Chapter 1
Systems and Network Biology to Investigate Epigenetic De-regulatory Mechanisms of MicroRNAs in Pancreatic Cancer

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Abstract Pancreatic cancer (PC) is highly resistant to conventional therapeutics, and it is a complex disease which is partly characterized by genetic and epigenetic de-regulation of many signaling pathways. Among the numerous de-regulated mechanisms aberrant expression of small non-coding MicroRNAs (miRNAs) are very important. Intense research in this area has lead to the identification and characterization of critically de-regulated miRNAs which raises our hope that targeting them may lead to clinically beneficial outcome of patients diagnosed with PC. Emerging evidence suggests that miRNAs are under a highly coordinated epigenetic regulation, which could be exploited for the development of novel therapeutics. For the success of miRNA based therapeutic regimens, a holistic approach may be required that takes into account the emerging epigenetic regulatory mechanisms. In this chapter, the aberrant epigenetic grooming of miRNAs especially in PC and computational strategies especially systems and network biology to target them are discussed.

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1.1 Introduction

Pancreatic cancer (PC) is a deadly disease with estimated 168,800 annual deaths worldwide translating to ~20 deaths every hour (Global Cancer Statistics 2012) [1]. PC is diagnosed at a very late stage when it is refractory to surgery or standard chemotherapy [2]. The median survival is 6 months and overall therapeutic response rate is less than 5 % [3]. These dismal statistics indicate that newer diagnostic biomarkers and therapeutically druggable avenues need to be urgently identified. PC is among the most complex and heterogeneous of malignancies carrying multiple de-regulatory signaling mechanisms [4]. Among the various critical pathways found to be altered in PC, the microRNA (miRNA) system are being well recognized [5]. The miRNAs comprise a class of short noncoding RNAs that are 18–25 nucleotides in length and they are found in all animal and plant cells. In 1993, the first miRNAs were recognized in Caenorhabditis elegans by Lee et al. [6]. In 2001, various small regulatory RNAs were discovered in plants and mammals and designated 'microRNA' [7–9]. Currently, >1,200 human miRNAs are registered in the miRBase database [10]. The miRNAs have been extensively studied for their involvement in RNA interference (RNAi) machinery to regulate gene expression post-transcriptionally, and they are known to contribute to diverse physiological and pathophysiological functions, including the regulation of developmental timing and pattern formation, restriction of differentiation potential [10], cell signaling [11], cardiovascular diseases [12] and carcinogenesis [13]. The biogenesis and RNAi functions of miRNA (i.e. how miRNAs are generated and processed into a mature form, and how they regulate gene expression) have been intensively investigated and well-described [14]. Furthermore, developments in miRNA-related technologies, such as miRNA expression profiling and synthetic oligoRNA, have contributed to the identification of miRNAs that are known to be involved in a number of physiological and pathological phenotypes. A Pubmed search for microRNAs returns >19,900 hits similarly key words microRNA and cancer return >4,500 research articles. Most interestingly, an evaluation of research publications from 2000 to 2011 shows an exponential increase in research (summarized in Fig. 1.1) indicating that the field is advancing rapidly. These studies have led to much deeper understanding of microRNA biogenesis, their regulatory control on different genes and strategies to target them for anti-cancer therapeutic benefits. However, some questions remain largely unanswered, such as how miRNA expression is controlled and which genes are regulated by each miRNA in a specific disease condition.

The revolution of epigenetics has revitalized cancer research, shifting focus away from somatic mutation toward a more holistic perspective involving the dynamic states of chromatin. Disruption of chromatin organization can directly and indirectly precipitate in genomic instability and transformation. Not surprisingly,

