researchers, especially experimentalists and pathologists, in choosing the optimal approach for their case of study.

2. INTRODUCTION

For many years the search for cancer biomarkers focused primarily on alterations in the status and expression of protein-coding genes conferring a survival advantage to cancer cells (oncogenes) or preventing cancer progress (tumour suppressors).

The first evidence that, besides protein coding genes, non coding RNAs (ncRNAs) and in particular microRNAs (miRNAs) can act as either oncogenes (oncomiRs) or tumour suppressors was the identification of *miR-15* and *miR-16* as potential cancer genes in the pathogenesis of chronic lymphocytic leukemia (CLL) (1). In more than half of CLL cases and in other malignancies, deletion of 13q14.3. was reported, suggesting the occurrence in this region of tumor suppressor genes involved in the initiation or progression of this disease. However, the identification of causal genes related to loss of 13q14.3. in CLL failed, until the discovery that the critical region contained two tightly linked miRNAs, *miR-15a* and *miR-16-1*, responsible of tumor suppression.

In the early 1990s miRNAs were discovered (2, 3) as small non-coding RNA molecules (approximately 22 nucleotides in length) that post-transcriptionally regulate gene expression by binding targets mRNAs and leading to inhibition of translation or mRNA degradation (4). MiRNA are transcribed from different genomic locations as long primary transcripts (pri-miRNA) by RNA polymerase (5). Frequently, to allow coordinated expression, some miRNAs are clustered in polycistronic transcripts. Once transcribed, the pri-miRNA is processed by the successive action of two enzymes, Drosha and Dicer, to generate the mature miRNA, recruited into an effector complex called RNA-induced silencing complex (RISC). In animals, the mature miRNA guides the RISC complex to the target mRNAs through imperfect base-pairing to multiple sites preferentially observed in 3'untranslated regions (UTRs). In the currently described binding model, Watson-Crick base-pairing to the 5' end of miRNAs, especially to the so-called "seed" that comprises nucleotides 2-7, is crucial for targeting (6).

MiRNA genes are frequently located in cancer susceptibility regions and at fragile sites, supporting their involvement in cancer disease (7). Since 2002, when *miR-15/16* involvement in CLL was described, expression data from a large panel of cancer cell types have confirmed aberrant miRNA expression in a variety of cancer diseases (8-10). More recent experimental evidences suggest that specific miRNAs may also have a role beyond the cancer onset and directly participate in cancer invasiveness and metastasis (11, 12). In fact, miRNA profiles can distinguish not only between normal and cancerous tissue but they can also successfully classify different subtypes of a particular cancer (13, 14). Moreover, due to their small size, miRNAs

are more stable than long mRNAs, allowing expression profiling from fixed tissues or other biological material. These results thus corroborate the interest in miRNAs as novel, minimally invasive and robust biomarkers. In the last years, the discovery of miRNAs in body fluids has opened the perspective to introduce miRNAs in clinics as biomarkers and putative therapeutic targets (15, 16).

Since miRNAs started to be largely studied, multiple reviews dealing with these small non coding RNAs in cancer have been proposed (17-19). Some of them provide an overview of the existing methods for miRNAs discovery (18). Some others take into account the different approaches for the prediction of miRNAs target transcripts (19). Finally, others are more centered on the medical aspects concerning microRNAs in cancer and they briefly report some examples of computational tools (17). Here we present a systematic and comprehensive overview of the current knowledge concerning the computational identification of miRNAs involved in cancer onset and subtyping, with possible theranostic employment. The aim of this review is thus to help researchers, especially experimentalists and pathologists, in choosing the optimal approach for their case of study. Indeed due to the complexity and heteregeneity of cancer diseases, computational approaches and system-oriented studies are becoming largely employed to complement some limitations of experimental studies (20). The description of the computational approaches is organized as follows: in the first part, we describe methods exploiting expression data from miRNAs and/or mRNAs. The second part is devoted to those methods that capture the effect of a joint miRNA-TF regulation. Then, some pipelines are introduced that make use of more recent types of genomic data such as PAR-CLIP data and methylation profiles. In the last part, more recent pipelines taking into account the miRNA-miRNA synergistic effect are described.

