RESEARCH ARTICLE

Systematical Analysis of Cutaneous Squamous Cell Carcinoma Network of microRNAs, Transcription Factors, and Target and Host Genes

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Abstract

Background: MicroRNAs (miRNAs) are small non-coding RNA molecules found in multicellular eukaryotes which are implicated in development of cancer, including cutaneous squamous cell carcinoma (cSCC). Expression is controlled by transcription factors (TFs) that bind to specific DNA sequences, thereby controlling the flow (or transcription) of genetic information from DNA to messenger RNA. Interactions result in biological signal control networks. Materials and Methods: Molecular components involved in cSCC were here assembled at abnormally expressed, related and global levels. Networks at these three levels were constructed with corresponding biological factors in term of interactions between miRNAs and target genes, TFs and miRNAs, and host genes and miRNAs. Up/down regulation or mutation of the factors were considered in the context of the regulation and significant patterns were extracted. Results: Participants of the networks were evaluated based on their expression and regulation of other factors. Sub-networks with two core TFs, TP53 and EIF2C2, as the centers are identified. These share self-adapt feedback regulation in which a mutual restraint exists. Up or down regulation of certain genes and miRNAs are discussed. Some, for example the expression of MMP13, were in line with expectation while others, including FGFR3, need further investigation of their unexpected behavior. Conclusions: The present research suggests that dozens of components, miRNAs, TFs, target genes and host genes included, unite as networks through their regulation to function systematically in human cSCC. Networks built under the currently available sources provide critical signal controlling pathways and frequent patterns. Inappropriate controlling signal flow from abnormal expression of key TFs may push the system into an incontrollable situation and therefore contributes to cSCC development.

Keywords: Cutaneous squamous cell carcinoma - miRNAs - transcription factors - signalling networks

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Introduction

MicroRNA (miRNA) is a small [21-24 nucleotides (nt)] non-coding RNA molecule found in multicellular eukaryotes which functions across various biological processes and is implicated in cancers (Johnson et al., 2005; Lu et al., 2005). It affects the expression of genes at a post-transcriptional level by binding to complementary sequences on target mRNAs, resulting in translational repression or target degradation and genes silencing (Calin et al., 2006). MiRNAs can contribute to cancer development and are abnormally expressed in cancer tissues (Volinia et al., 2006). MicroRNAs circulate in the bloodstream in a highly stable, extracellular form and are being developed as blood-based biomarkers for cancer and other diseases (Arroyo et al., 2011). MiRNA signature from the serum may serve as a noninvasive predictor for the overall cancer survival. Study reveals that miRNA act as oncomiR in cancer and may be a potential target in cancer therapy. Emerging evidence suggests that miRNAs affect the responsiveness of cells to signaling molecules such as transforming growth factor-beta, WNt, Notch and epidermal growth factor (Inui et al., 2010). Circulating miRNAs are thought to be promising application for diagnosing human cancers (Qu et al., 2011).

MiRNA expression is controlled by transcription factor (TF), a protein that binds to specific DNA sequences, thereby controlling the flow (or transcription) of genetic information from DNA to messenger RNA (Karin, 1990; Latchman, 1997). TF is capable of activating gene transcription independently or through indirect medium (Polakis, 2000). Regulation on the expression of gene at transcriptional level enables TF to exert an effect on malignant cell transformation (Libermann et al., 2006). Various genes were identified as key transcription factors which were frequently implicated in the development of cancers.

Certain miRNAs locate themsevles inside genes.

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Those genes are host genes of the miRNAs bounding to them. MiRNAs are transcribed in parallel with their host genes. Two types of transcriptions (exonic and intronic) were identified which indicate that miRNAs may require slightly different mechanisms of biogenesis (Rodriguez et al., 2004). Also the intronic miRNA and its host gene have a closer association than that of exonic miRNA and its corresponding host genes (Baskerville et al., 2005). Usually Intronic miRNA and its host gene are coordinately expressed and work together to conduct biological functions and affect the alteration of signaling pathways (Cao et al., 2010). The abnormal expression of host genes could contribute to the progression of cancer (Poliseno et al., 2010; Das et al., 2012).

