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Emerging role of transcription factor-microRNA-target gene feed-forward loops in cancer (Review)

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Abstract. Transcriptional regulatory networks are biological network motifs that act in accordance with each other to play decisive roles in the pathological processes of cancer. One of the most common types, the feed-forward loop (FFL), has recently attracted interest. Three connected deregulated nodes, a transcription factor (TF), its downstream microRNA (miRNA) and their shared target gene can make up a class of cancer-involved FFLs as ≥1 of the 3 can act individually as a bona fide oncogene or a tumor suppressor. Numerous notable elements, such as p53, miR-17-92 cluster and cyclins, are proven members of their respective FFLs. Databases of interaction prediction, verification of experimental methods and confirmation of loops have been continually emerging during recent years. Development of TF-miRNA-target loops may help understand the mechanism of tumorgenesis at a higher level and explain the discovery and screening of the therapeutic target for drug exploitation.

Contents

1. Introduction

Although theoretical concepts and technological approaches have made significant progress, the molecular basis of carcinogenesis and progression of various types of cancer remains to be understood. This deficit in knowledge hinders the development of effective therapies and progress in the treatment of cancers remains slow. As a result, the curability of cancers is still poor (1).

To promote the discovery of oncogenic pathways, investigators have assessed biological networks, such as transcriptional regulatory networks (TRNs). TRNs (also known as gene regulatory networks) can offer the possibility to improve the understanding of the topology and function of gene regulation of the cellular responses to environmental changes at a system level. One important local property of biological networks is the 'network motifs', first described by Milo *et al* (2). They are patterns of interconnections occurring in complex networks and may reflect a framework in which particular functions are achieved efficiently. Much experimental study has been devoted to understanding network motifs in TRNs, as they define the core of the regulatory machinery of cellular life and are largely responsible for information processing and decision making (3).

The transcription network is a collection of DNA segments in a cell, which interacts with each other indirectly (through their RNA and protein expression products) and with other substances in the cell, thereby governing the expression levels of mRNA and proteins. In the network, a gene serves as the source of a direct regulatory edge by producing an RNA or protein molecule that functions as a transcriptional activator or inhibitor of the target gene. The network consists of network motifs, such as feed-forward loops (FFLs), feed-back loops (FBLs) and single-input modules. FFLs have been shown to be one of the most important and promising classes of transcriptional network motifs (2,4,5).

The FFL, a three-node motif pattern, is composed of two input elements, one of which regulates the other, both jointly regulating a target gene. Each of the three interactions in the FFL can be either activating or repressing (6,7).

As the research is being driven by its promising prospects, the importance of post-transcriptional processes have become more evident than previously expected in the regulation of gene expression. Among the various mechanisms of TRNs, transcription factors (TFs) and a class of small RNAs, known as microRNAs (miRNAs or miRs), are frequently observed in numerous TRN motifs, joining transcriptional and post-transcriptional regulatory interactions together, so as to play their prominent roles in regulation. Additionally, as the research regarding cancers expands, FFLs composed of a TF, an miRNA and their equivalent target gene are becoming

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apparent and the number of studies with such FFLs of different components reported is continuously increasing (8-12).

All the possible FFLs involving miRNA, TF and target gene are shown in Fig. 1. The TF-miRNA-target gene FFLs, as the network motif of typical TRNs that we will discuss in the present review, are depicted in Fig. 1.

In molecular biology and genetics, a TF (sometimes known as a sequence-specific DNA-binding factor) is a protein that binds to specific DNA sequences, thereby controlling the flow (or transcription) of genetic information from DNA to messenger RNA (mRNA) (13). Characterized by containing one or more DNA-binding domains, which attach to specific sequences of DNA adjacent to the genes that they regulate, TFs are essential for the regulation of gene expression and are found in all living organisms. TFs can read and interpret the genetic 'blueprint' in the DNA. They bind to the DNA and help initiate a program of increased or decreased gene transcription. As such, they are vital for numerous important cellular processes (14-16).