3. COMPUTATIONAL APPROACHES TO DETECT MIRNAs INVOLVED IN CANCER ONSET AND SUBTYPING

Following the discovery of the crucial role of miRNAs in cancer, computational methods for the identification of microRNAs potentially driver of cancer onset or subtyping have been proposed. Here, with the term miRNA driver we refer to a miRNA whose overexpression promotes the transition of a cell from the normal state to the cancerous one (cancer driver miRNA), or from a cancer subtype to another (cancer subtyping driver miRNA). Depending on the type of data that they employ, six main categories of approaches can be distinguished: (i) Methods based on miRNA expression data; (ii) Methods based on mRNA expression data; (iii) Methods based on combined miRNA-mRNA expression data; (iv) Methods taking into account the miRNA-TF crosstalk; (v) More recent integrative works considering also other data types; (vi) Methods considering the miRNA-miRNA synergistic effect. These six points are treated in detail in the sections below, a summary of the main computational approaches employed in the proposed algorithms is presented in Figure 1 and an overview of the available tools for each category is summarized in Table 1.

3.1. Methods based on miRNA expression data

The role of microRNAs in cancer started to be explored computationally when the first profiling methods, able to measure the expression pattern of all known miRNAs, were made available (1, 21-27). At the beginning, the bioinformatic studies employing these expression data were aimed at investigating whether miRNAs expression could be used to distinguish tumour from normal tissue (26). Interestingly, such works proved that miRNA expression profiles could be surprisingly informative even when no robust mRNA marker could be identified. As a consequence, their encouraging classification power and their number, lower than that of mRNAs, made them suitable as tumor biomarkers. Many researchers started to explore computationally the involvement of miRNAs in cancer onset and subtyping through the analysis of their expression data with statistical tests as Student's t-test (8, 28-30), Wilcoxon signed-rank test (31-33), ANOVA (32, 34, 35) and Significance Analysis of Microarrays (SAM) (8, 36).

The output of such analysis is generally composed of hundreds of miRNAs, some of which are likely to be false positives. However, a biomarker set should better be composed of only few molecules in order to be practically used in clinics, thus differential expression analysis alone is not sufficient and a second step of prioritization is mandatory. Multiple strategies were proposed to reduce the list of candidate microRNAs. When it was possible, microRNAs were ranked based on the number of similar studies in which they were found differentially expressed (37). This strategy led, for example, to the detection of miR-21, miR-106b, miR-17, miR-18a and miR-20a as candidate diagnostic and/or prognostic biomarkers for gastric cancer. In other cases the functional consistency between the miRNA target genes and cancer-related genes was evaluated to quantify the association between miRNAs and the type of cancer under investigation (38). Data-driven approaches, based on the use of experimental measurements of other nature like epigenetics (39), or proteomics (40), or employing prior knowledge like networks (41, 42), were also employed for microRNA prioritization. This last strategy involves the reconstruction of an undirected weighted network, whose nodes and edges represent differentially expressed miRNAs and their correlations (when significant), respectively. In such a network, critical nodes were pointed out evaluating some centrality measures classically employed in network theory: degree, betweenness and clustering coefficient (43). Employing this approach, in (41) some candidate driver miRNAs in colorectal (*miR-195*, *miR-1280*, *miR-140-3p* and *miR-1246*) and in pancreatic (*miR-103*, *miR-23a* and *miR-15b*) tumors were identified. Among them, some were already known to regulate key oncogenic processes. *MiR-103* is associated with the TGF-β signaling and thus it contributes in maintaining tissue homeostasis and it plays a crucial role in the suppression of the proliferation in cancer cells. *MiR-23a* is involved in the KRAS-mediated signaling. Finally, the overexpression of *miR-1246* had already been proved to decrease the induction of apoptosis.

Given their ability to model the miRNA organization at a system-level, networks have been also employed alone to detect miRNA drivers. In some cases, following the procedure detailed above, a network whose nodes represent all the expressed microRNAs is reconstructed independently in tumor and normal tissue. Then the two obtained networks are compared to highlight cancer-associated microRNAs (44). Variants of the network reconstruction procedure were also explored, based on measures different from correlation (45), or combining miRNA expression with alternative sources of information as drug response and miRNA targets (46). In this last case, the approach led to the identification of 11 oncomiRs (e.g. miR-20a-5p, miR-27a-3p, miR-29a-3p, and miR-146a-5p) biomarkers for metformin response in breast cancer. Finally, an approach completely independent from the aforementioned ones is PROGmiR (47), which selects biomarker miRNAs having a prognostic potential based on the Kaplan-Meier overall survival.

Overall, methods based on miRNA expression data can be easily used to identify miRNA biomarkers based on their differential expression between normal and cancer tissues. The association between miRNA and clinical data can also provide risk stratification of patients. A main advantage of miRNA in this context is that, as they are very stable in formalined tissues, retrospective or prospective studies can be performed on samples collected in a clinical setting. The main drawback of analysis using only miRNA data is the difficulty to define their functional consequences on disease progression with confidence.