Cutaneous squamous cell carcinoma (cSCC) is the second-most common cancer of the skin. It is a malignant tumor of keratinocytes that tends to metastasize and leads to mortality (Xia et al., 2013). Co-regulation between miRNAs and TFs connect isolated regulatory relations. Computational predicted methods and experimentally validated databases provide associations between miRNA and targets, TF and miRNA, miRNA and host gene. Genes and miRNAs involved in cutaneous squamous cell carcinoma are collected and divieded into three levels of abnormally expressed, related and global. Abnormally expression is commonly observed on genes and miRNAs in tumor tissues presented as mutation or dysregulation. Its contribution to the carcinogenesis enhances the value of the network constructed with abnormally expressed genes and miRNAs (Abdel-Rahman et al., 2012; Chaudhry et al., 2012). Both abnormally expressed and related molecular components are found contributing to cSCC development while only the former is reported mutated or dysregulated. The global ones can be traced through interactions between them and the abnormally expressed or related factors but they have not been noticed functioning in cSCC.

Increasingly, factors are found implicated in cancers concerning their aberrant expression or therapeutic intervention (Moad et al., 2012; Zheng, 2013). However they were mostly inspceted in isolation while new insights on complicated gene regulated network may light on novel strategies for the diagnosis, therapy and prognosis (Xie, 2013).

Present study conducts network quest on TFs, miRNAs, target genes and host genes involved in cSCC to investigate their comprehensive interactions. The up/down regulations or mutations of abnormally expressed genes and miRNAs are discussed within the environment in which they interact with others. Networks at three levels are constructed and significant patterns are extracted to uncover possible signal controlling models in cSCC which may contribute to the understanding of the cSCC pathogenesis and therapy development.

Materials and Methods

Genes and miRNAs

The genes and miRNAs at abnormally expressed and related levels are manully extracted from literatures. Abnormally expressed genes and miRNAs were experimentally validated mutated or up/down regulated in cSCC compared to normal tissues. Related genes and miRNAs were reported funtioning during cSCC development but abnormally expression is not necessary.

Experimentally validated interactions between genes and miRNAs

MicroRNA can bind to one or more target sites to negatively regulate protein expression, subsequently controlling cellular activities. Collection of miRNA-target interactionswith experimental support is essential to thoroughly elucidating miRNA functions. The interactions were extracted from Tarbase 5.0 and miRTarBase. Tarbase 5.0 provides a collection of all experimentally tested miRNA targets describing each supported target site by the miRNA that binds it, the gene in which it occurs, the location within the 3' UTR where it occurs, the nature of the experiments that were conducted to validate it, and the sufficiency of the site to induce translational repression and/or cleavage (Sethupathy et al., 2006). The interactions in miRTarBase are validated experimentally by reporter assays, western blot, or microarray experiments with overexpression or knockdown of miRNAs (Hsu et al., 2011). With only entries of homo sapiens accepted, totally 6749 interactions between 426 miRNAs and 2029 genes were acquired.

The expression of miRNAs can be activated or repressed by transcription factors at the transcriptional level which therefore TFs can serve as upstream regulators of miRNA. A dataset of TF-miRNA regulating relations was achieved from the manually built database TransmiR which came from manually surveyed approximately 5000 reports in the literature experimentally support from 86 publications (Wang et al., 2010). Altogether 863 regulating relations between 220 miRNAs and 153 genes were included.

Research suggested that miRNAs are transcribed in parallel with their host transcripts. A list consisting of host genes with respective miRNAs was made based on miRBase (Kozomara et al., 2011) and NCBI available at http://www.ncbi.nlm.nih.gov/. There are totally 1137 host genes of 1209 miRNAs as 1419 entries.

Construction of networks at three levels

The present study applied a series of pre-designed procedures to extend the interactions between TFs, miRNAs, target genes and host genes. Interactions between miRNAs and target genes, TFs and miRNAs, host genes and miRNAs are described as follows.

U_1=[(M, G_T) | M targets G_T]

U_2=[(G_{TF}, M) | G_{TF} targets M]

U_3=[(G_H, M) | G_H is the host gene of M]

M refers to miRNAs. G_T represents the target gene of corresponding miRNA. G_{TF} is the transcription factor regulating miRNA. G_H is miRNA's host gene. Three datasets respectively display the ways in which miRNAs and genes interact with each other. These structures arrange the participants into pairs marked by interaction types. With the network searching procedures the original connections limited to direct interactions were extended to larger domains representing the complex of controlling signal pathways.

