Published in final edited form as:
Trends Mol Med. 2014 January ; 20(1): . doi:10.1016/j.molmed.2013.10.005.

Smoking and microRNA dysregulation: a cancerous combination

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Abstract

MicroRNAs (miRNAs) are post-transcriptional gene regulators that are differentially expressed in several patho-physiological conditions including cancer. They impact the disease course by modulating an array of putative target gene(s). Interestingly, there is a strong correlation between the various miRNAs target(s) and the smoking-regulated genes in cancer. This review article provides an insight into the current status of smoking-induced miRNAs and their genetic/epigenetic regulation in smoking associated cancers, with a major focus on lung cancer (LC). Further, it discusses the role of miRNAs in smoking-mediated oncogenic events in cancer and explores the diagnostic/prognostic potential of miRNA-based biomarkers and their efficacy as therapeutic targets.

Keywords

miRNA; smoking; cancer; epigenetics; signaling; diagnostic

MicroRNAs: the micro steering wheel of cancer

In the traditional “central dogma of genetics”, DNA codes for RNA and RNA codes for protein. With the advent of new genetic tools, scientists observed that a small proportion of RNA (1–3%) do not code for proteins, but regulate protein expression at the post-transcriptional level. These regulatory RNA molecules are merely 22 nucleotides in length, and therefore, termed microRNAs (miRNAs). The miRNAs regulate gene expression either by translational inhibition or by degradation of target mRNAs. The miRNAs are coded by their own gene families. To date, ~1600 distinct miRNA genes have been discovered in the

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human genome. A number of computational algorithms for miRNA target prediction have revealed that each miRNA family can target more than 200 different mRNAs [1,2]. Accordingly, it can be speculated that most cellular processes are regulated by these small non-coding RNAs.

Expression patterns of miRNAs are altered in several disease conditions including cancer [3]. The miRNAs influence cancer in two ways: by regulating the expression of protein-coding oncogenes and tumor suppressors, and by themselves acting as oncogenes (referred to as oncomirs) and tumor suppressors (referred to as mirsupps). The diversity of environmental mutagens, including UV light, common air pollutants, diesel particles, cigarette smoke (CS) and other complex organic mixtures modulate miRNA expression. In particular, the effects of CS on miRNA expression and their bio-regulatory machinery are of great scientific interest [4]. Cigarette-smoking is an important risk factor for multiple human pathologies [5,6]; among them lung cancer (LC) takes the lead with smokers having much higher risk than non-smokers [7]. We are now beginning to understand the role of smoke-induced altered miRNA expression in cancer. The majority of miRNA studies in smoking-related malignancies are focused on LC. Herein, we will review current knowledge about the involvement of miRNAs in smoke-induced cancers including LC, cervical, pancreatic, and squamous cell carcinoma with major emphasis on LC. Further, we will discuss the enigmatic potential of miRNAs as diagnostic, prognostic, and therapeutic target(s).

MicroRNAs at a glance: biogenesis and processing

MicroRNAs are transcribed as 5′-capped and 3′ polyadenylated transcripts (pri-miRNA) by RNA polymerase (Pol) II. In the nucleus, the processing of the pri-miRNA is carried out with the help of Ribonuclease (RNAse) III enzymes Drosha/Pasha to form pre-miRNA [8], followed by exportin-5-mediated transport from the nucleus to the cytoplasm [9]. Subsequently, the pre-miRNA is processed by a ‘Dicer’ (RNAse III family member) to form the mature ~22-nt miRNA duplex. One of the miRNA strands of the processed duplex is loaded onto the RNA-induced silencing complex (RISC), while its complementary strand undergoes degradation [10]. The core protein of RISC is the argonaute protein (Ago), of which four closely related members exist in humans [8]. Other RISC components are protein activators of the interferon-induced protein kinase (PACT) and human immunodeficiency virus transactivating response RNA binding protein (TRBP). In mammals, nucleotides 2 to 8 of the miRNA 5′-end constitute a “seed region” that binds imperfectly to a mRNA complementary recognition sequence at the 3′ UTR [1], whereas, in a few instances, they show complete complementarity to the target mRNA sequence. Depending on the base pairing between the miRNA and mRNA, a different mode of target mRNA decay is employed. Figure 1 provides a detailedschematic representation.

MicroRNAs implicated in cancer

Deregulation of miRNA expression was first shown in chronic lymphocytic leukemia [11]. Subsequently, in 2006, miRNA profile analysis revealed varied genetic network in normal/healthy and cancer patients [12]. While a normal miRNA genome was represented by a single organized miRNA network, in adenocarcinoma, networks mostly appeared to be built by several unconnected sub-networks, targeting multiple genes involved in cancer-related pathways [12].

Lung cancer—MiRNAs regulate multiple target genes, and as a consequence, they are involved in a myriad of pathobiological processes in LC (Figure 2). Their targets include a number of oncogenes (*Ras* family, *Myc* family, *PIK3CA*, *NKX2-1* and *ALK*), tumor suppressor genes (TSGs: *TP53*, *RB1*, *CDKN2)*, cell cycle-related genes (*Skp-2*, Cyclin family, *Cdk* family), anti-apoptotic genes (*BCL-2* family, *MCL1*, *XIAP*), angiogenic factors

(*VEGF* family, *VEGFR* family), metastasis-related genes (*E-cadherin*, *COX2*, integrin-α5, *HOX10b*), autocrine/paracrine loops (IGF axes), and *MMP* family and chemo-resistance genes (*FGF* family, *GSTP1*(glutathione S-transferase pi gene)). Furthermore, some tumor associated transcription factors (TFs) can regulate miRNA processing machinery and individual miRNA expression; for example, *STAT3* regulates mir-21, *TWIST1* trans-activates mir-10b, *MYC* is a negative regulator of let-7 and mir17–92 cluster, and *TP53* regulates the expression of mir-34 family members [13].

Pancreatic cancer—Various miRNAs exhibit differential expression during development of pancreatic cancer (PC). A progressive increase of mir-196a is observed from pancreatic intraepithelial neoplasia 1b (PanIN-1b, the precursor lesion of PC) (0%) to PanIN-2 lesions (20%) and to more advanced and aggressive PanIN-3 lesions (60%) [14]. On the other hand, mir-217 expression levels exhibited the opposite trend [14]. During malignant transformation in the transgenic KRAS(G12D) mouse model, downregulation of mir-148a/b, mir-375 and an upregulation of mir-10, mir-21, mir-100 and mir-155 expression was observed in premalignant lesions and invasive PC compared to the normal tissues [15].

MiRNAs directly impact epithelial-mesenchymal transition (EMT) in PC as modulation of mir-200a and mir-30 family members resulted in alterations in EMT-associated TFs *ZEB1*, *ZEB2*, vimentin, *SNAIL*, *SLUG*, and *TWIST* [16,17]. Additionally, mir-34a expression is associated with apoptosis, while mir-421, mir-301a, and mir-21 confer a proliferative advantage in PC *via* targeting various putative oncogenic target genes including *NFκB*, *SMAD4* and *PDCD4* [18,19]. The mir-142-5p is a promising predictive marker in resected PC patients for gemcitabine response [20]. Likewise, PC patients with lower levels of mir-10b expression associate with better response to therapy and better survival [21].