3.2. Methods based on mRNA expression data

As described in the introduction, miRNAs act on the target mRNAs by translational silencing or mRNA degradation. Thus, it has been demonstrated that mRNA abundance for the majority of the targeted genes is somewhat affected by miRNAs (48, 49). Therefore the activity of a microRNA can also be predicted from mRNA expression data. Methods predicting miRNA activity using mRNA data are particularly useful as mRNA expression data are already available for virtually all diseases. The large availability of these data thus makes in silico studies predicting miRNA activity and their potential as biomarkers extremely powerful. Predictions based on such studies can have outcomes both for biologists studying a specific tumor or pathologists to assess new biomarkers.

All the algorithms that try to capture the dysregulation of a microRNA using only mRNA expression data generally explore the expression behavior of its targets obtained using only one predictive database (e.g. TargetScan, PITA, PicTar and miRanda). Some examples are Sigterms (50) and CORNA (51) that perform an mRNA differential expression analysis and then test whether the set of differentially expressed genes is enriched in predicted targets of a particular miRNA. Another tool is MirAct (52), which infers the regulatory effect of a miRNA via a two-step procedure. First, a score measuring the activity of a miRNA in a sample is obtained by comparing the expression levels of its non-targets with those of its targets. Second, the changes of miRNA activity across different classes of samples are investigated by comparing the scores across samples. Finally, miR-Path (53) is a recently developed R package to identify cancer driver microRNAs. The algorithm first identifies the targets of each microRNA, using more than one predictive database, then it ranks the microRNAs based on the number of cancer pathways that are enriched in their targets. The outputs of all these algorithms are strongly influenced by the huge amount of false positives and the unknown amount of false negatives produced by the currently available microRNA target prediction algorithms. On the contrary, CoMeTa (The Co-expression Meta-analysis of miRNA Targets) (54) is less affected by the number of false positive miRNA targets, given that it selects bona fide miRNA target genes by ranking them according to their degree of co-expression. Of particular note is the fact that miR-519d, miR-190 and miR-340, predicted by CoMeTa to regulate the TGFβ pathway, were functionally validated.
All these algorithms do not take into account miRNA expression data, thus the association between the miRNA and the mRNA expression levels is not evaluated, loosing a key information which could provide evidence for the regulatory relationship between the miRNA and its putative mRNA targets.

3.3. Methods based on combined miRNA-mRNA expression data

As pointed out in the previous section, the use of combined miRNA/mRNA expression data may be a timely strategy to study the regulatory relationship between a miRNA and its putative targets, permitting to achieve a higher precision in the identification of biomarker miRNAs in cancer and to asses their functional significance. Nowadays the study of combined miRNA/mRNA expression data is easy thanks to the multiple data types from hundreds of cancer patients that are collected in repositories such as Gene Expression Omnibus (GEO) (55), The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov) and the International Cancer Genome Consortium (ICGC (56)). Therefore, numerous procedures have been developed in the last years to infer miRNA activities from these data. Based on their final aim, these works can be divided into two main categories: (i) those interested in miRNA-mRNA couples and (ii) the microRNA-centered ones. The aim of the methodologies belonging to group (i) is the identification of co-expressed miRNA-mRNA couples whose behavior is altered in cancer. Such couples are generally identified by combining miRNA target prediction with miRNA/mRNA expression profiles correlation (57-61), or by more sophisticated approaches integrating miRNA/mRNA expression with sequence complementarity (62-64), or with other strategies capturing the miRNA-mRNA pairs without taking into account target complementarity (65-68). Among the results obtained by these methods, the miR-29 family was identified to recurrently regulate the DNA demethylation pathway in (60) and a signature of four miRNAs (miR-320d, miR-139-5p, miR-567 and let-7c) was proposed for breast cancer grade classification in (61).

All these approaches, powerful for precise miRNA targets identification, have limited application in the context of miRNA biomarkers detection. In fact, given that they deal with miRNA-mRNA couples, their evaluation of the miRNA driving role is always conditioned by the activity of the associated mRNA. To overcome this limitation, methodologies that are centered on microRNAs, corresponding to group (ii), are needed. The most common and well-defined miRNA-centered procedure taking advantage of both miRNA and mRNA expression data involves a first step of microRNA differential expression analysis, in which active microRNAs are detected, followed by the evaluation of their regulatory effect on mRNAs, in which the intersection between the miRNA predicted targets and the group of regulated genes emerging from the expression data is computed. The set of mRNAs regulated by each microRNA is generally evaluated studying the anti-correlation between the expression values of the miRNA and those of its targets. A popular example of this kind of approach is MAGIA (69), but a similar procedure is also applied in other works (70-75). Interestingly, such approach led to the identification in (73) of two miRNA signatures (miR-16, miR-155, miR-125b, miR-374a and miR-16, miR-125b, miR-374a, miR- miR-374b, miR-421, miR-655, miR-497) predictive of triple negative breast cancer overall survival and distant-disease free survival, respectively. Alternative measures employed to capture the miRNA-target activity from expression data are: inverse expression (MMIA (76)), which is less stringent than correlation, penalized Cox regression (77) and Independent Component Analysis (ICA), which was proved to give more reliable results than correlation (78). In the last case eight microRNAs involved in type 1 diabetes regulation were identified, three of which (miR-124, miR-375 and miR-204) were already documented to have an important role in this disease.