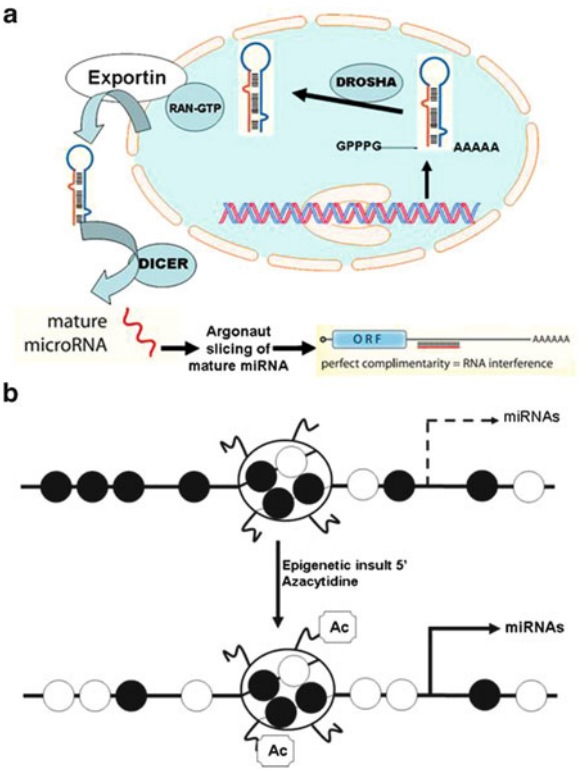

Fig. 1.1 miRNA biogenesis and epigenetic regulation: Generalization of a model involving epigenetic regulation of miRNAs in determining cell fate. A miRNA may be epigenetically silenced in the undifferentiated cell state by the combination of a methyl-DNA binding protein and associated chromatin-modifying repressor complex. In this state, the expression of the repressed miRNA is low whereas the expression of target mRNA transcript or protein coded for by the target transcript is high relative to the differentiated state. Upon an extrinsic or intrinsic signal cue for cellular differentiation, epigenetic repression of the target miRNA is released, and miRNA expression increases. The increase in miRNA expression then correlates with decreased stability or translation of target mRNAs. By epigenetically regulating one miRNA, the cell can thereby direct and fine-tune the expression of multiple miRNA-targeted mRNA transcripts during cell fate determination. The illustrated example provides a single direction in which this mechanism may potentially function, and it may be equally likely that the mechanism contributes to cell fate determination in the opposite manner as well. In this case, miRNA expression may become epigenetically silenced during differentiation. Subsequently, target mRNA translation would increase and proteins important for the differentiating cells would be expressed at higher levels than the undifferentiated cells

studies have shown that a greater majority of miRNAs are regulated epigenetically [15]. There are some studies that have focused upon the relationship between miRNA and its consequence on therapy resistance especially in complex diseases such as PC. We will first illustrate the current knowledge regarding the epigenetics–miRNA regulatory networks and its impact on inducing drug resistance in PC.

1.2 MicroRNA Biogenesis and Their Epigenetic Regulatory Mechanisms

Over the years, our understanding of microRNAs biogenesis has increased and this is in part due to increased understanding of the transcription, replication and translations process. The miRNA biogenesis originates primarily in the nucleus where the RNA polymerase II initially transcribes miRNAs into long segments of coding or noncoding RNA, known as pri-miRNAs that carry a polyadenylated cap. Following this, RNase type III, Drosha and the dsRNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8) (also called Pasha) (collectively called as microprocessor complex) capture short sets of the pri-miRNAs measuring approximately 70–100 nucleotides in length and containing a stem-loop becomes pre-miRNAs [19]. Pre-miRNAs form a complex with exportin-5 (XPO-5) and RAN-GTP, and undergo nuclear export to cytoplasm. Following this a Dicer or type III dsRNAse further processes the pre-miRNAs to a double-stranded miRNA duplex (ds-miRNA). This ds-miRNA duplex is incorporated into a RNA-induced silencer complex (RISC)-loading complex (RLC) in an ATP-dependent manner [20]. One strand (the passenger strand) of the miRNA is removed from the RLC, whereas the other guide strand remains in the complex to form a mature RNA-induced silencer complex (RISC) and serves as a template for capturing target miRNAs. The mature RISC represses gene expression post-transcriptionally and the core (catalytic) component or RNase III domain of a Argonaute protein cleaves the RISC to generate highly complementary target miRNAs [21]. For partially complementary targets, the RISC complex deadenylates target miRNAs and this, in turn, reduces the stability of target microRNAs [22]. Additionally, the RISC complex has also been shown to repress the translation of target genes under most conditions (Biogenesis summarized in Fig. 1.1a). However, the mechanisms underlying miRNA turnover in human cells remains unclear. Instead of re-reviewing the existing knowledge on miRNA processing and their targets, the focus of this article is to highlight the disease specific role of miRNA epigenetics which is described below.