Cervical cancer—Human papillomavirus (HPV) infection corresponds to about half of all cervical cancer (CC) incidence. HPV encodes three oncogenes, E5, E6, and E7, out of which viral oncoproteins E6 and E7 modulates the expression of several miRNAs including mir-15/16 clusters, mir-203, mir-mir-34a, and mir-218 through E6-*p53* and E7-*pRb* pathways [22]. In addition, E6-mediated mir-34a [23] and mir-218 inhibition [24] is an early-onset event in the development of CC. Gain of copy-number of the chromosomal 5p region results in an overexpression of Drosha in CC and in turn, a significant upregulation of several cancer-associated miRNAs that have the potential to regulate several protein-coding genes important for CC development, with mir-31 showing the maximum alteration [25]. Among others of note, mir-372 exerts its anti-oncogenic role through the downregulation of the cell cycle genes *CDK2* and *cyclinA1* [26], whereas, mir-886-5p down-regulates the expression of the pro-apoptotic gene Bax [27].

Head and Neck squamous cell carcinoma (HNSCC)—In addition to CC, HPV status also affects the miRNA expression patterns in head and neck squamous cell carcinoma (HNSCC) [28]. Quite similarly to CC, certain miRNAs (mir-15a/mir-16/mir-195/mir-497 family, mir-143/mir-145 and the mir-106-363) are specifically detected in HPV-positive HNSCC cases [28]. Additionally, significant upregulation of mir-21 and mir-26b is observed in plasma and tissue samples of HNSCC patients compared to the healthy subjects [29]. Circulating levels of these miRNAs are reduced in post-operative cases with good prognosis, whereas, these levels remain high in expired post-operative cases. These results suggest the potential of these miRNAs as prognostic markers for HNSCC. MiRNAs may also have a survival benefit; for example, enhanced chemoresistance (mir-21 activated via *STAT3/NANOG*-signaling CD44-activated HNSCC cell) and angiogenesis (mir-34a by modulating the *E2F3*, *survivin* and *VEGF* levels) of HNSCC cells [30,31]. Downregulation of various tumor suppressive miRNAs (mir-99, mir-1, mir-135b, mir-107) leads to aggravation of

HNSCC by targeting several genes including *TAGLN2*, *HIFα*, protein kinase Cε, *moesin*, and *laminin-332* [32–36].

MicroRNAs and epigenetics

The analysis of miRNA genomic sequences associates most of them with CpG islands, which suggests epigenetic regulation of these miRNAs genes [37] (Figure 3a). A recent study has mapped fifty-five epigenetically silenced miRNAs in non-small-cell lung cancer (NSCLC) [38]. In addition, some transcription factors (TFs) regulate miRNA expression by recruiting epigenetic factors to their promoter regions. For example, *AML1/ETO* fusion oncoprotein binding to the pre-mir-223 region epigenetically silences the expression of the mature miRNA through the recruitment of various chromatin remodeling enzymes [39].

Hypermethylation can silence tumor suppressor miRNAs, whereas DNA hypomethylation causes upregulation of putative oncogenic miRNAs. Hypermethylation of mir-148a, mir-34b/c, and mir-9 has been reported in cell lines derived from lymph node metastases [40]; these miRNAs suppress metastasis by targeting *c-MYC*, *CDK6*, *E2F3*, and *TGIF2* transcripts. Studies have reported promoter hypermethylation-mediated silencing of miRNAs in different cancers, including in acute lymphoblastic leukemia, breast cancer, colon cancer and prostate cancer [41] (Figure 3a). Conversely, hypomethylation and overexpression of oncogenic mir-200a and mir-200b was demonstrated in PCleading to the downregulation of its downstream target genes like *ZEB2*, and retention of *E-cadherin* expression [42]. Methylation of mir-127 and mir-124a genes affects the expression of two oncogenic proteins, *BCL6* and *CDK6*, respectively, which are not methylated in normal tissues [43].

Investigations have revealed that certain miRNAs (epi-miRNAs) themselves counteract CpG methylation, and regulate the components of epigenetic machinery, creating a tightly controlled feedback mechanism. For example, the silencing of family of miRNAs (mir-29a, mir-29b and mir-29c) during lung cancer development, leads to the upregulation of its target genes such as *DNMT3A* and *DNMT3B*. Further, ectopic expression of mir-29-mediates direct repression of *DNMT3A* and *DNMT3B* transcripts in LC cell lines, leading to the activation of TSGs such as *FHIT* and *WWOX* [44]. Another known epigenetically modulated miRNA, mir-148, has been shown to directly target *DNMT3B* [45].

Histone modification is another epigenetic mechanism that can affect miRNA expression as shown in breast cancer cells [46]. *NFκB p50-C/EBPβ* repressor complex has been demonstrated to bind to the let-7i promoter and promote histone-H3 deacetylation, leading to silencing of its expression [47]. The repression of *HDAC1* is directly mediated by mir-449a [48], whereas *HDAC4* expression is indirectly regulated (suppressed) by epi-miRNAs such as mir-1 and mir-140 by modulating the histone acetylation enzyme expression [49]. Not only miRNAs, but also Dicer RNase III family nucleases, known for miRNA processing and maturation, have been validated for the maintenance of DNA methylation at H4K20me3- and H3K9me3-enriched heterochromatin [50]. In Dicer−/− cells, the mir-290 family miRNAs fail to mature, thereby abrogating the retinoblastoma-like-2/E2F-mediated transcription of active DNMTs, *DNMT1*, DNMT3A and DNMT3B[51].

Smoking and lung cancer

The airway epithelium constitutes an essential tissue barrier protecting the lung from inhaled environmental challenges. CS, containing more than 60 mutagens, is known to substantially compromise airway function by increasing bronchial epithelial permeability [52]. Moreover, mutagens can bind and modify DNA, marking the lung cancer genome with characteristic mutations [53]. In bronchial epithelial cells, miRNAs are proven to modulate various

smoke-induced genes [4]. miRNA alterations in a CS-exposed *in vitro* model of human bronchial epithelial cells are demonstrated to closely resemble those in the bronchial epithelium of human smokers [54]. These discoveries of smoking-mediated miRNA deregulation in cancer have provided a new paradigm of smoking-mediated regulatory mechanisms in cancer.

Smoking-modulated miRNAs

Nicotine dependence is a major cause of CS-mediated pathogenesis. Significant progress has been made in identifying susceptible genes for nicotine dependence. The mir-504 is known to increase the dopamine receptor DRD1 gene expression that is associated with tobacco dependence. This demonstrate that miRNA variations directly influence the risk of developing tobacco addiction [55].

Comparison of miRNA expression profiles among LC patients with or without smoking history revealed that two miRNAs, mir-138 and let-7c, are significantly downregulated in nonsmokers with LC. On the other hand, LC patients with a smoking history showed significant alterations in 36 miRNAs; including upregulation in mir-210, mir-191, mir-155, mir-128, mir-129, mir-148a, mir-7, mir-17, mir-102, mir-20a, mir-146, mir-200a, mir-106, mir-99b, mir-199a, mir-9, mir-214, mir-136, mir-24 and mir-142, and downregulation in mir-30a, mir-145, mir-30d, mir-218, mir-9, mir-29b, mir-204, mir-30b, mir-125a, mir-224, mir-124, mir-96, mir-208, mir-193b, mir-223 and mir-188 [56]. Further, several histologic types of NSCLC have a chromosome inversion-induced *EML4-ALK* fusion gene. It is observed to be associated with non-smokers and occurs in mutual exclusion to *EGFR* and *KRAS* mutations [57].