Given that these approaches evaluate the miRNA activity based not only on its expression but also on its regulatory effects, they tend to be more accurate than those described in the previous sections. However they do not capture all those cases in which a miRNA up-regulates its target mRNAs, phenomenon that has been abundantly observed (79-82). To take into account also this regulatory mechanism, bioinformatics strategies that do not use miRNA-mRNA sequence complementarity have been developed. Some of them apply the procedure described above without using target prediction, i.e. miRNA differential expression followed by correlation analysis with all the expressed mRNAs (83, 84). In this case both positive and negative correlations are considered. Such basic strategy proved is effectiveness by identifying miR-648 as a novel candidate miRNA biomarker in prostate cancer (84). Some modifications of this pipeline have then been also proposed. Hua *et al*. (85) substitute correlation with summed Pearson Correlation Coefficient (sPCC) that is less affected by signal distortions than correlation. Engstrom *et al*. (86) and Genovese *et al*. (87) replace correlation with Mutual Information (MI), which does not assume a linear relationship between miRNA and mRNA expression values. Other methods substitute miRNA differential expression analysis with alternative approaches (87-89). Genovese *et al*. (87) and Zadran *et al*. (88) employ information-theoretic approaches, Context Likelihood of Relatedness modeling algorithm and Surprisal analysis, respectively. Finally, Sehgal and coauthors (89) substitute miRNA differential expression analysis with a selection based on the prognostic significance, more advisable for clinical application. The use of measures alternative to correlation led in (87) to the identification of a novel regulation of TGF-β signaling via Smad4 by miR-34a.

The advantage of the above methods in respect to those that intersect predicted targets with expression correlation is that they are more general and thus they can capture more complex miRNA-mRNA regulatory events. However, given that they are less specific, they tend to be considerably prone to false positive predictions. In this regard, a good compromise is represented by the combined use of miRNA targets prediction and miRNA/mRNA expression analysis without computing the intersection of the two. Examples of these approaches are represented by Context-Specific MicroRNA analysis (CosMic) (90), TargetRunningSum (91) and MicroRNA Master Regulator Analysis (MMRA) (92, 93). The first two pipelines use a strategy closely related to gene set enrichment analysis (GSEA) to calculate the enrichment of the top ranked sequence-based predicted targets by the top ranked correlated genes. MMRA performs a more complex procedure involving four sequential steps, each aimed at progressively reducing the number of candidate microRNAs: (i) differential expression analysis to highlight microRNAs with subtype-specific expression; (ii) target transcript enrichment analysis, to further select those microRNAs whose predicted targets are enriched in the associated subtype mRNA signature; (iii) network analysis, in which an mRNA network is constructed around each microRNA using ARACNE and tested for enrichment in signature genes; (iv) identification of microRNAs whose expression ‘explains’ the expression of subtype signature genes, using stepwise linear regression (SLR) analysis. The pipelines described so far have been typically applied to distinguish tumour from normal tissue or to test the involvement of microRNAs in a pathway based on expression profiles derived from cell lines under stimulation. On the contrary, MMRA and TargetRunningSum are the first designed for miRNA biomarker identification in tumor subtypes, a comparison characterized by much lower variations. Moreover, as done only for CoMeTa so far, the results of both CosMic and MMRA were experimentally validated in cancer cell lines by microRNA silencing experiments. In particular, the control of migration by miR-20a, miR-212 and miR-671-5p, identified by CosMic, was validated in MCF10A cells after EGF stimulation. On the other hand, miR-429, miR-200b, miR-203 and miR-194, predicted by MMRA to drive the stem-like aggressive and poor prognosis colorectal cancer (CRC) subtype, were functionally validated in HT29, NCIH508 and SW403 CRC cell lines, suggesting the use of these miRNAs as biomarker and/or therapeutic molecules in the aggressive CRC subtype. Therefore MMRA is the only pipeline in its category able to identify candidate miRNA driver of cancer subtypes whose role has been functionally verified.