The most well-known TF, p53, regulates genes, such as *p21*, *cdc25c*, *bax* and *puma,* which are elucidated to have an indispensable role in cell cycle arrest, apoptosis or cell senescence, and all these can eliminate or reverse the presence of progenitor cancer cells in the body (17). p53 function appears to be crucial in tumors: i) p53 limits the first steps of transformation by preventing the proliferation of cells with damaged genomes or dysregulated growth. ii) p53 may act as an emergency brake at later stages of tumor progression, by preventing cells from accumulating multiple mutations and developing an invasive phenotype. Taken together, these mechanisms explain the effects of *TP53* mutation in numerous types of human cancer, detectable sometimes as an early event in precursor lesions or as a later event at the transition from *in situ* to invasive cancer (18). Cancer associated miRNAs, such as miR-34, miR-221 and miR-15/16, harbor p53 consensus binding sites and are already confirmed to be regulated by p53 and thus control downstream genes, including *Bcl2*, *p27*, *E2F3* and *CDK6*, to carry out anti-tumorigenesis function (19). In 2008, Brosh *et al* (20) reported an FFL constructed by p53, E2F and miR-106b/93/25 polycistron, of which the target gene *E2F* was also a TF.

Numerous other TFs, such as WT1, TAL1/SCL and Myc, have also been proven to be associated with their regulating miRNA or target gene in cancer (21,22).

Increasing attention has focused on miRNAs as they have been indicated in various types of human cancer. miRNAs are small, evolutionarily conserved, endogenous non-coding RNAs of 18-25 nucleotides (nts) in length that have an important function in gene regulation by pairing to the mRNAs of protein-coding genes to direct their post-transcriptional repression. Their silencing effects are exerted by cleavage of their target mRNAs and by inhibition of their translation. Each miRNA can target a large number of genes (mRNAs) and each mRNA can be targeted by several miRNAs. It is generally observed that miRNAs only have a minor influence on the protein levels of their targets; however, miRNAs can have a profound influence on cell-fate determination. It can even change a phenotype by modulating a single miRNA. miRNAs are now known to repress thousands of target genes and coordinate normal processes, such as developmental timing, pattern formation, embryogenesis, differentiation, organogenesis, growth control and cell death. This discovery established a new paradigm of
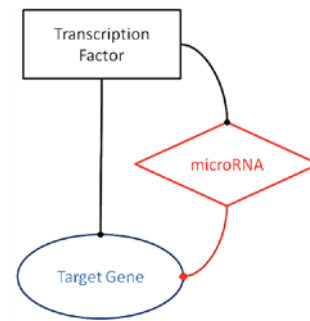

Figure 1. Typical feed-forward loops (FFLs). Representation of the FFL discussed in the present study. The square box represents the master transcription factor, the diamond-shaped box represents the microRNA involved in the circuit, and the oval box represents the downstream protein-coding target gene. Inside this circuit, the black line indicates transcriptional activation/repression, while the red line indicates post-transcriptional repression.

gene regulation (23-29). The alteration of miRNAs also contributes to a range of human pathologies, including cancer. Different associations between miRs and cancer have accumulated since the first evidence of an oncogene, KRAS, being targeted by an miRNA, the let-7 family, was reported in 2005 (30).

Beyond the impact of somatic, genetic and epigenetic lesions, the altered expression of miRNAs in cancer can arise through the aberrant activity of TFs that control their expression. Of note, the same TFs are often targets of miRNA-mediated repression, which gives rise to complex regulatory circuits (24).

Following this, as the understanding of miRNAs improved due to high-throughput miRNA expression profiling, bioinformatic prediction and other advanced technologies, their functions as regulators in signaling pathways and transcription networks have been revealed step by step. The maps of the networks have been completed gradually.

Directed by the theoretical discovery, research on signal flow inside the cell has increased. Researchers have carried out various experiments on the typical transcription network motifs. Subsequently, more hypotheses and their respective confirmative experiments were conducted and the TF-miRNA-target gene FFL motif theory was proven repeatedly, particularly in various types of human cancer (31-34).

2. Reported TF-miRNA-target gene FFLs in cancer

One of the best-characterized oncogenic miRNAs is miR-17-92, a polycistronic miRNA cluster also designated by He *et al* (35) as oncomiR-1 in 2005. This was the first time that the concept of an 'oncogenic miRNA (oncomiR)' became apparent. As more miRNAs have been identified to act as oncogenes, tumor suppressors and important modulators in cellular pathways have been divided into two classes: Increased activity of oncomiRs leads to inhibition of tumor-suppressor genes, facilitating cell proliferation and tumor progression. Decreased activity of tumor-suppressor miRNAs (tsmiRs) leads to increased oncogene translation, contributing to tumor formation (36,37).