The miRNA genes are known to be recurrently located at fragile sites (FRAs) as well as in minimal regions of amplification/loss of heterozygosity and common breakpoint regions [58]. While mir-21 and mir-205 are located in the genetic region that is amplified in LC, mir-126 is in the region deleted in LC [59]. Mir-21 also happens to be located at the fragile site FRA17B while mir-27b is located at FRA9D and mir-32 at FRA9E [59]. The miRNAs downregulated by CS exposure, especially in the early period, are mainly tumor suppressor miRNAs. *Cyclin F*, a direct target of let-7, is significantly upregulated by CS [60]. CS smoke may induce mutations like single nucleotide polymorphisms (SNPs) in miRNA genes, leading to their downregulation. Also, SNPs are known to block the miRNA biosynthesis [61]. Thus these polymorphisms may in turn render high susceptibility in miRNAs towards CS. It is anticipated that SNPs within miRNA target sites will emerge as a gold mine for molecular epidemiology. CS-induced, differentially expressed miRNAs bind to 3′UTR regions of a number of genes and regulate their expression, suggesting that these miRNAs may play a pivotal role in the pathogenesis of smoking-related diseases (Table 1).

MicroRNAs as prognostic/diagnostic tools

Tumor profiling studies in LC increasingly demonstrate the existence of unique miRNA signatures that can distinguish healthy controls from LC cases (80.6% sensitivity and 91.7% specificity), as well as the types and subtypes of LC [62]. Such differential miRNA expression patterns exist not only in tumors, but also in plasma, serum, sputum and exosome samples of patients; therefore, these could well serve for minimally invasive screening and as triage tools for further diagnostic evaluations [63].

Some studies have already identified such miRNA signatures, including mir-486, mir-375, mir-374a and mir-200b, and these could be useful for detecting LC at an early stage [63,64]. Among others of interest are: mir-101, mir-126, mir-199 and mir-34; their expression is reduced in early-stage neoplastic transformation of the lungs of F344 rats that are treated

with nicotine-derived nitrosamine ketone (NNK) [65]. In contrast, mir-210 is overexpressed in the late stages of LC [66]. Other studies indicate that mir-155, mir-29c, mir-146b, mir-221, let-7a, mir-17-5p, mir-27a, mir-106a are potential non-invasive biomarkers for early detection of LC [67]. Further, a strong correlation between high serum mir-21 and tumor-node/lymph node metastasis is observed in NSCLC patients [68]. The miRNA profiles (miR-21 and tumor miR-200c) could be usefulfor the prediction of LC outcome/ relapse after surgical resection [69].

Successive and cumulative miRNA expression alterations have been characterized with respect to the transition in the histological stages of lung squamous carcinogenesis starting from the normal lung tissue of nonsmokers to the invasive squamous cell carcinoma (SCC) of smokers [70]. Most miRNAs follow a bimodal expression pattern; first, they undergo a downregulation during the early stages of morphological modifications of bronchial epithelium (mirsupps), and second, they are upregulated in the advanced stages of cancer (oncomirs) (Figure 3b).

Genetic/epigenetic alterations

Genome-wide association studies have identified a link between SNP variation at the 15q24–15q25.1 region and susceptibility to LC [71]. This region harbors two genes that code for nicotinic acetylcholine receptor alpha (*nAChR*) subunits [72]. Similarly, TP53 mutations are common in lung cancers of smokers, with high prevalence of G:C-to-T:A transversions and A:T-to-G:C transitions. These G-to-T transversions are usually caused by polycyclic hydrocarbons and nitrosamines [73]. In contrast, G:C-to-A:T transitions were found in LC patients with nonsmoking histories [74].

Mutation analysis within the *KRAS* and *EGFR* genes utilizing single tumor samples revealed that these mutations are mutually exclusive. While the frequent occurrence of *KRAS* mutations in LC is associated with a heavy lifetime exposure to tobacco smoke, there is a strong inverse relationship between cigarette smoking and the incidence of *EGFR/HER2* mutations [75]. A recent study showed an increased risk for NSCLC among moderate smokers due to a significant downregulation of let-7, which has a binding site in 3′UTR of *KRAS*, leading to an upregulation of *KRAS* [76]. Therefore, let-7 appears to directly down-regulate *KRAS* oncogene expression. The occurrence of mutations in exon 19 or 21 of the *EGFR* gene is associated with low exposure to CS, but for unknown reasons, mutations in exon 20 of the EGFR gene, associated with a decreased sensitivity to *EGFR* tyrosine kinase inhibitors, are common in smokers [77]. The mir-21 is an established *EGFR*-regulated anti-apoptotic factor in LC in never smokers, while the downregulation of mir-128b, a putative regulator of *EGFR*, is associated with smoking [4,56].

CS-mediated miRNA alterations can be explicated to be an outcome of epigenetic disruption rather than as initiating mutational events, and that would support the fact that CS acts through continued exposure rather than a single, initiating event [78] (Figure 3a). Mounting evidence implicates the direct attribution of CS with aberrant expression/activity of epigenetic regulators and the expression of their target gene(s) during the initiation and progression of LC [79]. In NSCLC, aberrant methylation targets include the promoter regions of the *p16*, *p14*, *FHIT*, *RASSF1*, retinoic acid receptor β (*RARβ*) and *DAPK* genes that are closely related to smoking status [80]. High frequencies of methylation of the *p16* promoter leading to its inactivation have been reported in NNK- or CS-induced rat lung tumorigenesis, and also in the bronchial epithelium and sputum of smokers [81]. *FHIT* methylation is known to cooperate with the *p16* methylation in the progression of LC [82]. Methylations of promoter DNA of *DAPK* and *ECAD* have been identified as early events, whereas DNA methylation at *p16* and *MGMT* are identified as late events [83]. High prevalence in CS-induced epigenetic alterations is also seen in genes such as h-cadherin

(*CDH-13*, the mouse homologue of human H-cadherin), estrogen receptor-α (*ER-α*), progesterone receptor (*PGR*) and runt-related transcription factor-3 (*RUNX-3*) in mouse lung tumors [84].

Certain sequence variants of *DNMT1, DNMT3A and DNMT3B* are associated with mutagen sensitivity induced by the tobacco carcinogen benzo[*a*]pyrene diol epoxide (BPDE) in smokers [85]. CS also induces CpG island demethylation of prometastatic oncogene synuclein-gamma (*SNCG*) in LC cells through *DNMT3B* [86]. On the other hand, CS mediates post-translational modifications of histone deacetylase (HDAC) leading to decreased expression of *HDAC1*, *HDAC2*, and *HDAC3* proteins and stimulation/release of IL-8 [87]. Tobacco-carcinogen-induced transformation of human lung epithelial cells [88] is associated with epigenetic silencing of mir-205 and mir-200 leading to EMT and stem cell-like properties in lung epithelial cells [88]. A recent study demonstrates epigenetic suppression of mir-487b leading to an upregulation of oncogenes *BMI1*, *SUZ12*, *WNT5A*, *MYC* and *KRAS* in pulmonary carcinogenesis [89]. Therefore, the prevalence of hyper-methylation is significantly higher in smokers as compared to non-smokers [90].