Even though the biogenesis of miRNA has been intensively studied and is well-described, the regulation of miRNA expression remains largely unclear. In early studies, promoter regions had been determined for only a small subset of miRNAs while in-silico predictive studies have provided blueprints of the promoter regions of miRNAs [16]. However most of these predicted miRNA promoters have yet to be confirmed in wet-laboratory experiments. The miRNAs are classified as either ‘intragenic’ or ‘intergenic’, according to whether the miRNA is localized in a genomic region transcribed by a gene, or not. The means by which miRNA expression is regulated appears somewhat complicated. Earlier, it was established that epigenetics controls a number of genes in cancer and other diseases while recent evidence shows that epigenetics and miRNAs control each other forming a regulatory circuit that maintains normal physiological functions. One can envision that a disruption of this regulatory circuit can manifest into various diseases, such as cardiovascular diseases and cancer.

The earliest study on epigenetic control of miRNA were published in seminal paper by Saito and colleagues where it was established that the expression of miR-127 is regulated epigenetically [17]. In this study, using a bladder cancer cell model, it was shown that targeted demethylation resulted in the activation of certain microRNAs (miR-127). Specifically, the DNA methylation level and histone modification status at identified promoter regions of miR-127 was correlated significantly with mature miR-127 expression. These findings paved the way for numerous subsequent studies documenting epigenetic modulation of miRNAs in different cancer models.

1.3 Epigenetic Modulation of miRNAs in PC

With respect to PC, a number of miRNAs have been correlated to drug resistance and poor overall survival. Our laboratory was among the first to demonstrate the expression profile of microRNAs (miRNAs) in the plasma of patients diagnosed with PC (n = 50) compared with healthy volunteers (n = 10) [18]. In this study, 37 different miRNAs were found to be down-regulated and 54 were up-regulated in the plasma from patients with PC. The expression of miR-21 was significantly higher, and the expression of let-7 family (especially let-7d) and miR-146a was significantly lower in PC. Most interestingly, the expression of miR-21 was correlated with poor overall survival, and the expression of let-7 was inversely correlated with survival in this pilot study with mixed patient population. Additionally, we observed miR-21 family was markedly over-expressed in chemo-resistant PC cell line models, which was consistent with the plasma data from PC patients. This was a proof of principle study suggesting that identifying and validating the expression of miRNAs in newly diagnosed patients could possibly serve as potential biomarker for tumor aggressiveness, and such miRNAs could be useful for the screening of high-risk patients, and may also serve as targets for future drug development. We have also verified the de-regulated miRNA signatures in RNA samples derived from fine needle aspirate of PC patients [19]. These studies have verified the role of deregulated miRNAs and their association with deregulated signaling in PC. The next question is how miRNA expression is deregulated, which is discussed in the following paragraphs.

The first study on miRNA epigenetic regulation in PC came from Maitra's group where they have used two human pancreatic cancer cell lines – MiaPACA-2 and PANC-1 to verify the effect of demethylating agents, 5-aza-2′-deoxycytidine (5-Aza-dC) or the histone deacetylase inhibitor, trichostatin A, as well as the combination of the two [20]. Control and treated cell lines were assessed using a custom microarray platform. Fourteen miRNAs were found to be up-regulated by two-fold or greater in each of the cell lines following exposure to both chromatin-modifying agents, including five miRNAs that were found to be common (miR-107, miR-103, miR-29a, miR-29b, and miR-320) to both MiaPACA-2 and PANC-1 cell lines. The differential over expression of miR-107 in the treated cancer cell lines was

confirmed by Northern blot assays. Methylation-specific PCR assays for assessment of CpG island methylation status in the 5′ promoter region of the miR-107 primary transcript demonstrated complete loss of methylation upon exposure to 5-Aza-dC. Interestingly, forced expression of miR-107 in MiaPACA-2 and PANC-1 cells reduced *in vitro* growth, and this was associated with repression of the putative miR-107 target, cyclin-dependent kinase 6. These studies provided functional basis for the epigenetic inactivation of this miRNA in pancreatic cancer.

In another epigenetic study on miRNAs in PC, Mees and colleagues used a total of 16 human PDAC cell lines in murine orthotopic PDAC models [21]. Using a standardized dissemination score, local invasion and metastatic spread were assessed. The authors detected CD40 as a relevant target gene for differentially expressed miRNAs observed in highly invasive and metastatic PDAC only. A significant overexpression ($P < 0.05$) of CD40-related miRNAs such as miR-224 and miR-486 was detected in highly invasive and metastatic PDAC models, whereas CD40 mRNA expression was not significantly altered. Intriguingly, CD40 protein expression at cell surfaces of these highly invasive and metastatic PDAC was significantly reduced ($P < 0.01$). Most importantly, epigenetic alterations with up-regulated CD40-targeting miR-224 and miR-486 were found to be related to reduced-CD40 protein expression at cell surfaces in highly invasive and metastatic PDAC. From these studies, the authors have concluded that miRNA-regulated CD40 expression seems to play an important role in progression of PDAC. These data also suggested a diagnostic and therapeutic potential for CD40 and its targeting miRNAs in PDAC.