Since the same TF can act as an activator or a repressor under different conditions, its directly-regulating downstream miRNA can be either an oncomiR or a tsmiR regardless of whether the TF is an oncogene or a tumor repressor. For instance, Myc, the c-Myc oncogenic TF, is known to directly

upregulate a pro-tumorigenic group of miRNAs known as the miR-17-92 cluster, however, the predominant consequence of Myc activation is widespread repression of miRNA expression (38). The involved miRNAs, including miR-26a, miR-150 and miR-195/miR-497, whose tumor-suppressing properties are to be confirmed (39-41).

TF-miRNA-target gene FFL circuits with its TF as an oncogene in cancer. In 2005, O'Donnell *et al* (42) reported that the loop consisting of c-Myc, miR-17-5p and miR-20a cluster and E2F1 modulates cellular proliferation in P493-6 cells. c-Myc simultaneously activates E2F1 transcription and limits its translation by upregulating miR-17-5p and miR-20a, allowing a tightly controlled proliferative signal.

Burk *et al* (31) reported an FFL in 2008. In the FFL, zinc-finger E-box binding homeobox 1 (ZEB1) directly suppressed the transcription of miRNA-200 family members, miR-141 and miR-200c, which suppress target gene transforming growth factor-β2 (TGFβ2) and strongly activate epithelial differentiation so as to repress the epithelial-mesenchymal transition (EMT) in pancreatic, colorectal and breast cancer cells. As less-suppressed TGFβ2 in return upregulates ZEB1, ZEB1 triggers this miRNA-mediated FFL that stabilizes EMT and promotes invasion. TF ZEB1 itself is a crucial inducer of EMT in various human tumors and was shown to promote invasion and metastasis of tumor cells (43).

Following this, KRAS was proved to be a target for several miRNAs and *KRAS* activation indicated the repression of several miRNAs. For example, in pancreatic cancer with mutant *KRAS*, RAS-responsive element-binding protein 1 (RREB1) represses the miR-143 and miR-145 promoter and at the same time KRAS and RREB1 are targets of miR-143 and miR-145, revealing a feed-forward regulatory circuit that increases the effect of RAS signaling (44).

El Baroudi *et al* (45) made a summary of simple and mixed FFLs involving c-Myc in 2011. There are various complex circuits with c-Myc involved, such as the MYC/PTEN/miR-106b, miR-93, miR-25, miR-19a, miR-22, miR-26a, miR-193b and miR-23b circuit, acting as a noise-buffering circuit to guarantee a steady level of the PTEN protein as a tumor-suppressor gene. The MYC/retinoblastoma 1 (RB1)/miR-106a, miR-106b and miR-17 circuit has a critical role in the pathogenesis of solid cancer by repressing the transcription and translation of tumor-suppressor gene *RB1*. The MYC/vascular endothelial growth factor (VEGF)/miR-106b, miR-106a, miR-93, miR-34a, miR-20a, miR-17, miR-16 and miR-15a circuit, can be classified as a coherent or incoherent loop, depending on the different functional roles of VEGF ranging from cell migration to apoptosis.

In the year 2013, Polioudakis *et al* (46) confirmed that miR-22, activated by the TF Myc when quiescent cells enter proliferation, could inhibit the Myc transcriptional repressor MXD4, mediating an FFL to elevate Myc expression levels in HeLa cells and human foreskin fibroblasts.

Also in 2013, Zhao *et al* (41) published a study that identified a MYC-miRNAs-EZH2 FFL linking overexpression of MYC, EZH2 and miR-26a repression in aggressive B cell lymphomas.

TF-miRNA-target gene FFL circuits with its TF as a tumor repressor in cancer. He *et al* (47) reported that the loop formed by p53 and miR-34a-c promotes cell cycle arrest and

inhibits inappropriate cell proliferation in 2007. They proved direct regulation of the association between p53 and miR-34a, miR-34a and its target genes *CDK4* and *MET*; however, in 2011, Hwang *et al* (32) established the exact p53-regulated FFL: Regulation of cancer-invasion-promoting gene *MET* by wild-type p53 consists of miR-34-dependent and -independent mechanisms. p53 activates miR-34, which represses *MET* and p53 can repress *MET* itself.

Untypical or uncertain reported FFLs are associated with cancer. In 2008, Lin *et al* (48) showed that TF c-Myc directly activates transcription of the 3 subunits of eIF4F (eIF4E, eIF4AI and eIF4GI), which is thought to be the rate-limiting phase of translation. Increased eIF4F levels result in stimulation of c-Myc mRNA translation specifically. This FFL involving c-Myc and eIF4F that serves to link transcription and translation could contribute to the effects of c-Myc on cell proliferation and neoplastic growth. The following year, the investigators published another study confirming the FFL association and highlighted that the regulators of the transcription and translation that affect Myc function (such as Mad1 or antisense approaches) or eIF4F activity (such as mammalian target of rapamycin) are expected to act as rheostats during normal growth and development to fine-tune the outcomes of the Myc/eIF4F FFL, representing promising targets for cancer therapy (49).