The abnormally expressed network is constructed with abnormally expressed miRNAs, abnormally expressed genes and miRNAs' host genes across three types of interactions with mutations and dysregulations taken into consideration. The related network is the extension of the abnormally expressed one with related genes and miRNAs included.

Results

Abnormally expressed MiRNAs in cSCC

Over the past several years it has become clear that alterations in the expression of microRNA (miRNA) genes contribute to the pathogenesis of most - if not all - human malignancies. These alterations can be caused by various mechanisms, including deletions, amplifications or mutations involving miRNA loci, epigenetic silencing or the dysregulation of transcription factors that target specific miRNAs (Croce, 2009). MiR-34a expression increases with keratinocyte differentiation, while it is suppressed in skin and oral SCCs, SCC cell lines, and aberrantly differentiating primary human keratinocytes (Lefort et al., 2013). MiRNA expression profiles detected by microarray miRNA expression profiling with a non-adjusted p≤0.01 revealed three up-regulated (hsa-miR-135b, hsa-miR-424 and hsa-miR-766) and six down-regulated (hsa-miR-30a*, hsa-miR-378, hsa-miR-145, hsa-miR-140-3p, hsa-miR-30a and hsa-miR-26a) miRNAs in cSCC (Sand et al., 2012). Hsa-miR-21 and hsa-miR-31 were identified significantly up-regulated. Up-regulation of hsa-miR-205 was newly found as well (Bruegger et al., 2013). Comparing cSCC with healthy skin, miR-31, miR-135b, miR-21, and miR-223 were identified up-regulated in cSCC cancerous tissue. On the contrary, hsa-miR-375, hsa-miR-125a, hsa-miR-125b, hsa-let-7a, hsa-let-7b, hsa-let-7c, hsa-let-7d, hsa-let-7f, hsa-let-7g, hsa-miR-99a, hsa-miR-99b, hsa-mir-100, hsa-mir-143 and hsa-mir-101 were revealed significantly down-regulated (Bruegger et al., 2013).

Abnormally expressed genes in cSCC

Down-regulated genes. Real-time RT-PCR confirmed that FOXN1 and FGFR3 were suppressed in SCCs (Mandinova et al., 2009). Analysis of IRF6 expression in a large series of SCCs showed a strong down-regulation that correlated with tumor invasive and differentiation status (Botti et al., 2011). RPL15 and LGTN were down-regulated and the expression of p15(INK4b) was significantly reduced (Dang et al., 2006; Moad et al., 2009). The expression of KGF receptor (KGFR) mRNA was lower in cutaneous SCCs than in normal skin samples (Toriseva et al., 2012). Analysis by quantitative reverse transcription-PCR revealed that PKC-delta RNA was reduced an average of 90% in the SCCs tested, consistent with PKC-delta down-regulation at the protein level (Yadav et al., 2010). Loss of CDKN2A were found in SCCs (Hameetman et al., 2013). Researches imply that an important component of the early stages in squamous carcinoma progression may be a modest decrease in RalA gene expression that magnifies the effects of decreased E-cadherin expression by promoting its degradation (Sowalsky et al., 2010). Several laboratories reported altered I kappa B kinase alpha (IKK alpha) protein localization, downregulated IKK alpha, and IKK alpha gene deletions and mutations in human SCCs of the skin, lung, esophagus, and neck and head (Park et al., 2011).

Up-regulated genes. While the normal epidermis showed a negative to weak-positive expression of MUC4, its expression was significantly upregulated in SCCs where the intensity of staining correlated negatively with tumour grade and positively with age (Chakraborty et al., 2010). CD200 gene and message were upregulated in SCC stroma (Belkin et al., 2013). E2F1 and E2F7 were both found overexpressed in cSCCs compared with normal epidermis (Belkin et al., 2013). With detection by quantitative real-time reverse transcriptase polymerase chain reaction, DGCR8, AGO1, AGO2, PACT, and TARBP1 expression levels were found significantly higher in the SCC than the healthy controls (Sand et al., 2012). Several genes involving the TP53 pathway, anti-apoptotic pathways, signal transduction, structural loss and DNA replication, including BCL2A1, MUC4, PTPN11 (SHP2) and FGF9, were confirmed upregulated in SCC and suggested to be warranted further study regarding their role in disease pathogenesis (Kathpalia et al., 2006). CNN2, COX4l1, COX5B, COX7C, CRLF3, CTSC showed increased expression (Dang et al., 2006). In cSCC.Matrix metallopeptidase 13 (MMP13) was identified as a direct target suppressed by miR-125b (Xu et al., 2012). Keratin 6 (KRT6), KRT16 and KRT17 are upregulated in SCCs (Hameetman et al., 2013). Increased