Recently Kitamoto and colleagues in their study presented epigenetic regulatory effects of miRNAs on Mucins (specifically, MUC17) that is recognized to be aberrantly expressed in PC [22]. Using gene expression analysis, epigenetic regulation investigations such as promoter methylation, histone modification and miRNA expression, the authors showed that near the transcriptional start site, the DNA methylation level of MUC17-negative cancer cell lines (e.g. PANC1) was high, whereas that of MUC17-positive cells (e.g. AsPC-1) was low. Histone H3-K9 (H3-K9) modification status was also closely related to MUC17 expression. Their results indicated that DNA methylation and histone H3-K9 modification in the 5′ flanking region could play a critical role in MUC17 expression. These cell line observations were correlated with hypomethylation status as observed in patients with PDAC. From these studies, it was concluded that the hypomethylation status in the MUC17 promoter could be a novel epigenetic marker for the diagnosis of PDAC. Most importantly, the result of miRNA microarray analysis showed that five potential miRNA candidates existed, suggesting that the MUC17 might be post-transcriptionally regulated by miRNA targeting to the 3′-untranslated region of its mRNA.

Apart from the above studies, miR-34 family has been well investigated for epigenetic regulations in PC. In this direction Vogt and colleagues found frequent concomitant inactivation of miR-34a and miR-34b/c by CpG methylation in PC and other solid tumors [23]. The authors proposed that miR-34a down-regulation and CpG methylation can serve as diagnostic marker for this disease. Supporting this study, Nalls and coworkers investigated the functional significance of miR-34a in

PC progression through its epigenetic restoration with chromatin modulators, demethylating agent 5-Aza-2′-deoxycytidine (5-Aza-dC) and HDAC inhibitor Vorinostat (SAHA) [24]. The authors observed re-expression of miR-34a in human PC stem cells (CSCs) and in human PC cell lines upon treatment with 5-Aza-dC and SAHA which was strongly associated with inhibition of cell proliferation, cell cycle progression, self-renewal, epithelial to mesenchymal transition (EMT) and invasion. In PC CSCs, modulation of miR-34a induced apoptosis by activating caspase-3/7. Most interestingly, treatment of pancreatic CSCs with the chromatin-modulating agents resulted in the inhibition of Bcl-2, CDK6 and SIRT1, which are considered the putative targets of miR-34a. The miR-34a up-regulation by these agents also induced acetylated p53, p21(WAF1), p27(KIP1) and PUMA in pancreatic CSCs. Inhibition of miR-34a by antagomiR abrogated the effects of 5-Aza-dC and SAHA, suggesting that 5-Aza-dC and SAHA regulates stem cell characteristics through miR-34a.

In CSCs, SAHA inhibited Notch pathway, suggesting its suppression may contribute to the inhibition of the self-renewal capacity and induction of apoptosis. Interestingly, treatment of pancreatic CSCs with SAHA resulted in the inhibition of EMT with the transcriptional up-regulation of E-Cadherin and down-regulation of N-Cadherin. Expression of EMT inducers (Zeb-1, Snail and Slug) was inhibited in CSCs upon treatment with SAHA. The 5-Aza-dC and SAHA also retarded the *in vitro* migration and invasion of CSCs. From these comprehensive studies, the authors have demonstrated the role of miR-34a as a critical regulator of pancreatic cancer progression by regulating CSC characteristics. The restoration of its expression by 5-Aza-dC and SAHA in CSCs was suggested to not only provide mechanistic insight and therapeutic targets for PC but also identify promising methodologies to boost patient response to existing chemotherapies or as a stand-alone cancer drug by eliminating the CSC characteristics. Collectively, these studies have confirmed the critical role of epigenetics in the regulations of miRNAs in PC. In the following passages the emerging computational technologies that are helping in better understanding the complexity of epigenetic mechanism of miRNAs in cancer in general including PC is discussed.