Signal transduction cascades

The hallmark pathways, such as self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, inflammation/immune surveillance, tissue invasion and metastasis, sustained angiogenesis, limitless replicative potential in normal cells and disruption of cellular energetics, results in tumorigenicity [91]. CS is responsible for the changes in the cellular environment that produces a selection pressure favoring the emergence of cells acquired with the aforementioned traits [6,92]. To date, the role of smoking-mediated miRNA dysregulation in lung tumorigenesis is not well explored. On the basis of CS exposure-induced aberrant expression and deregulation of miRNAs in lung cancer, it is tempting to speculate that a pool of such miRNAs may contribute to the diversity of smoking-mediated cellular processes in lung tumorigenesis. Box 1 shows the speculated miRNAs that might play a pivotal role in the smoking-mediated pathogenesis.

Box 1

Hypothesized role of miRNAs in smoking-mediated oncogenic signaling

Lung tumor tissues of smokers predominantly show the overexpression of ErbB ligands, namely *TGF-α* [106] and amphiregulin/*HER* levels [107]. The A Disintegrin and Metalloprotease (ADAM) family members, *ADAM17* and A*DAM8*, have been suggested to be the major ErbB pro-ligand proteases [108] that shed off and release signaling receptors. The ErbB ligand expression is increased in both lung tumor tissue and the serum of patients [107], while ADAM expression is induced by CS *in vitro* [109]. CS directly impacts tumor development by modulating the mutational status of *KRAS*, *p53*, *EGFR* [110] as well as triggering effectors of the *EGFR*, *KRAS*, *STATs* and protein kinase B/*Akt*. Yet another level of control for the KRAS pathway, which has only recently been discovered, is provided by the expression of miRNAs. let7a inhibits proper splicing of the KRAS mRNA and prevents the translation to the KRAS protein [76]. *KRAS* in turn triggers a cascade of mitogen-activated protein kinases (*MAPK*) to induce a proliferative signal through the inhibition of the tumor suppressor gene, retinoblastoma (*Rb*). CS also affects the downstream pathways such as p16 promoter methylation, proteosomal activation and genetic alterations in *KRAS*, *p53*, *FGIT*, *CDKN2*, *CDK6* and *MYC*. In addition, CS alters the cellular redox status leading to *HIFα* production and *NFκB* activation influencing TLR-mediated pathways. Once activated, *NFκB* can mediate the suppression of the negative effector of Akt, phosphatase and tensin homologue deleted from chromosome 10 (*PTEN*), leading to Akt activation [111].

Nicotine/NNK may stimulate the anti-apoptotic signals *via* nAChR and activate STATs and Akt via the inhibition of *p53*. NNK also induces the nuclear accumulation of *DNMT1* protein through *AKT/GSK3β/E3* ubiquitin ligase signaling. Furthermore, these NNK-induced DNMT proteins bind to promoters of various TSGs, such as *SLIT* genes, and result in promoter hypermethylation, ultimately leading to tumorigenesis. Benzo[*a*]pyrene, a constituent of CS, has the potential to upregulate the expression of various EMT-related genes, such as fibronectin, *TWIST*, *TGFβ*, and the basic fibroblast growth factor. Moreover, *HOXD10* represses the genes involved in cell migration and extracellular-matrix remodeling; it is found downregulated in tumors that show increased malignancy. Studies have also demonstrated nicotine-mediated upregulation of transcriptional repressors of E-cadherin, such as zinc finger E-box-binding homeobox 1 and 2 (*ZEB1* and *ZEB2*) and *SNAIL*, which are implicated in EMT and metastasis. Figure I depicts the schematics for various smoking-modulated targets in LC as well-established targets for several smoking-induced miRNAs.

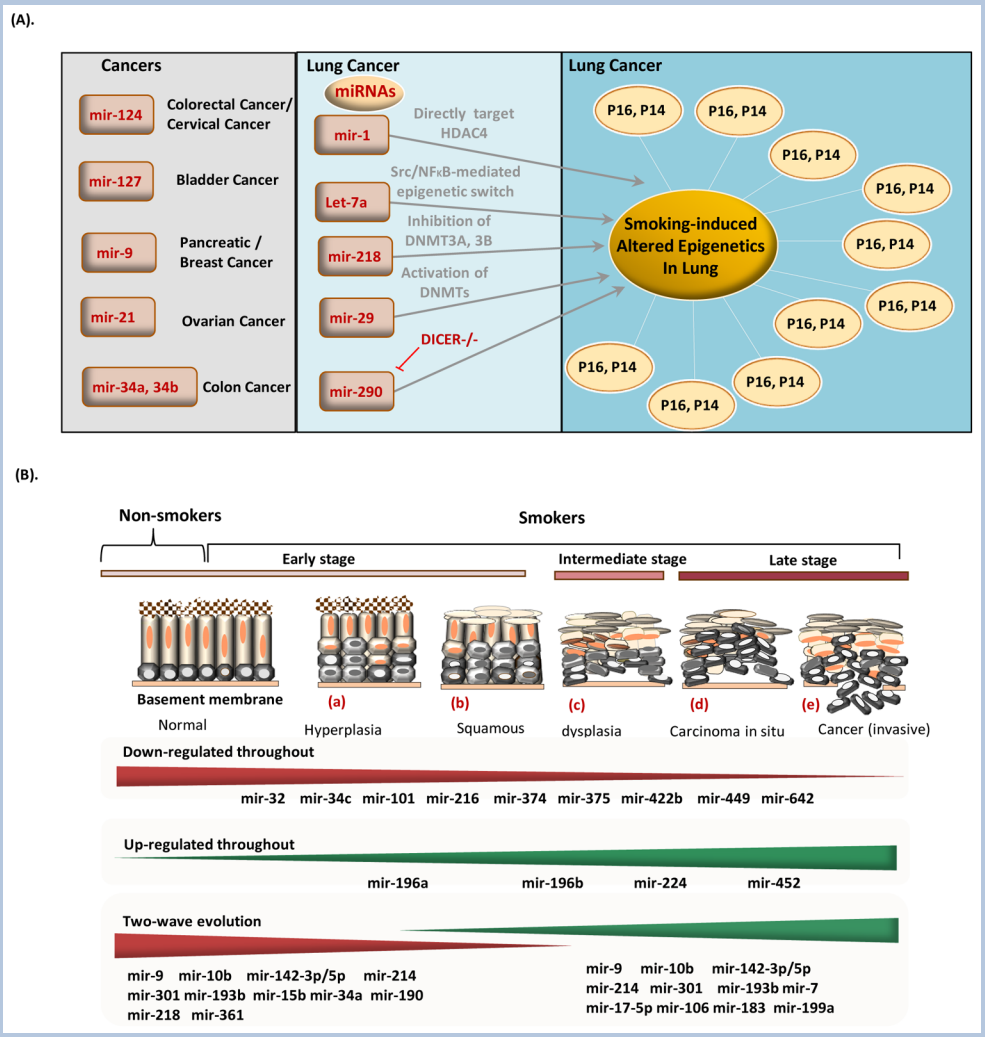

Figure I. miRNAs for smoking-modulated gene targets

Both, CS-induced nAChR-mediated pathways as well as CS-induced ADAMs-mediated EFG receptors (EGF) shedding of trigger several downstream effectors in lung cancer. CS also alters the cellular redox status leading to *HIFα* production influencing toll like receptor (TLR)-mediated pathways. Further, these downstream effectors including

EGFR, *KRAS*, STATs, mTOR, *NFκB*, *ZEB1/ZEB2* and protein kinase B/Akt stimulate proliferative, metastatic/EMT and anti-apoptotic signals. MiRNA let7a has recently been discovered as another level of control for the KRAS pathway, inhibiting the proper splicing of KRAS mRNA and preventing protein translation. Overall, CS is known to directly impact the tumor development by modulating several downstream pathways such as promoter hypermethylation, proteosomal activation and genetic alterations in *KRAS*, *p53*, *FGIT*, *CDKN2*, *CDK6*, *PTEN*, *TWIST*, *TGFβ* and *MYC* that are potential targets for various miRNAs.