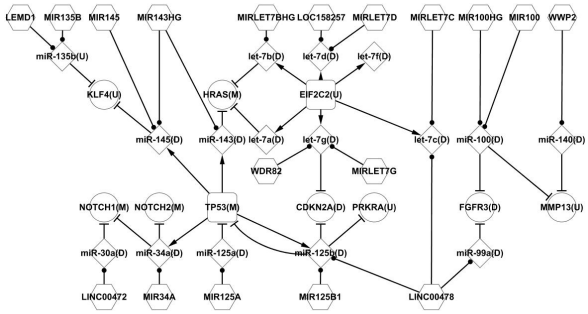

Figure 1. Abnormally Expressed Network of TFs, miRNAs, Target Genes and Host Genes Involved in cSCC

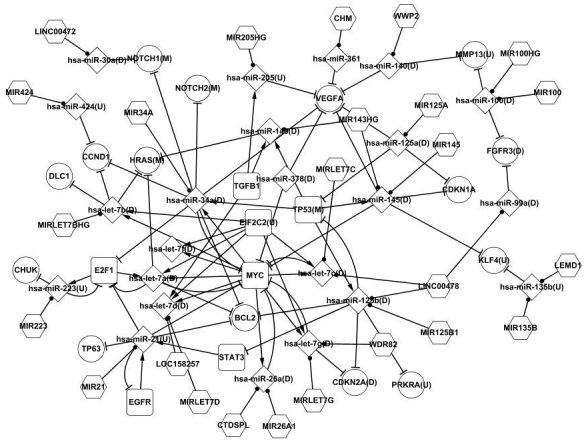

Figure 2. Reltated Network Extending the Abnormally Expressed Network with components at Related Level

Table 1. MiRNAs Targeting and Regulated by TP53 at Three Levels

miRNA			Gene	miRNA		
Global	Related	Abnormally expressed		Abnormally expressed	Related	Global
hsa-miR-125a	hsa-miR-125a	hsa-miR-125a	TP53	hsa-miR-125b	hsa-miR-125b	hsa-miR-200c
hsa-miR-125b	hsa-miR-125b	hsa-miR-125b		hsa-miR-143	hsa-miR-143	hsa-miR-107
hsa-miR-1285				hsa-miR-145	hsa-miR-145	hsa-miR-125b
hsa-miR-15a				hsa-miR-34a	hsa-miR-34a	hsa-miR-143
hsa-miR-16						hsa-miR-145
hsa-miR-221						hsa-miR-155
hsa-miR-222						hsa-miR-192
hsa-miR-25						hsa-miR-194
hsa-miR-30d						hsa-miR-200a
hsa-miR-612						hsa-miR-200b
						hsa-miR-200c
						hsa-miR-215
						hsa-miR-29
						hsa-miR-29b-1
						hsa-miR-29b-2
						hsa-miR-29c
						hsa-miR-34
						hsa-miR-34a
						hsa-miR-34b
						hsa-miR-34c
						hsa-miR-519d

Table 2. The Genes Regulating or Targeted by miR-125b at Three Levels

Gene			miRNA		Gene	
Global	Related	Abnormally expressed		Abnormally expressed	Related	Global
AKT1	TP53	TP53	hsa-miR-125b	CDKN2A	BCL2	ABCC4
CDX2				PRKRA	CDKN2A	AKT1
NFKB1				TP53	PRKRA	ATXN1
STAT4					STAT3	BAK1
TP53					TP53	BBC3
						BCL2
						...

expression of KLF4 protein and mRNA were found in squamous cell carcinoma cell line studies and fresh skin tissue respectively, using western blotting and semi-quantitative real time polymerase chain reaction (Chen et al., 2008). Overexpression of epidermal growth factor receptor (EGFR) has been shown in SCC (Yin et al., 2013). TCTP was demonstrated overexpressed in cSCC cells, compared to normal skin keratinocytes (Wu et al., 2012).