1.4 Why Computational Approaches Are Needed to Study miRNA Epigenetics?

Till date most of the studies have employed differential expression of miRNAs to identify candidates for subsequent epigenetic evaluation. This approach introduces bias that limits the number of epigenetically regulated miRNAs that can be identified. For example, tissue-specific miRNAs that are influenced by DNA methylation may be equally methylated in normal tissues and tumors, resulting in a lack of differential expression between normal and cancerous specimens. Furthermore, miRNAs with low expression levels cannot be reliably identified because the differences in the expression between normal and cancer or between baseline and demethylated

conditions will likely fall below conventional cutoffs limit that is ideally set between 2 and 1.5 fold. Finally, residual methylation persists in both pharmacologically and genetically demethylated cells [25]. Such methylation may be critical in maintaining cell viability, potentially through persistent repression of key miRNAs. This would result in such miRNAs not being identified through the expression-based strategies.

As elegantly summarized by Griffith Jones and colleagues, there are three critical steps in the discovery of miRNA-mediated gene regulatory signaling namely (a) discover co-expressed genes; (b) discover conserved cis-regulatory signature in UTRs of co-expressed genes as evidence of miRNA-mediated control; and finally (c) identify the miRNA seed sequence from miRBase that is complementary to the *cis*-regulatory signature [26]. Baliga and co-workers have developed a very user friendly analysis system, miRvestigator that allow users the flexibility to use any method to select a gene set that are likely to be co-regulated by a common factor [27]. The miRvestigator uses a *de novo* motif discovery algorithm that models miRNA binding in a probabilistic manner. Unlike earlier algorithm models which worked on a sorted gene list, miRvestigator expects a specifically selected subset of co-expressed genes identified using classification methods such as hierarchical clustering, biclustering and others. The miRvestigator scans the 3′-UTR sequences of query genes for an overrepresented sequence motif using the Weeder software package.

The complexity of the action of miRNAs calls for comprehensive, integrative systems level approaches to examine the effect of miRNAs. To this end, genomic and genome-related approaches in the study of miRNA have been appreciated recently. Integration of genome-related approaches with physiological and clinical approaches has been shown to be valuable for further elucidating the role of miRNAs in systems and personalized medicine. Notably, advanced genomics, proteomic, epigenomic techniques have begun to be utilized in the analysis of widespread effects of miRNAs [28]. Other approaches that have been used include large-scale sequencing of miRNA and potential miRNA targets, mRNA expression profiling, and bio-informatics modeling [29]. Sequencing of cleaved fragments of mRNAs has been used to identify miRNA targets, the applicability of which would depend on the extent to which miRNAs induce mRNA cleavage in a given species.

To overcome the discovery bias introduced by expression-based identification strategy, hollistic analysis is needed that directly utilizes global DNA methylation patterns to identify miRNAs regulated by DNA methylation of cancer cells. This requires computational tools that can not only identify miRNAs related to disease but also meaningfully unwind the complex interactions they regulate that result in a disease phenotype (Fig. 1.2 showing the complexity of miRNA network exemplified by let7 miRNA). Initial computational studies were restricted to mapping genome-wide DNA methylation in cancer cell lines using methyl CpG binding domain (MBD)-isolated Genome Sequencing (MiGS) [30]. Additionally miRNAs with proximal DNA methylation as candidates have also been identified that were cross-referenced the list of candidates with miRNA expression data as supporting evidence. Building such mapping studies, researchers have been able to perform