Overall, the approaches based on the combined use or miRNA and mRNA expression described in this section allow not only to strengthen miRNA prediction as a biomarker but also to reconstruct the effects of the miRNA deregulation on the

cellular functions. They can thus lead to the identification of central pathways deregulated in cancer, having direct consequences in the development of new therapeutic strategies. Moreover, the outputs of such approaches can help identifying key regulatory mechanisms involved in drug resistance that can be targeted in the context of drugs combination.

3.4. Methods taking into account the miRNA-TF crosstalk

The translation of an mRNA into protein is a multi-step process regulated at the transcriptional and post-transcriptional level by Transcription Factors (TFs) and microRNAs, respectively. Considering that TFs and miRNAs play a prominent role in transcription, their own and target sequences represent one of the major location of cancer-driver alterations. At the same time, given that they share a common regulatory logic, it is straightforward to hypothesize that they are able to cooperate. Therefore cancer onset or subtyping is more frequently characterized by a dysregulation in the synergistic miRNA-TF crosstalk rather than by the alteration of a single factor. As a consequence, to robustly identify miRNA biomarkers, it is important to consider the coordinated miRNA-TF activity, which is generally modeled using networks. MiRNAs, mRNAs, and TFs are the three classes of nodes in these networks, while edges are usually drawn integrating co-regulations inferred from miRNA/mRNA expression data with database derived interactions (e.g. TF-gene interaction form JASPAR, TRANSFAC and ECRbase ; miRNA-gene interactions from PITA, miRANDA, TargetScan 5.0., RNAhybrid and Pictar, Microcosm, microrna.org, DIANA-microT, miRDB, RNA22 ; TF-miRNA interactions form mirGen2.0. and TransmiR). Moreover, a sign is associated to each graph edge corresponding to activation/inhibition depending if this connects a molecule that influences positively/negatively the level of another one. In this context, the different algorithms differ for the technique used to combine the database-derived and data-driven interactions. MAGIA^2 (94), extension of the MAGIA algorithm described in the previous section, and mirConnX (95) reconstruct two independent networks. One derived from matched microRNA/mRNA expression data according to Person, Spearman correlation and mutual information. The other consistently retrieved from multiple interaction databases. The two networks are then integrated computing the intersection for MAGIA^2 and through a weighted sum function for mirConnX. Interestingly, mirConnX proved to be performing particularly well in the detection of cancer associated miRNA-TF interactions. For example, it identified a feed-forward loop among SMAD TFs, let-7 d and HMGA2 gene, which is central in the regulation of epithelial to mesenchymal transition (EMT). These approaches can be employed only if a matched microRNA/mRNA expression dataset is available. Alternative methods, applicable also to unmatched miRNA/mRNA expression data, perform miRNA and mRNA differential expression analysis and then reconstruct the network whose nodes are represented by the differential molecules and whose links are derived from databases. MAGIA^2 (supporting both procedures) and the pipelines by Ying *et al*. (96) and Samantarrai *et al*. (97) follow this idea. Interestingly, the work by Ying and coauthors put light on the role of miR-16 in triggering an accumulation of cells in G0/G1 through the silencing of multiple cell cycle genes and thus they suggested this miRNA as candidate biomarker for ovarian cancer. The integration of database and data-driven derived information has been finally performed also in other two completely new ways (98, 99). Gene4x (98) combines mRNA expression data with database knowledge (including protein-protein interactions) through the use of multi-networks. The approach proposed by Yu and coauthors (99) uses a linear regression model taking into account both miRNA/mRNA expression and predicted regulatory relationships. Of note is the fact that multiple candidate biomarker microRNAs have been suggested for colorectal, pancreatic, lung and gastric cancer in (98). For example, MiR-337 was suggested as biomarker for survival in pancreatic cancer and indeed its overexpression had already been shown to induce the suppression of cell proliferation and invasion in pancreatic cancer. MiR-153 was identified as prognostic marker in the same cancer and indeed it was already known to inhibit PDAC cell migration and invasion by targeting SNAI1.