By studying head and neck squamous cell carcinoma in 2009, Cohen and Rosner (50) and Cohen *et al* (51) identified an FFL in cell cycle regulation involving protein kinase Cα (PKCα) that activates mitogen-activated protein kinase (MAPK), as well as cyclin E translation via inhibition of miR-15a. Of note, while one arm of the network entails classic transcriptional regulation of cyclins by the MAPK pathway, the other arm involves regulation of miR15a inhibiting cyclin E translation. The FFL is constitutively driven by PKCα activation, leading to the unabated proliferation inherent to cancer cells. Although the specific elements may differ, their results suggest that FFL networks could play a fundamental role in controlling DNA synthesis and cell cycle progression in tumor cells. In this FFL, PKCα is not typically a TF, the mechanism of how it downregulates miR-15a remains to be elucidated.

Using a coculture model system, Rokavec *et al* (52) showed a feed-forward inflammatory signaling circuit in breast cancer in 2012. The circuit was composed of miR-200c, p65, c-Jun N-terminal kinase 2 (JNK2), heat-shock factor 1 (HSF1) and interleukin 6 (IL-6). Suppression of miR-200c by IL-6 constitutively activates p65/RelA and JNK2, and the latter phosphorylates and activates HSF1. In turn, HSF1 triggers demethylation of the IL-6 promoter that facilitates the binding of p65 and c-Jun, which together drive constitutive IL-6 transcription, promoting transformation in human cancer cells and in a mouse model of ErbB2-driven breast cancer.

3. Finding and confirming a specific TF-miRNA-target gene FFL

Research has accumulated in this field of the typical TF-miRNA-target protein coding gene formed FFL involved in cancers. During the past decades, investigators have developed an accession of procedure to predict, investigate and verify the interaction of the 3 elements of a special FFL circuit. A series

Table I. Examples of common databases for miRNA-TG and TF-TG interactions.

Databases	URL
miRNA target prediction databases	
TargetScan	http://www.targetscan.org/
miRanda	http://www.microrna.org/
PicTar	http://pictar.mdc-berlin.de/
RNA22	https://cm.jefferson.edu/rna22/Interactive/
DIANA	http://diana.imis.athena-innovation.gr/DianaTools/index.php
Databases on validated microRNA targets	
TarBase	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index
miRWalk	http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/mirnapredictedtarget.php
miRBase	http://www.mirbase.org/
Motif search database for TF binding site	
TRANSFAC	http://www.gene-regulation.com/
JASPAR	http://jaspar.genereg.net/
TRED	http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home
DBTSS	http://dbtss.hgc.jp
TRRD	http://wwwmgs.bionet.nsc.ru/mgs/gnw/trrd/

miRNA, microRNA; TG, target gene; TF, trancription factor.

of databases appeared with respectively different algorithms to offer bioinformatic support of predicted connections.

miRNAs repress the translation of target genes by binding, in a Watson-Crick complementary manner, to 7-nt long sequences present at the 3'-untranslated region (3'-UTR) of the regulated genes. The binding usually involves 2-8 nts of the miRNA, known as the 'seed'. The large amount of research associated with the discovery of TF binding sites suggest that transcriptional and post-transcriptional regulatory interactions could be predicted *in silico* by searching over-represented short sequences of nts present in promoters or 3'-UTRs and by filtering the results with suitable evolutionary or functional constraints.

Independent computational evidence for the regulatory interactions of the TF-miRNA-target gene FFL can be extracted from the ECRbase, miRBase, PicTar and TargetScan databases, with relevance to cancer of the TF-miRNA-target gene FFL as deduced from their intersection with the oncomiR and cancer gene census databases. In addition, Gene Ontology enrichment provides detailed information regarding the joint targets of the loop (5).