Mutated genes. Increased number of Tp53 mutations were in the squamous cell carcinoma samples compared with perilesional or control samples (Boukamp et al., 2005; Loeb et al., 2012). More than half of all SCCs contain TP53 tumor suppressor gene mutations; such TP53 gene alterations are also common in the AK and may work synergistically with ultraviolet light to account for its malignant character (Butani et al., 2005). NOTCH1 or NOTCH2 mutations were identified in similar to 75% of cutaneous SCCs (Wang et al., 2011).From the three ras genes, Harvey-(Ha), Kirsten-(Ki) and N-ras, mutations in Ha-ras predominate in the general population with the mutations characteristically seen at codons 12, 13 and 61-all localized opposing UV-sensitive CC sites (Boukamp et al., 2005).TERT promoter mutations with UV-signatures are frequent in non-melanoma skin cancer, being present in around 50% of basal and squamous cell carcinomas(Griewank et al., 2013).

Abnormally expressed network

The abnormally expressed network consists of 28 genes including two TFs, eight target genes and eighteen host genes and sixteen miRNAs. Two TFs regulate ten miRNAs through ten regulating relations presented. Thirteen miRNAs target nine genes through seventeen targeting relations.

TFs are presented as round rects, miRNAs are diamonds, target genes are eclipses and host genes are hexagons. Delta arrow shows a TF regulating a miRNA. Arrow in T shape gives a targeting relation between a miRNA and a target gene. Circle arrow displays a gene's hosting a miRNA. Labels marked (D) indicates the down-regulation and (U) is the up-regulation. Mutated factors are annotated with (M).

Related network

The related network is built with seven TFs, 20 target genes, 27 miRNAs and 30 host genes. Its visual criterion including types of interactions, components identities and expressions is similar to that of the abnormally expressed network. Nodes without mutated or dysregulated label represent genes and miRNAs not been found abnormally expressed in cSCC.

Global network

The global network is the summation of the three experimentally validated interactions between genes and miRNAs. It provides a comprehensive data source for investigation on molecular components' interactive regulations in diseases including malignant tumors.

Discussion

The abnormally expressed network is built with genes and miRNAs observed mutated or dysregulated in cSCC and it is centralized by TP53 and EIF2C2.

The extensively studied tumor-suppressor gene, TP53, is frequently mutated in human cancers (Soussi and Beroud, 2001; Petitjean et al., 2007). A large amount of data is available on the functional impact of missense mutations in TP53 and on mutation patterns in many different cancers (Petitjean et al., 2007). Researchers suggest that immunohistochemical analysis of TP53 protein expression and fluorescent in situ hybridization of TP53 gene could be applied as screening tool for microinvasion of oral squamous cell carcinoma (Heah et al., 2011). In the abnormally expressed network TP53 regulates four miRNAs (hsa-miR-34a, hsa-miR-125b, hsa-miR-143 and hsa-miR-145). Hsa-miR-34a targets NOTCH1 and NOTCH2. Hsa-miR-143 targets HRAS. Hsa-miR-145 targets KLF4. An uncommon interacting regulation exists between TP53 and hsa-miR-125b in which TP53 regulates hsa-miR-125b and hsa-miR-125b targets TP53 simultaneously.

The involvement of miR-125 family is diffusely uncovered in lung, breast and lymph cancer. MiR-125 can increase the survival of immature hematopoietic cell populations (Raver-Shapira et al., 2007). MiR-125b is an important negative regulator of TP53 and TP53-induced apoptosis during development and during the stress response (Le et al., 2009). TP53 and hsa-miR-125b bond together into a balance with mutual restraints in which change from either side influences each other. MiR-125b was implicated in differentiation, cell proliferation, and mobility (Nam et al., 2008). Breast cancer primary tumors and cell lines show evidence of a decreased level of miR-125b expression, suggesting that lack of miR-125 may impair differentiation capabilities of cancer cells (Iorio et al., 2005). Minh T. N. Le et al propose that miR-125b buffers and fine-tunes TP53 network activity by regulating the dose of both proliferative and apoptotic regulators, with implications for tissue stem cell homeostasis and oncogenesis (Le et al., 2011). Also there was an inverse relationship between the expression of miR-125b and MMP13 Knockdown of MMP13 expression phenocopied the effects of miR-125b overexpression. These findings provide a novel molecular mechanism by which MMP13 is up-regulated in cSCCs and indicate that miR-125b plays a tumor suppressive role in cSCC (Xu et al., 2012).