The regulatory networks, obtained with the procedures described above, are usually composed of over-represented sub-network patterns known as network motifs (100, 101). Among the various motifs mixed feed-forward loops (FFLs) are those playing a pivotal role in gene regulation and known to have an important role in cancer development (102). A typical mixed FFL consist of a TF that regulates the transcription of the miRNA and both the TF and the miRNA regulate a common set of target gene (103-106). Given that the study of mixed FFLs has emerged as a powerful tool to understand specific biological events, multiple research groups have investigated their involvement in cancer. Sun *et al*. (107) have first reconstructed the regulatory network of genes, TFs and microRNAs known to be involved in Glioblastoma (GBM) and then they have extracted significantly over-represented mixed motifs. With such approach, Sun *et al*. suggested six key miRNAs (miR-124, miR-137, miR-219-5p, miR-34a, miR-9, and miR-92b) involved in the Notch signaling pathway in GBM. Among them the most noteworthy one is miR-34a, which regulates proteins involved in cell cycle, apoptosis, differentiation and cellular development. Afshar *et al*. (108) developed integraMiR a new tool for regulatory network reconstruction and detection of overrepresented FFLs. On the other hand, there are methods that do not reconstruct the network, but only explore all possible FFL combinations. Some of them reconstruct the FFLs using only databases information (109, 110). In (110) the authors provide a comprehensive database of all possible FFLs involving MYC, a TF of crucial importance in several biological processes, using only experimentally validated interactions. In particular, the authors centered their discussion on three main FFL circuits: (i) MYC-PTEN-(miR-106b, miR-93, miR-25, miR-19a, miR-22, miR-26a, miR-193b, miR-23b) suggested to act as a noise buffering circuit that guarantees a steady level of PTEN, a tumour suppressor gene which plays an important role in various cancer related pathways; (ii) MYC-RB1-(miR-106a, miR-106b, miR-17) which controls the expression of the retinoblastoma protein (*RB1*), a tumour suppressor shown to be dysfunctional in many types of cancer and (iii) MYC-VEGF-(miR-106b, miR-106a, miR-93, miR-34a, miR-20a, miR-17, miR-16, miR-15a) involved in cell migration and apoptosis. Jiang at al. (111) and Yan *et al*. (112) studied the FFLs active in multiple cancer types. In (111) network motifs were reconstructed in 13 tumor types using predicted interactions. 26 of those motifs were

then prioritized having a significant biological activity in at least 5 tumor types, where the biological activity was investigated through node differential expression and edge differential co-expression between tumor and normal tissue. In (112), instead six tumor types were examined for FFLs presence through the tool dChip-GemiNI developed by the authors in the same paper. DChip-GemiNI reconstructs the FFLs using different predictive databases and then selects significant FFL motifs through integration of network motifs and expression data. The significance of a motif is determined through the use of a network motif score (NMS) and false discovery rate (FDR). The NMS is a function of multiple scores, including TF and miRNA binding scores to their target sequences, differential expression P-values of the FFL components between normal and cancer tissues, and TF and miRNA's target enrichment in differentially expressed genes and miRNAs. The major limit in the use of the methods described above for miRNA biomarkers identification is that they describe interactions that are more complex than those of the other approaches. Indeed given that they try to capture regulatory processes jointly controlled by a miRNA and a TF their activity cannot be controlled by only looking at the behavior of the miRNA.

The employment of the approaches proposed in this section for biomarkers identification is limited by the fact that they take into account mechanisms that are too complex to be used in the clinics for cancer detection. However, their power is due to the fact that they can substantially help cancer management by predicting key driver mechanisms with possible therapeutic application.

3.5. More recent integrative works considering also other data types

The studies described so far are based on the use of expression data alone, they thus ignore all those effects that arise from the interaction among different genomics levels. However, cancer is a complex disease characterized by multiple levels of dysregulation, for this reason the generation of integrative approaches can be employed to better elucidate the role of small non-coding RNAs in cancer. With the current availability of large collections of multi-omics data, it is now possible to compare cancer and normal profiles in multi-omics dimension. The integrative studies designed for microRNA biomarker identification realized so far combine methylation with miRNA/mRNA expression data. Volinia S. and Croce C.M. (113) employed methylation and miRNA/mRNA expression data from TCGA to construct an integrated prognostic signature, composed of 7 miRNA (such as hsa-miR-328, hsa-miR-484, and hsa-miR-874) and 30 mRNA genes, for invasive breast cancer. RNAs were selected if being significant in the survival analysis in at least two prognosis related subgroups and if their DNA methylation profile was significantly associated with patients overall survival. Finally the identified signature was tested in eight independent datasets proving to perform better than previous ones for risk stratification. Rajamani D. and Bhasin M.K. (114) employed a network-based approach to integrate the same omics profiles (mRNA, miRNA, DNA methylation) in pancreatic ductal adenocarcinoma (PDAC). In particular, first multidimensional disease signatures were obtained using rank-based meta-analysis, then these signatures were integrated in a network constructed using knowledge-based interaction information. Finally key regulators were identified from the network based on centrality measures. Yang *et al* (115) investigated the role of microRNAs in the regulation of the ovarian cancer (OvCa) mesenchymal subtype through a multivariate linear regression model searching for differentially expressed genes whose expression was correlated with copy number alteration, DNA methylation, or associated miRNA expression. With such approach, eight miRNAs were predicted to be crucial in the regulation of the OvCa mesenchymal subtype. Follow-up functional experiments validated the role of miR-506 in inhibiting cell migration and in preventing TGFβ-induced epithelial-mesenchymal transition by targeting *SNAI2*, a transcriptional repressor of E-cadherin.

