

# MiR-326: Promising Biomarker for Cancer

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**Abstract:** MicroRNAs (miRNAs) are small non-coding and highly conserved RNAs that act in biological processes including cell proliferation, invasion, apoptosis, metabolism, signal transduction, and tumorigenesis. The previously identified miRNA-326 (miR-326) has been reported to participate in cellular apoptosis, tumor growth, cell invasion, embryonic development, immunomodulation, chemotherapy resistance, and oncogenesis. This review presents a detailed overview of what is known about the effects of miR-326 on cell invasion, metastasis, drug resistance, proliferation, apoptosis, and its involvement in signaling pathways.

**Keywords:** MicroRNA, miR-326, tumor suppressor, cancer, metastasis, oncogene

## Introduction

With short sequence length, non-coding and endogenous microRNAs (miRNAs) can regulate gene expression by binding to the 3' untranslated region of target messenger RNAs (mRNAs), thus suppressing translation or degradation of the mRNA. MiRNAs participate in several significant biological processes, including cell differentiation, proliferation, apoptosis, and host response against viral infections.<sup>1-3</sup> All known or assumed protein-coding genes are reportedly expressed only in a small proportion of the entire genome, and miRNAs evidently constitute approximately 1-2% of all genes in worms, flies, and mammals.<sup>2</sup> A single miRNA can regulate expression of many genes.<sup>4</sup> Overall, gene expression that is regulated by miRNA may significantly contribute to overall regulation.

The goal of this review was to describe miRNA-326 (miR-326) activity in tumors, as miR-326 is downregulated in most tumors. Low expression of miR-326 is dramatically related with unfavorable prognosis, tumor development, metastasis, and progression. For example, downregulated miR-326 is positively correlated with the risk of metastasis in patients with gastric cancer, prostatic carcinoma, esophageal squamous cell carcinoma, and non-small lung cancer (NSCLC).<sup>5-8</sup> In this review, we summarize the promising effects of miR-326 on cell invasion, metastasis, drug resistance, proliferation, apoptosis, and its involvement in signaling pathways.

## miR-326 in Invasion and Metastasis

Tumor invasion and metastasis processes are influenced by multiple factors. The progress of early tumor development into metastasis includes invading from the extracellular matrix to the stromal layers and then transferring to distant organs via infiltration from blood vessels to remote parenchymal tissues.<sup>9,10</sup> Studies have shown that miR-326 is involved in tumor cell invasion and metastasis in NSCLC, gastric cancer, breast cancer (BC), cervical cancer, osteosarcoma, glioma, colorectal cancer,

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endometrial cancer, prostatic carcinoma, and esophageal squamous cell carcinoma.<sup>5,6,11–18</sup>

In NSCLC, upregulated expression of miR-326 limits tumor metastasis by targeting a disintegrin and metalloprotease 17 (Adam17), nucleosome-binding protein 1 (NSBP1), and paired-like homeobox 2a (Phox2a).<sup>11,19,20</sup> As a member of the tumor necrosis factor converting enzyme family, Adam17 is an indispensable regulator of tumor metastasis.<sup>21,22</sup> Adam17 can adjust the expression of epidermal growth factor receptor by activating Notch1.<sup>23</sup> Studies found that miR-326 can inhibit NSCLC cell invasion, possibly by downregulating Adam17 expression level.<sup>19,23</sup> NSBP1 can regulate gene transcription by binding chromatin. It is distinctly expressed in various tissues and is a potential oncogene in diverse tumors.<sup>24,25</sup> Li et al reported that miR-326 hampered NSCLC cell invasion via inhibiting the protein level of NSBP1.<sup>20</sup> Phox2a essentiality was demonstrated in neurogenesis and in recent years its role in cancer has been described. Wang et al found that miR-326 slowed NSCLC metastasis by targeting Phox2a.<sup>11</sup> In gastric cancer, low expression of miR-326 was associated with clinical stage, tumor depth, lymph nodes, and distant metastasis. Survival analysis indicated that low expression of miR-326 was a prognostic factor for poor outcome for gastric cancer patients. Downregulation of miR-326 promoted metastasis by targeting Fascin 1 (FSCN1).<sup>6</sup> As an actin-binding protein, FSCN1 is often upregulated in different cancers, and overexpression of FSCN1 promotes tumor invasion and metastasis.<sup>26–28</sup> In BC, miR-326 was reported to repress cell metastasis by targeting B7-H3, an immunomodulin belonging to the B7 family. B7-H3 is highly expressed in various tumors and is correlated with adverse prognosis.<sup>29,30</sup> In cervical cancer, restoration of ETS domain-containing protein (ELK1) was reported to eliminate cell invasion due to miR-326 mimics, indicating that miR-326 suppressed cell invasion and metastasis by targeting ELK1.<sup>31</sup> As a member of the ETS oncogene family, ELK1 affects cytoskeleton transformation and tumor invasion.<sup>32,33</sup> As a conserved protein, nin one binding 1 (NOB1) affects RNA metabolism and protease function.<sup>34,35</sup> In osteosarcoma, glioma and colorectal cancer, miR-326 can target NOB1 to inhibit metastasis.<sup>16,36,37</sup> TWIST1, a transcription factor, affects tumorigenesis and tumor progression, especially in metastasis.<sup>38</sup> Analysis from data in *TargetScan* and *MicroRNA.org* identified TWIST1 as a potential target of miR-326, and luciferase reporter assays were consistent with the targeting of TWIST1 by miR-326. Forced expression of miR-326 significantly decreased the levels of