All five miRNAs (miR-125a, miR-125b, miR-143, miR-145 and miR-34a) targeting or regulated by TP53 found involved in cSCC are validated abnormally expressed.

STAT3 and BCL2 are direct targets of miR-125b without being identified abnormally expressed.

MiR-34 family, the transcription of which is actived by TP53, regulates cell cycle progression, cellular senescence and apoptosis. Based on previous observation, it is proposed that a positive feedback loop, in which TP53 induces expression of miR-34a which suppresses SIRT1, increasing TP53 activity (Yamakuchi et al., 2009). MiR-34a is a direct proapoptotic transcriptional target of TP53 that can mediate some of TP53's biological effects. Its expression some human cancers was inferred may contribute to tumorigenesis by attenuating TP53-dependent apoptosis (Raver-Shapira et al., 2007). Mir-34a targets NOTCH1 and NOTCH2, the pathway of which can act in a tumor-suppressive or tumor-promoting fashion, depending on cellular context and whether cooperating (proto)-oncogenes are present (Huber et al., 2005). Summarized findings show that miR-34a suppresses brain tumor growth by targeting c-Met and Notch and suggest that miR-34a could serve as a potential therapeutic agent for brain tumors (Li et al., 2009). NOTCH1 is also targeted by down-regulated miR-30 which is demonstrated protecting podocytes by targeting NOTCH1 and TP53 and the loss of it facilitates podocyte injury (Wu et al., 2014).

MiR-143 and miR-145 are frequently implicated in cancers. They consistently display reduced steady-state levels of the mature miRNA at the adenomatous and cancer stages of colorectal neoplasia (Michael et al., 2003). TP53 enhances the post-transcriptional maturation of miR-143 and miR-145 with growth-suppressive function in response to DNA damage (Suzuki et al., 2009).In the network miR-145 and miR-135b jointly target KLF4. Mir-143 targets HRAS together with let-7a.

All the four miRNAs regulated by TP53 are identified down-regulated in cSCC which may be due to the mutation of TP53. Study pointed out that miR-342-3p tends to have significantly lower expression in the TP53 mutant breast tumors (Enerly et al., 2011). KLF4 is up-regulated with two oppositely expressed precursors. It shows that the down-regulation of miR-145 overweighs the up-regulation of miR-135b. HRAS, NOTCH1 and NOTCH2 are observed mutated in cancer tissues. KLF4 and PRKRA are up-regulated while CDKN2A is down-regulated. The up-regulation of PRKRA lines with expectations because of miR-125b's down-regulation. CDKN2A is down-regulated even with the down-regulation of both hsa-miR-125b and hsa-let-7g which act as controlling miRNAs of CDKN2A. It may suggests additional factors under cover which influence the expression of CDKN2A. Similar case occurs on down-regulated FGFR3 which is targeted by miR-99a and miR-100.For the mutated genes, NOTCH1 is targeted by the hsa-miR-30a and HRAS is controlled by extra factors of down-regulated hsa-let-7a and hsa-miR-7b. All these three miRNAs were identified down-regulated in cSCC. Down-regulated hsa-miR-125a targets TP53 but unlike hsa-miR-125b it is not found regulated by TP53.

The expression levels of EIF2C2 in cSCC were observed significantly higher than the healthy controls in the research. Besides in cSSC, EIF2C2 is amplified and overexpressed in head and neck SCC cell lines and primary tumors (Chang et al., 2010). In abnormally expressed network EIF2C2 is not targeted by any abnormally expressed miRNAs. EIF2C2 regulates six miRNAs (7a/b/c/d/f/g) from hsa-let-7 family. Ethal-7 (let-7), a founding member of the miRNA family, is required for timing of cell fate determination in C. elegans. In humans, various let-7 genes have been reported to map to regions deleted in human cancers and let-7 miRNAs are suggested to be tumor suppressors (Johnson et al., 2005).

In cSCC all the six involved miRNAs from let-7 family are down-regulated. This observational result is in consonance with logical inference because of EIF2C2's suppressing effect on the miRNAs as their regulator. Hsa-let-7a and hsa-miR-7b target HRAS together with hsa-miR-143 regulated by TP53. Hsa-let-7g targets CDKN2A together with miR-125b.The two sub-networks with TP53 and EIF2C2 as roots respectively hold controlling signal pathways ending at HRAS and CDKN2A.

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