Establishment of the miRNA-target gene association. Significant progress has been made in computational algorithms for miRNA target prediction during the last decade. Currently, there are databases such as TargetScan, miRanda, PicTar, RNA22 and DIANA for target gene prediction (53-55) (Table I). When miRNA and its potential target gene emerge from bioinformatics, gain- and loss-of-function methods, most commonly transfection, are ready to be applied to evaluate the effect of the miRNA in respective human cell lines. Immunology or molecular biology experiments, such as western blot analysis, quantitative polymerase chain reaction (qPCR), and miRNA and mRNA microarray expression profiling, are also involved to verify the linking dynamic expression changes of the miRNA, or its mimics and its target genes' mRNA and protein. For microarray analysis, the

stand-alone software tool CoExpress (freely available at www.bioinformatics.lu/CoExpress), could be chosen to perform interactive detection of correlated profiles in large expression data sets. Finally, a luciferase reporter assay will confirm associations between the miRNA and its targets (56). Publications of validated miR-target correlations are recorded in TarBase.

Establishment of the TF-target gene association. In an effort to further dissect the molecular pathways regulated by a particular TF, several genome-wide screens have been used to identify its transcriptional targets. The transcription regulation databases include TRANSFAC, JASPAR, TRED, DBTSS and TRRD (57-61). Among these databases mentioned, TRANSFAC is the most commonly used (Table I). First, researchers use bioinformatics to select consensus DNA elements situated in the 5' regulatory region of genes and subsequently they measure TF binding to those sequences *in vivo* by, most commonly, quantitative chromatin immunoprecipitation (ChIP). Other experimental methods include using the luciferase reporter gene, electrophoretic mobility shift assays and DNase footprinting. Recently, as the technology of microarray and new generation sequencing develop, high-throughput methods based on ChIP are emerging. These advanced approaches are ChIP-chip and ChIP-seq, which are expensive but extremely promising. Classic ChIP results from electrophoretic analysis of PCR amplification products, and this method can only observe some specific target genes. However, the emergence of ChIP-chip and ChIP-seq technology has made the observation on the whole genome of protein and DNA combination possible (62-66).

Establishment of the TF-miRNA association. Based on bioinformatic prediction, using a miRNA microarray containing different miRNAs and a set of miRNA qPCR assays to validate the microarray results, the correct miRNAs can be identified that are induced by a special TF *in vitro*, whether upregulated or downregulated with the TF amplification. The TransmiR

database can also be used, which is a TF-miRNA regulatory database built by researchers from Peking University (Beijing China) who manually surveyed ~5,000 reports in the literature and identified 243 TF-miRNA regulatory associations, which were supported experimentally from 86 studies (67).

In 2012, Yan *et al* (68) described a novel method for integrating gene and miRNA expression profiles in cancer using FFLs consisting of TFs, miRNAs and their common target genes. This was the dChip-GemiNi (Gene and miRNA Network-based Integration) method, available at http://www.canevolve.org/dChip-GemiNi/usergemini.php. It statistically ranks computationally predicted TF-miRNA-target gene FFLs by their explanatory power to account for differential gene and miRNA expression between two biological conditions, such as normal and cancer. Compared to existing approaches, GemiNI also computationally derives information regarding TF-target gene and miRNA-mRNA interactions. The integrated modeling of expression data and FFLs better identifies cancer-related TFs and miRNAs.

All the connections of these TF-miRNA-target gene FFLs were based on experimentally validated interactions referenced in the Ingenuity Knowledge Base, based on which the powerful software Ingenuity Pathway Analysis (IPA) was built.

4. Conclusions

As the concepts of transcription network motifs and TF-miRNA-target gene FFLs emerge, our understanding of the molecular deregulatory mechanism of the cancer step delves further into the unknown field. Researchers could study the complicated deregulation system involving numerous elements at a higher level. Associated TF-miRNA-target gene FFLs can be confirmed and collected to form a regulatory network, similar to a jigsaw puzzle. Key nodes may be used therapeutically as a target for drugs or as the drug itself. More experimental analyses *in vivo* and more accurate network constituent data analyses may lead to the discovery of crucial principles of cancer, which indicates curability and hope of overcoming malignancy.

However, since miRNAs can also regulate other non-coding RNAs (for example, long non-coding RNAs), which have a role in cancer development and *vice versa* (69), and TFs are also involved in the regulation of other cancer-associated non-coding RNAs. Each member as a node of a special FFL can be another motifs' indispensable element, which indicates that the overlaying of TF-miRNA-target gene FFLs should put other types of motifs (such as FBL) into consideration. These two elements make the profile of transcription networks more complex and have a larger role than expected, which is currently unknown. New strategies to identify and characterize the entire targets of individual miRNAs and TFs, with an improved high-throughput, and to determine how they function in combination to regulate specific targets, will be required to understand their action on cancer pathology.

Acknowledgements

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