Integration of multi-omics data is becoming nowadays a widely employed approach to decipher the complexity of diseases such as cancer. Using miRNA data along with other data types can define comprehensive prognosis for patient that would not be achieve with single data sets. It is foreseeable that muli-omic based prognosis as the potential to be the most accurate methodology to precisely risk stratify patient and will play an important role in therapeutic strategy choices. On the other side, one difficulty in determining the microRNAs activity is represented by the wide set of potential target genes. To more accurately determine miRNA targets, Photoactivatable-Ribonucleoside–Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP)-based methodologies (116), able to experimentally identify microRNA–target interactions in a genome-wide manner, have been designed in the last years. However the current collection of miRNA targets identified through the PAR-CLIP technology is restricted to a subset of the expressed microRNAs. For this reason the data obtained with this experimental technique have been recently combined with predictive approaches to increase the accuracy of the reconstructed miRNA-target interactions. Farazi and coauthors (117) identified miRNAs controlling subtype-specific pathways in breast cancer by training a regression model to rank and select miRNA-target interactions from TargetScan predictions that display similar characteristics to AGO2-PAR-CLIP targets and then prioritized miRNA regulatory activity based on association with expression of respective validated targets. The results of this approach suggest that miR-17, miR-19a, miR-25, and miR-200b show high regulatory activity in the triple-negative, basal-like subtype, whereas miR-22 and miR-24 do so in the HER2 subtype. Hamilton *et al*. (10) identified a pan-cancer, coregulated oncogenic microRNA 'superfamily' by integrating the atlas of AGO-PAR-CLIP-based microRNA targets with microRNA/mRNA expression, copy number variation (CNV) and exome-sequencing datasets.

3.6. Methods considering the miRNA-miRNA synergistic effect

These few decades of research on miRNA regulation have evidenced that in cancer, as in other contexts, miRNAs frequently act in a combined manner (118). The availability of large datasets derived from high-throughput experiments has allowed researchers to start investigating the synergistic relationships among miRNAs. Some computational methods have then

been designed to predict genes and pathway jointly regulated by a set of microRNAs. The network-based methods that we have described above for the prioritization of differentially expressed microRNAs (41, 44-46) represent a first example of such kind of methodologies. Given that from the structure of the miRNA-miRNA network, used to prioritize miRNAs, it is also possible to elucidate their combined effect. Some methods have then been specifically designed for the identification of synergic microRNAs regulatory modules (119-121). Zhang *et al* (119) designed mirSRN (miRNA synergistic regulatory network), to infer miRNA synergism in human molecular systems by considering both downstream miRNA targets and upstream TF regulation. Xu *et al*. (121) constructed a miRNA–miRNA functional synergistic network via co-regulating miRNA modules satisfying three conditions: common targets, enriched in the same gene ontology category and close proximity in the protein interaction network. The same authors compared the miRNA synergistic network obtained on normal and tumor tissue observing an increased frequency of synergism in the disease network. Two well-known examples of cooperating microRNA sets are represented by miRNA families and genomically clustered microRNAs (122). MiRNA families are composed of miRNAs that share the same seed region and thus can be captured by all those approaches that evaluate synergism by looking at the number of common targets between groups of microRNAs. On the other hand, what is striking about miRNA clusters is that they frequently contain representatives from different miRNA families, meaning that the miRNAs of a given cluster can target different mRNAs, but surprisingly they have been shown to jointly regulate proteins in close proximity of the PPI network (123), or belonging to the same pathway (124). The role of some well-known miRNA clusters in cancer has been largely studied experimentally (118). However more than half of the total human microRNAs are organized into genomic clusters. If this is not a random event, we can thus hypothesize an involvement of other clustered miRNAs in cancer. The systematic investigation of the role of miRNA genomic clusters in cancer can be only realized computationally. By now, only two computational works exist about miRNA clusters (125, 126). They are both based on miRNA differential expression analysis, but no methodologies have yet been designed for the identification of biomarker miRNA clusters employing more sophisticated steps as those described above for single miRNA pipelines.