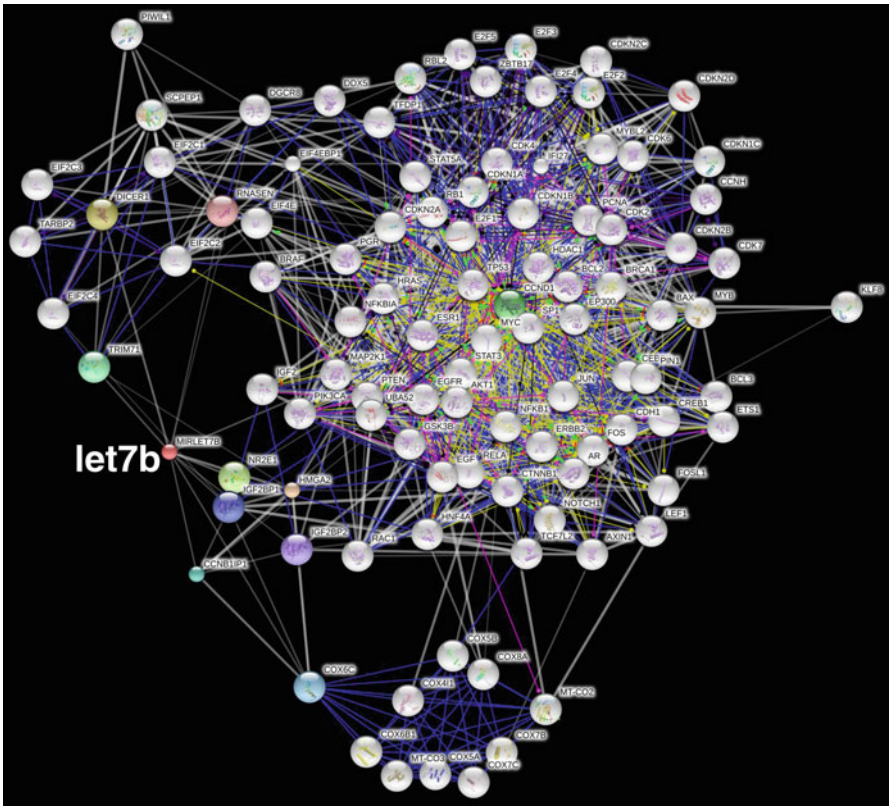

Fig. 1.2 Complex miRNA interaction network requires systems and network level understanding. A single miRNA can regulate and/or influence 100s of proteins. Figure showing interaction network of let7 miRNA (commonly found aberrantly expressed in different cancers). Nodes are either colored if they are directly linked to the input or white (nodes of a higher iteration/depth). Edges, i.e. predicted functional links, consist of up to eight lines: one color for each type of evidence. Network was developed using String 9.0

functional analysis of methylation regulated miRNAs in cancer. Using such approaches, Hongli Yan and colleagues have successfully identified both known and novel DNA methylation-regulated miRNAs [31]. They found 64 miRNAs to be robustly methylated in HCT116 cells; 18 of them were located in imprinting regions or already reported to be regulated by DNA methylation. For the remaining 46 miRNAs, expression levels of 18 were consistent with their DNA methylation status. Being consistent with their observations, another study showed that interacting proteins in the human PPI network tend to share restricted miRNA target-site types rather than random pairs [32]. Interestingly, a computational method named mirBridge that assesses enrichment of functional sites for a given miRNA in the annotated gene set showed that many epigenetically regulated miRNAs coordinately regulate multiple components of signaling pathways and protein complexes related to cancer [33].

Ju-Ichi Satoh and colleagues have identified a coordinated regulation of gene expression by transcription factors and miRNAs at transcriptional and epigenetic levels in cancer-associated miRNA targetome networks [34]. Importantly, a recent study showed that the genes with more transcription factor-binding sites have a higher probability of being targeted by epigenetically modified miRNAs regulations and have more miRNA-binding sites [35]. These observations support the general view that the human miRNAome and miRNA epigenome play specialized role in the regulation of oncogenesis. Therefore, the miRNA-based therapy designed to simultaneously target multiple cancer-associated networks and pathways might serve as the most effective approach in suppressing the oncogenic potential in a wide range of cancers. This can only be achieved if computational sciences are taken into consideration for the design of therapeutics strategies involving these multi-targeted small molecules.

1.5 Conclusions

PC is a deadly disease that is considered by far incurable among all other human malignancies. The disease is in a dire need of modern therapeutics as well as technologies to better understand the basic tenets of its origin. The miRNAs have been touted as the future of personalized therapy against cancer including PC and a number of miRNA targeted strategies are advancing towards clinical assessment for different cancers. However, a number of challenges remain especially with the discovery of epigenetic regulation of miRNAs that adds to a second tier of regulatory control on these small molecules. This is in addition to the by far incomprehensive level of complexity involved in the number of targets each miRNA regulates. Such large scale interaction networks can not be feasibly evaluated using reductionist molecular biology approach. Therefore, computational approaches would allow interrogating a variety of biological knowledge that can be extracted from the complexity of miRNA interactions. Integrated analysis of expression patterns comes in handy when the molecules under study have complex biological functions, such as those of miRNAs. Since the target mRNAs are regulated in a ‘one-to-many’ and a ‘many-to-one’ manner and the degree of regulation vary case-by-case or becomes context dependent. When accumulated miRNA–mRNA interactions identify biological functions, it will be necessary to look at those interactions comprehensively and recognize them as part of a gene regulatory network. Therefore, we suggest that further weight should be given to high-throughput analyses combined with computational approaches, as an effective methodology to achieve a systems-level understanding of complex biological functions of epigenetically groomed miRNAs. This will aid in the design of newer strategies that could selectively modulate the miRNAs for therapeutic benefit in order to realizing the dream of better and improved treatment outcome of patients diagnosed with deadly diseases such as PC.

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