Pharmacological standpoints

By modulating the metabolic clearance of anti-cancer therapies, and therefore, the efficiency of chemo-preventive agents, CS can alter cancer metastasis/recurrence potential [93]. CS-mediated alterations in miRNA levels have been shown to modulate the sensitivity towards various therapies. A large study was performed by Izzotti *et al*. on the chemoprevention of various smoke-induced alterations of miRNAs in the rat lung utilizing several dietary agents [78]. These chemo-preventive dietary agents in LC are known to modulate the functioning of angiogenesis and TGF-β–related response. However, the effects of chemo-preventive agents are counteracted by the pro-angiogenic effect of CS, which is exerted by miRNA-mediated upregulation of pro-angiogenic genes [60]. Various studies have demonstrated that the expression pattern of miRNAs is a good indicator for chemo-sensitivity (Table 2).

The short-interfering RNAs (siRNAs) have entered phase I/II trials for LC and have shown good therapeutic potential [94]. Such oligo-based therapeutics should also facilitate the clinical use of miRNAs due to their high comparability with a target. Usage of miRNAs over siRNAs may provide many advantages; including long-term activity/stability, high RNA promoter-compatibility, negligible toxicity and most importantly, the ability of affecting multiple targets with a single hit. MiRNA-based cancer therapy is a current priority, and attempts are being made to manipulate them either by inhibiting oncogenic miRNAs using anti-microRNA oligonucleotides or by reintroducing miRNAs lost in cancer.

Ebert *et. al*. demonstrated the use of synthetic miRNAs sponges, containing repeated miRNA antisense sequences as the binding site for specific miRNAs, acts as specific inhibitor for miRNA by preventing their association with endogenous targets [95]. Antisense oligonucleotide (ASO) is a potent technique to selectively manipulate the expression of genes *in vitro* and *in vivo*. The anti-miRNA oligonucleotides (AMOs) are chemically modified single-stranded RNA analogues that bind to specific miRNAs via Watson-Crick base pairing and effectively silence endogenously expressed miRNAs [96]. Examples of common AMO modifications include the addition of a 2′-hydroxyl group or methyl group, locked nucleic acid (LNA), also referred to as inaccessible RNA, and a phosphorothioate backbone modification (antagomir) that improves its stability and its binding affinity to miRNAs [97]. Inhibition of mir-122 expression in mice with ASO modified with 2′-MOE phosphorothioate resulted in reduced plasma cholesterol levels [98]. In addition to its role in modulating cholesterol homeostasis, mir-122 is also involved in promoting Hepatitis C Virus (HCV) RNA abundance [99]. Notably, in a recent phase 2 study, HCV-infected patients showed good tolerance of a LNA-modified antimir-122 drug miravirsen (SPC3649), and produced a long-lasting suppression of viremia [100]). Indeed, this may be the first miRNA-based therapeutic brought to the marketplace. Alternatively, therapy may be directed against oncogenic mRNAs using a synthetically designed double-stranded mimic miRNA which would directly target the mRNAs (agomiR) [101].

Efficient *in vivo* delivery of antagomir/agomirs, *in vivo* stability, target specificity and systemic toxicity are among major challenges for efficient delivery of miRNA [96].

Adenoviral vector-mediated delivery of mir-122 showed anti-tumor activity due to the induction of apoptosis and/or cell cycle arrest [102] and reduced the expression of three other oncogenic miRNAs (mir-16, mir-192 and mir-194) in eleven different tissues or organs [96]. Systemic delivery of mir-34a through liposome-polycation-hyaluronic acid nanoparticle formulation modified with a tumor-targeting single-chain antibody fragment (scFv) demonstrated anti-cancer effects in the murine B16F10 melanoma model [103]. Similarly, cationic lipoplex loaded with mir-133b had ~30% better accumulation in lung tissues that was ~50-fold higher than siPORT NeoFX transfection agent. Additionally, cationic lipoplex were found to be promising delivery carrier for the mir-133b-based therapeutics in the treatment of LC patients [104]. Agomirs of the tumor suppressor miRNAs, such as mir-34a and let-7, complexed with a novel neutral lipid emulsion, preferentially targeted lung tumors produced in a KRAS-activated mouse model of NSCLC and showed a therapeutic benefit [101].

Re-expression of TSGs silenced by methylation is another promising molecular strategy for the treatment of LC. Using the demethylating agent 5-aza-2V-deoxycytidine (DAC) with the histone deacetylase inhibitor, sodium phenylbutyrate, results in a significant decrease in tobacco-carcinogen-induced LC via genetic DNMT1 reduction [105]. Another study has demonstrated reactivation of mir-127 by epigenetic therapy leading to downregulation of the proto-oncogene BCL-6 at the protein level [43].

Concluding remarks

The persistence of irreversible molecular alterations induced by cigarette-smoking is the key for many normal cells, particularly lung cell, commitment toward hyperplasia and tumorigenesis. Several studies suggest that CS-induced miRNAs have the ability to activate multiple chain-reactions and feedback pathways involved in the pathogenesis of cancer, and therefore, may actively collaborate with smoking-induced neoplastic transformation in multiple cancers.

Each miRNA has been allied to its sole contribution toward differential regulation of its target gene expression; however, the scenario is complicated for two reasons (Box 2). First, their activity is exerted in a one-to-many fashion, with each miRNA targeting hundreds of different target mRNA molecules. Second, there is redundancy among miRNAs, where a single mRNA molecule can be targeted by more than one miRNA. This gives rise to a complex regulatory network in which biologic effects and properties of a particular miRNA fail to follow a linear explanation. Nevertheless, the ability of a single miRNA to influence the expression of several hundred different mRNAs can help in designing miRNA-based therapies to combat complex diseases like cancers.

Box 2

Outstanding questions

- What are the exact differential mechanisms by which cigarette-smoke modulates miRNA expression in normal and pathological conditions?
- What are the criteria for selecting therapeutic miRNAs considering their promiscuity with multiple target(s) and vice-versa?
- What are the temporal sources and clinical significance of circulating miRNA?

The escalation in knowledge of CS-induced molecular alterations and the regulatory networks connecting them with supporting miRNA co-conspirators adds excellence in the identification of key miRNA in LC. Emerging techniques in the fields of epigenetics and

gene expression profiling could revolutionize clinical approaches across the spectrum of cancers by identifying potential molecular markers for early detection/prognosis and response to treatments. In light of this connection between miRNAs, smoking and cancer, further studies on smoking modulated miRNA will pave the better therapeutic target(s).