4. CONCLUSIONS AND PERSPECTIVES

Since 2002, when the involvement of *miR-15/16* in CLL was described, bioinformatic approaches contributed greatly in elucidating the role of miRNAs in cancer. One of the causes of the high interest in miRNAs activity in cancer is given by the fact that these tiny molecules are attractive candidates for employment as biomarkers. In fact, miRNAs are known to be very stable in formalined tissues, which are the common source of samples for biomarker analysis, and they are present in body fluids, which makes their analysis possible by less invasive methods and hence more practical in clinical settings. However, the full potential of miRNAs should not be exhausted in their use as biomarkers. Future research should increment the development and delivery of miRNA-based drugs. In this respect, some miRNAs have already shown promising results (127). Moreover, to increase the efficacy of currently used non-miRNA treatments, miRNAs can be employed to overcome resistance by acting on the multiple genes associated with a chemoresistant phenotype. Outstanding challenges remain to be overcome in order to achieve this goal. The two main problems in miRNA-based therapies are their poor cellular uptake due to their size and negative charge, as well as the off target effects due to the fact that a miRNA affects hundreds of transcripts in different tissues. To overcome these limitations nanoparticles and polymers as well as virus-based approaches are starting to be employed and overall, targeting miRNAs to reprogram regulatory mechanisms in cancer remains a strategy with strong potential and chances for success.

The achievement of such a goal in a less timing and resource demanding way can be performed thanks to the support of systems biology. In fact, elucidating the involvement of microRNAs in cancer onset and subtyping can help in the prioritization of the key miRNAs having a promising power in theranostics. In this review the state-of-art computational approaches for the detection of driver microRNAs in cancer have been comprehensively described. Concerning the existing methods, those combining miRNA expression with other measurements (e.g. mRNA expression, methylation) are those giving better performances. In particular, the approaches combining miRNA and mRNA expression gave the best performances leading to output miRNAs whose key role was also validated in functional experiments. The overall results seem thus to indicate that integration of different data types is the key to improve the future development of miRNA-based theranostic tools.

The majority of the approaches here proposed are devoted to elucidate the involvement of single microRNAs in cancer. However, miRNAs have been shown to frequently act in a combined manner, both in cancer and in other contexts. In particular, a well-known example of cooperating microRNA sets is represented by genomically clustered microRNAs. What is striking about miRNA clusters is that their components can target different mRNAs that surprisingly jointly regulate proteins in close proximity of the PPI network (118), or belonging to the same pathway (119). The systematic investigation of the role of miRNA genomic clusters in cancer can be only realized computationally and by now no sophisticated methodologies have been designed for the identification of biomarker miRNA clusters. For this reason, the design of methodologies centered on genomically clustered microRNAs should probably represent the next step to achieve in computational biology in order to improve the actual identification of miRNAs in theranostics.

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Figure 1. Summary of the main computational modules employed. Summary of the main computational tasks performed by the six chategories of algorithms presented in the paper. The grid reports on the rows the six tool chategories ((i) Methods based on miRNA expression data; (ii) Methods based on mRNA expression data; (iii) Methods based on combined miRNA-mRNA expression data; (iv) Methods taking into account the miRNA-TF crosstalk; (v) More recent integrative works considering also other data types; (vi) Methods considering the miRNA-miRNA synergistic effect) and in columns the computational modules (miRNA differential expression analysis, network miRNA-miRNA, survival analysis, functional analysis, mRNA differential expression analysis, miRNA targets prediction, network miRNA-mRNA, TFs activity, miRNA-mRNA correlation computation, integration of other data). A violet square is present in position (i,j) if the computational module j has been employed in at least one publication of the tool chategory i.

Table 1. Summary of the main discussed tools

Method category	**Main tool available to run discussed in this review**	**Main procedures not yet implemented in a tool discussed in this review**
(i) Methods based on miRNA expression data	PROGmiR, SAM, ANOVA, Wilcoxon test, t-test	Volinia *et al*. (44), Piepoli *et al*. (41), Zhang *et al*. (39)
(ii) Methods based on mRNA expression data	Sigterms, CORONA, MirAc, miR-Path, CoMeTa	
(iii) Methods based on combined miRNA-mRNA expression data	MAGIA, MMIA, CosMic, TargetRunningSum, MMRA	Cava *et al*. (61), Liu *et al*. (66), Sehgal *et al*. (89), Hua *et al*. (85), Engstrom *et al*. (86), Genovese *et al*. (87)
(iv) Methods taking into account the miRNA-TF crosstalk	MAGIA2, mirConnX, Gene4x, integraMiR, Dchio-GemiNI,	Ying *et al*. (96), Samantarrai *et al*. (97), Yu *et al*. (99), Sun *et al*. (107)
(v) More recent integrative works considering also other data types		Volinia *et al*. (113), Rajamani *et al*. (114), Yang *et al* (115), Farazi *et al*. (117), Hamilton *et al*. (10)
(vi) Methods considering the miRNA-miRNA synergistic effect	mirSRN	Xu *et al*. (121)

The main methods/algorithms/tools discussed in the text are here summarized. On the rows are reported the main six categories of approaches described in the text, while the two columns correspond to tools available to run and procedures not yet implemented in a tool, respectively.

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