TWIST1.<sup>39,40</sup> In endometrial cancer, in vitro assays revealed that knockdown of TWIST1 inhibited tumor cell invasion.<sup>17</sup> In prostatic carcinoma, miR-326 functioned as a tumor suppressor by negatively regulating Mucin1 (MUC1). Previous studies indicated that MUC1 is involved in the regulation processes of several specific miRNAs on tumor cell proliferation and invasion.<sup>41</sup> Furthermore, the miR-326/MUC1 axis inhibits prostatic carcinoma cell invasion partly via suppressing c-Jun amino terminal kinase (JNK) signaling activation.<sup>5</sup> In esophageal squamous cell carcinoma, Su et al reported that vascular endothelial growth factor-C reduced miR-326 expression and increased Src substrate cortactin (CTTN) protein level and invasive abilities, suggesting vascular endothelial growth factor-C upregulated CTTN expression through Src-mediated downregulation of miR-326.<sup>18</sup> Additionally, the expression of miR-326 was correlated with poor prognosis in esophageal squamous cell carcinoma patients.<sup>8</sup>

Thus, several studies have shown that miR-326 plays a role in inhibiting invasion and metastasis in a variety of tumor cells, but the specific mechanism has not been elucidated. More in-depth experiments should be performed to identify appropriate targets to tackle metastatic issue.

## miR-326 in Cell Proliferation and Apoptosis

Tumor cells can exert complex effects on apoptosis and proliferation of malignant tumor cells.<sup>42</sup> Numerous genes may be involved in the regulation by miR-326 on cell apoptosis and proliferation, such as CyclinD1 (CCND1), fibroblast growth factor 1 (FGF1), son of sevenless homolog 1 (SOS1), and neuroblastoma RAS viral oncogene homolog (NRAS).<sup>7,43,44</sup>

As a pivotal cell cycle regulatory protein, CCND1 expression and cellular localization varies in human tumor cells. It is a gate-keeping protein that regulates conversion through the cell cycle restriction point between the G1 phase and the S phase. Consequently, changes in CCND1 gene amplification, posttranscriptional or posttranslational modifications, rearrangements, and variant polymorphisms can give rise to abnormal protein expression and increase risk of tumorigenesis.<sup>45,46</sup> In NSCLC, miR-326 may inhibit tumor proliferation by targeting CCND1. Sun et al found that miR-326 could suppress cyclin D1, thus facilitating the expression level of p57 and p21, which might explain the proliferation-inhibition property of miR-326.<sup>7</sup> Kaplan–Meier survival

analysis revealed that patients with low expression levels of miR-326 had shorter overall survival compared to patients with high expression levels of miR-326. These results demonstrated that down-regulation of miR-326 was associated with poor prognosis in NSCLC. As a member of the fibroblast family, FGF1 can facilitate the repair of damaged blood vessels and FGF family members can promote cancer cell growth and intensify chemotherapy resistance. FGF1 is overexpressed in a variety of tumors including NSCLC, ovarian, prostate, and breast cancers.<sup>47,48</sup> Studies have shown that miR-326 restrained FGF1 expression to modulate cell proliferation and apoptosis.<sup>43</sup> FGF family members target the fibroblast growth factor receptor (FGFR) to enhance tumor cell proliferation and invasion by controlling endothelial cells and pericytes in tumors.<sup>49,50</sup> The signaling pathways downstream of FGFR, i.e. mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), Ras, and JNK pathways, can promote cancer cell proliferation and distant dissemination, with participation in every step from tumorigenesis to oncogenesis.<sup>51,52</sup> In NSCLC, FGF1 increases the phosphorylation level of FGFR to activate PI3K and JNK, so decreased FGF1 regulated by miR-326 upregulation may inhibit the malignant behaviors of glioma cells by weakening the activities of PI3K/Protein kinase B (PI3K/AKT) and MAPK kinase (MEK) 1/2 pathways.<sup>53,54</sup> As a dual guanine nucleotide exchange factor for Ras and Rac1, SOS1 can convert inactive Ras-guanosine diphosphate to active status.<sup>55</sup> EGFR can regulate Ras to affect tumor cell apoptosis, metastasis, and tumorigenesis.<sup>56,57</sup> Additionally, nerve growth factor (NGF) can regulate cell proliferation through SOS1 stimulation and Ras signaling. NGF may promote endothelial cell migration and growth and influence cancerous angiogenesis.<sup>58</sup> NRAS, which belongs to the Ras family, is widely expressed in diverse cells, and NRAS activation may participate in SOS1 stimulation and be related to NGF and EGFR signaling. According to a previous study, SOS1 and NRAS are abundant in the NGF and EGFR signaling pathways.<sup>59</sup> Therefore, miR-326 might regulate tumor cell proliferation and angiogenesis through the NGF and EGFR signaling pathways. However, the detailed mechanisms underlying the regulation of these targeted genes by miR-326 have not been elaborated.