Acknowledgments

Financial Statement: The authors on this work are supported, in part, by grants from the National Institutes of Health (TMEN U54 CA163120, EDRN UO1 CA111294, RO1 CA131944, RO1 CA133774, SPORE P50 CA127297, RO3 CA167342, and RO1 CA78590).

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HIGHLIGHTS

- The unique miRNA-based signatures distinguish healthy controls from cancer cases with high sensitivity and specificity.
- Differential expression pattern of miRNAs in various malignancies varies with smoking status of individuals.
- Smoking mediates its impact by modulating tumor-suppressive miRNAs during neoplastic transformation and oncogenic miRNAs at later stages of cancer.
- MiRNAs are likely involved in smoking-induced modulation of signaling cascades in various cancers.

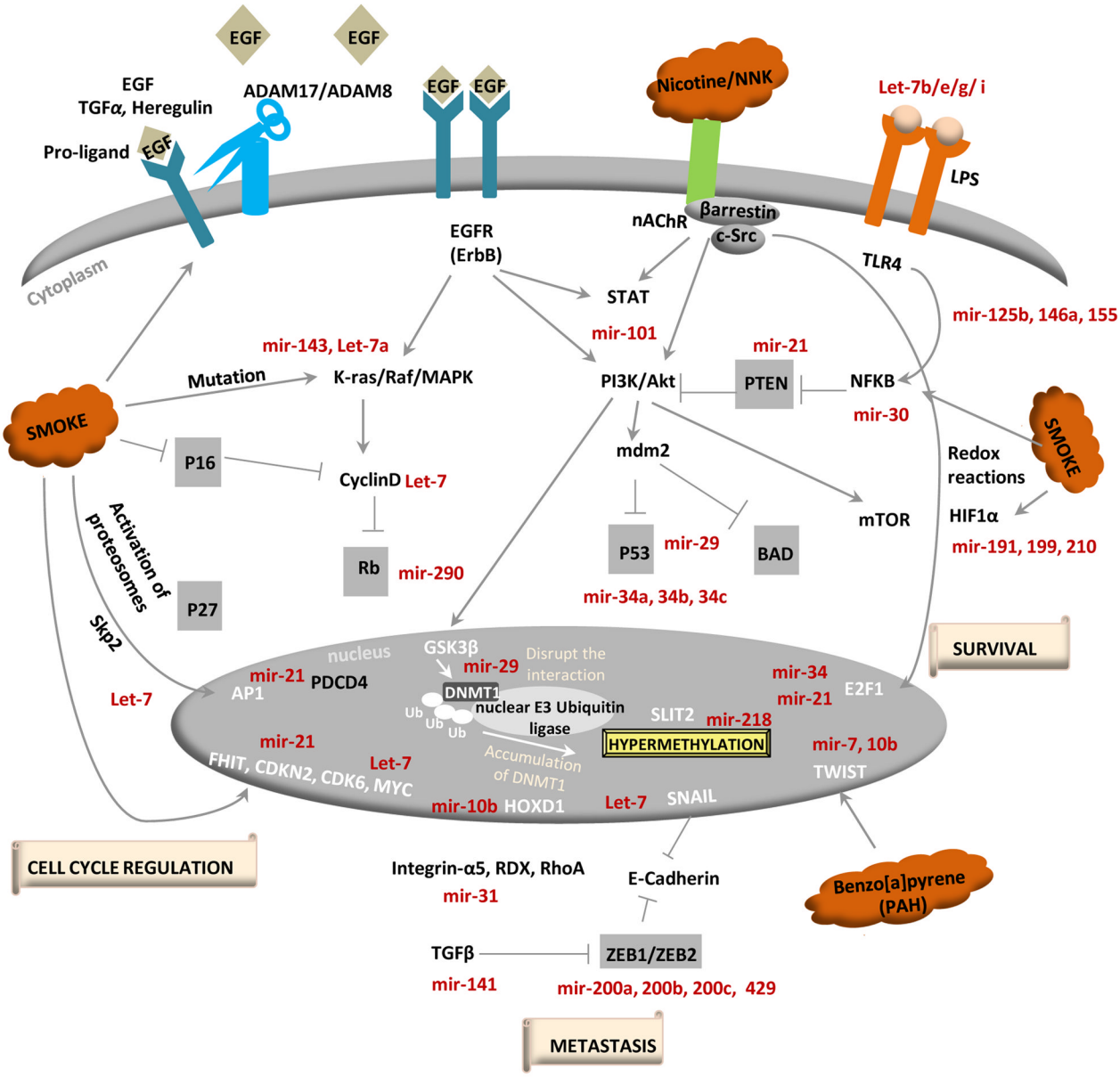

Figure 1. Three-tiered route of miRNAs to cancer

(I) Biogenesis and processing. The pol II enzyme directs the synthesis of primary miRNA transcripts (pri-miRNAs) that are post-transcriptionally modified by the addition of a 5′-cap and a 3′-poly (A) tail and cropped by a dsRNA-specific ribonuclease Drosha/Pasha into the precursor miRNA (pre-miRNA) (70 nt hairpin structure). The pre-miRNA is then transported to the cytoplasm by the nuclear export factor Exportin-5 and then maturated further (into 22 nt structure) by another RNase III enzyme, Dicer. This duplex is unwound, and the complementary strand is subjected to degradation, while the miRNA strand is incorporated into the RNA-induced silencing complex (RISC), which can mediate both translational repression and mRNA transcript cleavage depending on the extent of homology between the miRNA and its target mRNA. **(II)** The miRNA-binding-mediated mRNA fate. (A) The miRNA that has perfect complementarity to target mRNA, processes the mRNA and leads to its cleavage via the siRNA pathway. The miRNA, which has partial

complementary to the mRNA, causes translational inhibition either by (B) inhibiting ribosomal elongation upon recruitment of a protease that degrades the nascent polypeptide chain or, (C) by interacting with RISC and the translation initiation complex protein eIF4E, leading to a ribosome-free miRNA:mRNA structure that is directed to the processing bodies (P-bodies). There it interacts with the Ccr4:Not1 deadenylase complex and usually tends to undergo the miRNA-mediated decay, whereas, in some instances, the mRNA:miRNA complex may be stored in the P-body, and following an appropriate stimulus, might re-enter the cytoplasm for renewed translation. **(III)** Disruption of miRNA-directed regulation. This is caused by epigenetic/genetic modulations of the miRNA expression levels, defective miRNA-processing apparatus and defective mRNA translational machinery. The loss of tumor suppressor miRNAs may increase the translation of oncogenes, and thus, the formation of oncogenic proteins. On the other hand, upregulation of oncogenic miRNAs may block tumor suppressor genes that further enhance tumor development.

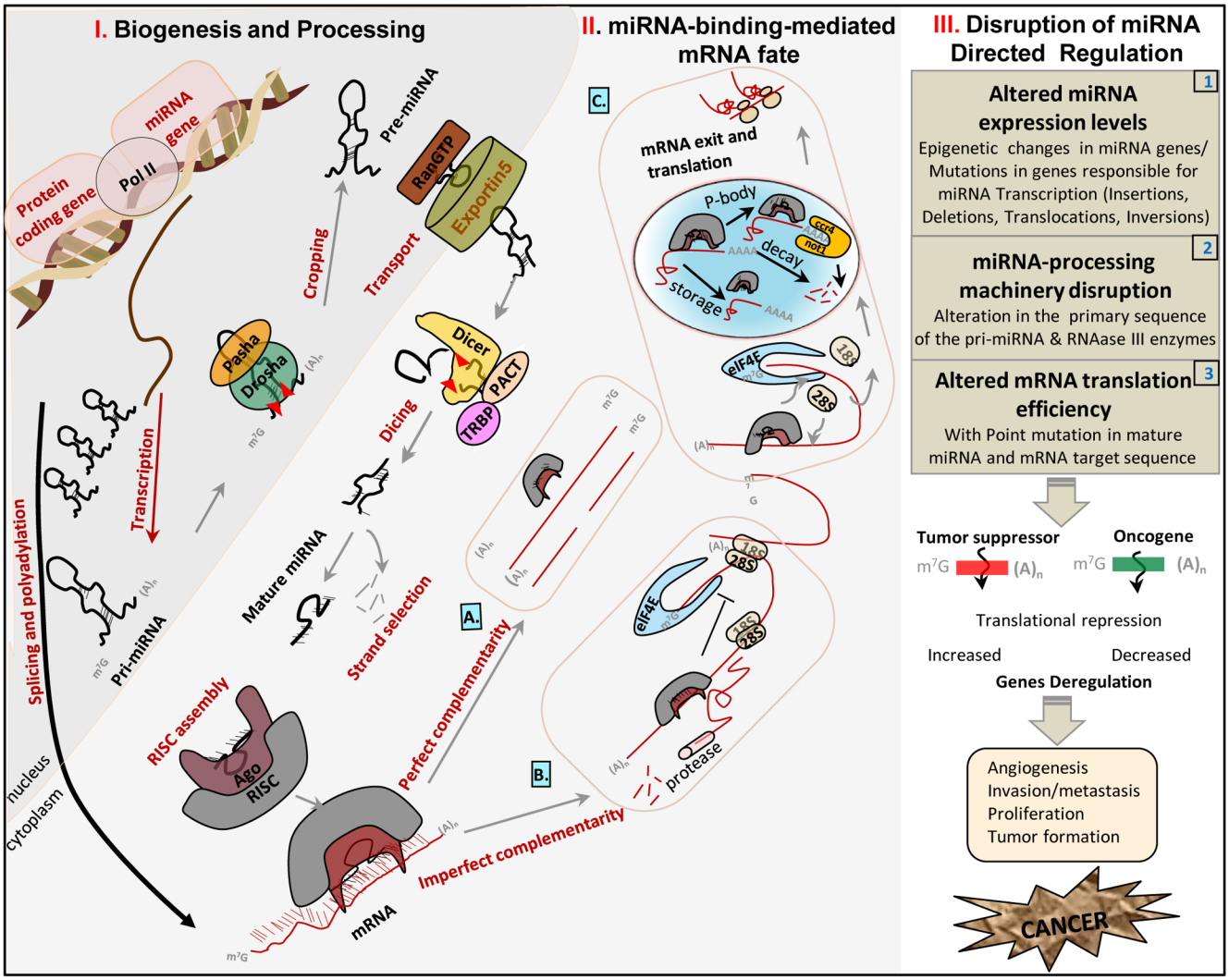

Figure 2. MiRNAs in cellular events of cancer

Essential stochastic alterations in cell physiology, including acquired self-sufficiency in growth signals, insensitivity to anti-growth signals, disruption of cellular energetics, inflammation/immune surveillance, evasion of apoptosis, sustained angiogenesis, limitless replicative potential and metastasis, are termed as the “hallmarks of cancer.” This is the schematic summation of several bodies of evidence demonstrating the miRNAs and their potential targeting genes affecting the individual hallmarks of lung cancer. The miRNAs upregulated and downregulated are depicted in green and red, respectively. The Venn diagram shows miRNA expression trends common and/or specific to lung, pancreatic and cervical cancer, based on the literature review of expression profiling studies of the well-established miRNAs. Interestingly, it shows a high rate of miRNAs deregulation in common with other smoking-associated cancers including pancreatic and cervical cancer. Direct-causal relationships have been observed between smoking and several types of cancers, but the majorities of miRNA studies have been focused on lung cancer. Cervical cancer is a comparatively new field in terms of miRNA-based research. It is hoped that this review will inspire smoking-induced miRNAs expression profiling studies in pancreatic and cervical cancer, which remains completely unexplored at this time.

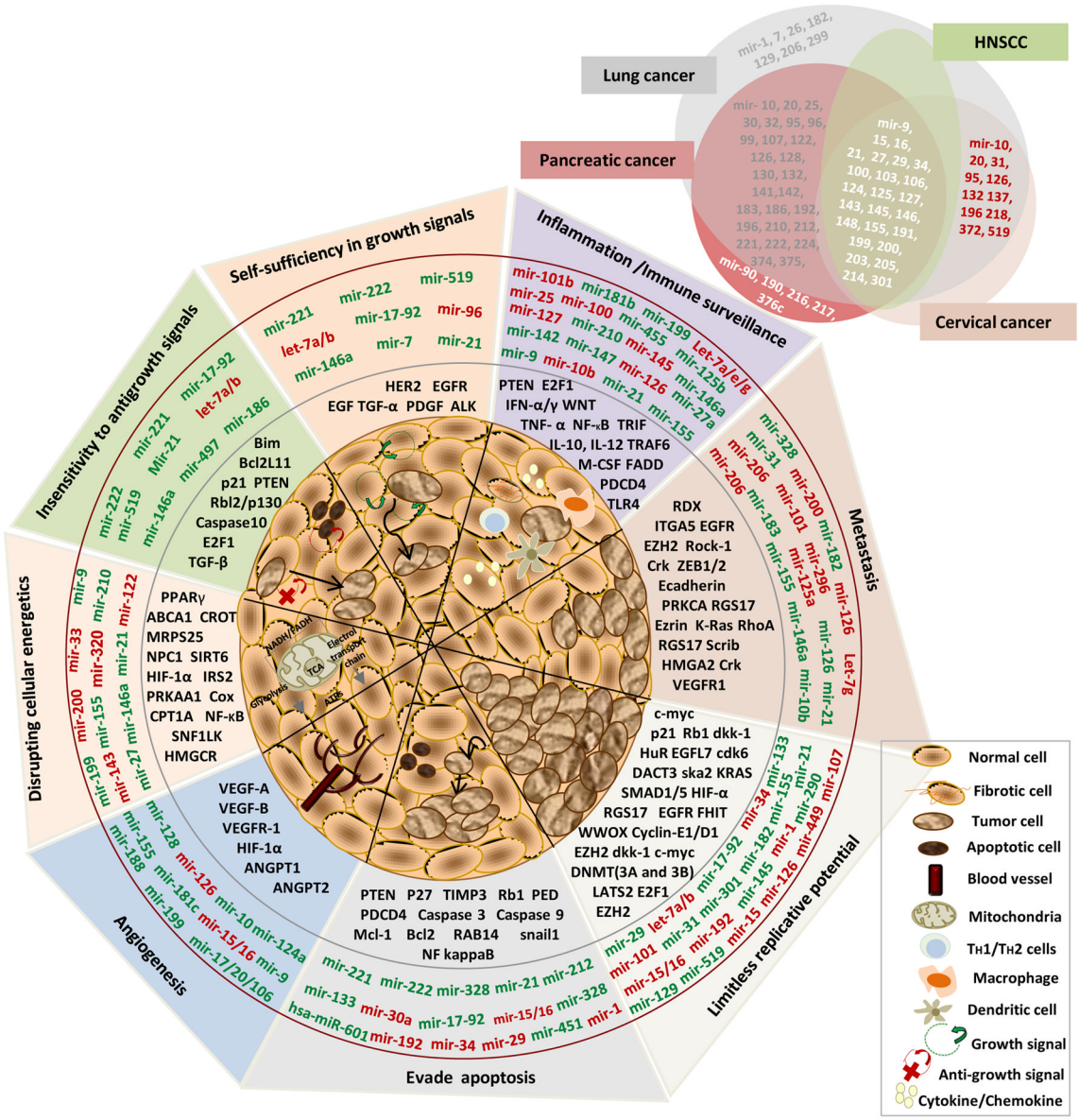

Figure 3. MiRNAs: stage-specific signatures and epigenetic regulators in smokers with lung cancer

(A) An emerging body of evidence supports miRNAs-mediated impact on epigenetic regulation of various malignancies (Left panel). Smoking-induced miRNAs modulate DNA methylation through the activation of various DNA methyltransferases (DNMTs) such as *DNMT1*, *DNMT3A* and *DNMT3B* in cooperation with histone tail modifications through reduced histone deacetylase (HDAC) activity (Middle panel). Among common targets of smoking-induced aberrant methylation in lung cancer, noteworthy are the promoter regions of the *p14*, *p16*, retinoic acid receptor β (*RARβ*), ras association domain family 1A (RASSF1), fragile histidine triad (*FHIT*) genes, death associated protein kinase (*DAPK*), h-cadherin (*CDH-13*, the mouse homologue of human H-cadherin), estrogen receptor-a (*ER-a*), progesterone receptor (*PGR*) and runt-related transcription factor-3 (*RUNX-3*) (right panel). **(B)** Schematic representation of successive and cumulative alterations of miRNA expression and their association with stages of lung squamous carcinogenesis (SCC) starting

from the normal tissue of non-smokers/normal bronchial tissue of smokers (a) and following hyperplasia, (b) metaplasia (widely dispersed cells, with a regular maturation from the basal region to the top and keratinization and low nuclei/cytoplasmic ratio), (c) dysplasia (hypercellularity of the epithelium with incomplete maturation, high nuclei/cytoplasmic ratio and micro papillary invasion of capillaries), (d) *in situ* carcinoma (marked pleomorphism of the cells with irregularity and prominent nucleoli) and (e) invasive SCC. Some miRNAs show a linear evolution in the level of expression, and their expression progressively decreased or increased from normal bronchial tissues of non-smokers to smokers SCC (mir-32, mir-34c). Other miRNAs behaved differently at successive stages (mir-142-3p, mir-9); some were modified from a specific stage (mir-199a, mir-139), or modified at one specific step of lung carcinogenesis (mir-199a, miR139). The expression levels of most miRNAs evolved through all successive stages: from the normal bronchi of non-smokers to invasive SCC in smokers, and showed a unique pattern, that is, an initial decrease in tumor suppressors during the earliest morphological modifications of bronchial epithelium, and at the later stages of lung carcinogenesis, an increase in oncomirs. Most miRNAs were initially downregulated, in agreement with the hypothesis that miRNA downregulation often occurs in cancer where tissue is losing its normal differentiation.

Table 1

List of potential cigarette-smoke mediated deregulated miRNAs in lung cancer correlated with their known target genes.

miRNAs	Gene Target(s)[a]
Tumor suppressor miRNAs	
mir-7	Pak1, EGFR
mir-10b	RhoC, HOXD10
mir-26	Cyclin D2, cyclin E2, TGF
mir-29	TCL-1, MCL1, DNMT3s, YYI
mir-30	EFG, NF-κB inhibitors, CDC40, SNAIL
mir-34a/b/c	SIRT1/CD44, cyclins, p53, YYI
mir-99	mTOR/FGFR3
mir-101	EZH2, Mcl-1, AKT
mir-122	ADAM17, RhoA, RAC1, cyclin G1, p53
mir-124	CDK6
mir-126	Crk
mir-128	E2F3a, BMI1, EGFR
mir-129	Cdk6
mir-143	FNDC3B, KRAS
mir-145	TNFSF10, EGFR, IGF-1R
mir-199	HIF-1alpha
mir-200	ZEB1, ZEB2
mir-204	Bcl-2, Mcl-1
mir-218	SLIT2/3, KIT, RET, BCL9, DCUN1D1 and PDGFRA
mir-223	C/EBPα, NFI-A
mir-224	CDC42 and CXCR4
Let-7	HMGA2, E2F2, c-Myc, KRAS, p21
Oncomirs	
mir-17/mir-20a	p21, STAT3, c-Myc, HIF-1α, Tsp1
mir-21	TPM1, PDCD4, PTEN, NFKB, maspin, RECK, TiMP3, SPRY2, EGFR, HER2/neu, Bcl-2, AP1, MYC, FHIT
mir-24	E2F2, MYC, CDC2, DK4, FEN1, XIAP
mir-31	RhoA, LATS2, PPP2R2A, RDX
mir-96	FOXO1
mir-106	p21, p73
mir-125	ERBB7 (gene for EGFR), IGFR
mir-130	GAX, HOXA5
mir-146	NF-κB
mir-155	TP53INP1, p21, SMAD1, SMAD5
mir-191	HIF-1α
mir-210	ISCU, NDUFA4, SDHD, COX10, EFNA3, MNT, HIF-1α

miRNAs	Gene Target(s)[a]
mir-221/222	p27(CDKN1B), p57(CDKN1C), PUMA
mir-294	Zinc finger protein 697, AT-rich interactive domain 4A

[a] Experimentally verified in the literature (database:miRTarBase).

Table 2

List of miRNAs associated with resistance/sensitivity to radiation and chemotherapy in lung cancer.

Therapy	miRNAs	Refs
Associated with Resistance to Radiation/Chemotherapy		
Paclitaxel	mir-135a	[112]
Docetaxel	mir-152, (*mir-192* [a], 424 and 98)	[113,114]
Cisplatin	mir-134	[115]
Etoposide	mir-379	[115]
Doxorubicin	mir-495	[115]
Radiotherapy	*mir-130a* [a], *mir-106b* [a] mir-19b, mir-22, mir-15b *mir-17-5p* [a] *and mir-21*[a]	[116]
Associated with Sensitivity to Radiation/Chemotherapy		
Paclitaxel	*mir-101*[a]	[117]
Docetaxel	*mir-200b* [a], mir-194 and *mir-212* [a]	[113]
Cisplatin	mir-497 (reduces BCL2), pre-mir-630, *pre-mir-181a* [a] (P22, P53 activation), mir-200c	[118,119]
Cetuximab	*mir-200c* [a]	[120]
Doxorubicin	mir-1	[121]
Gefitinib	*let-7a* [a], *hsa-mir-126* [a], *and hsa-mir-145* [a]	[122]
Radiotherapy	*mirNA-126* [a], *mirNA-let-7a* [a], mirNA-495, mir-451, *mir-128b* [a]	[116]

[a]Smoke-regulated miRNAs (denoted in italics).