## Involvement of miR-326 in Signaling Pathways

### Hedgehog/Gli (Hh/Gli) Signaling Pathway

Changes in the Hh/Gli pathway can occur through both non-canonical and canonical mechanisms.<sup>60</sup> The activation of

the canonical Hh/Gli pathway is controlled by the receptor Patched that inhibits the movement of Smoothed.<sup>61</sup> Sonic hedgehog (SHH) protein ligand can bind to Patched, thus moderating Smoothed inhibition, allowing activation of Hh/Gli that leads to Gli2 transcription factor activation and movement to the nucleus.<sup>62</sup> Gli2 can promote the transcription activity of Hh/Gli target genes, including Patched 1 and Gli1. Additionally, focal deletions, mutations, or gene amplifications that encode pathway components like Gli2, Smoothed, and Patched 1 have been shown to be involved in crucial oncogenic events in SHH-driven medulloblastoma (SHH-MB).<sup>63–66</sup> Other mechanisms of activation of non-canonical Hh/Gli pathways include p53/17p gene deletion, aberrant PI3K/Akt/S6 activation, histone methylation, and post-transcriptional modification of Gli1.<sup>60,67–69</sup> Additionally, miRNAs are crucial regulators of Hh/Gli signaling.<sup>70</sup> Suppressed expression of miR-326 and its host gene Arrestin B1 are characteristics of cancer-stem cells derived from SHH-MB. Overexpression of miR-326 and Arrestin B1 can inhibit the Hh/Gli pathway by targeting numerous activator components of this signaling pathway that Gli1, Gli2, and Smoothed require for cancer-stem cells activity.<sup>71</sup>

### PI3K/Akt Signaling Pathway

As part of the Receptor Tyrosine Kinases signaling pathway, the PI3 kinase pathway has a significant function in stimulating tumor cell growth and proliferation. The members of this pathway alter the progress of cell malignant transformation through active participation in cellular biological activities such as cell differentiation, proliferation, cell migration, invasion, trafficking, and glucose homeostasis.<sup>72,73</sup> Preternatural signaling via the PI3 kinase pathway leads to a change in the expression level of transcription factors. Activation of the PI3 kinase pathway is characteristic of malignancies.<sup>74</sup> Dimerization and autophosphorylation occur due to complexing of growth factor ligands with membrane receptor tyrosine kinases, and the PI3 kinase is activated by catalytic conversion of phosphatidylinositol (3,4)-bisphosphate lipids to phosphatidylinositol (3,4,5)-trisphosphate.<sup>75</sup> PKB/Akt exhibits high affinity to phosphatidylinositol (3,4,5)-trisphosphate, allowing its infiltration of the plasma membrane. Phosphorylation of T308 by phosphoinositide-dependent kinase 1 initiates excitation, which is then completed by phosphorylation on the S473 residue, possibly due to the action of diverse proteins, such as mTOR.<sup>76</sup> Phosphorylated Akt then can induce downstream pathways that control cell proliferation and survival, including the phosphorylation and activation of MDM2 E3 ubiquitin

ligase transcription factor, NF- $\kappa$ B, and mTOR kinase, and the inactivation of pro-apoptotic protein BAD and FOXO1 transcription factor to promote tumorigenesis.<sup>77,78</sup> In addition to the protein coding genes, other mechanisms like aberrant activity of the PI3K pathway may affect miRNAs whose expression levels are influenced by the PI3 kinase signaling.<sup>79</sup> For example, miR-326 expression level is restrained by abnormal PI3 kinase signaling, resulting in downregulation in glioblastoma.<sup>15,80</sup>

### RAS/ERK Signaling Pathway

In the RAS/extracellular signal-regulated kinase (RAS/ERK) pathway, Ras-family guanosine triphosphatases isoforms bind to RAF-family serine/threonine kinases, RAFs then interact with the dual-specificity MEK1 and MEK2, and then the MEKs together with MAPKs ERK1 and ERK2 participate in signaling for pivotal biological activities, including cell survival, proliferation, and differentiation leading to the development of carcinogenesis.<sup>56</sup> MEK can phosphorylate ERK1 and ERK2 (ERKs), which promotes the formation of homodimers, which are more stable than the labile heterodimers. The function of the dimerized ERKs has not yet been determined, and may affect diverse cell processes.<sup>81</sup> Dimerization can regulate ERK activity levels depending on the mono- or bi-phosphorylated state of the monomers.<sup>82</sup> Some studies have linked the phosphorylation state with the subcellular distribution of ERK. Dimerization was proposed as essential for ERK's nuclear translocation because mutations that alter dimerization of ERK2 reduced nuclear access.<sup>83,84</sup> However, the activities of ERKs to dimerize and then interact with nuclear pore proteins via the same structural motifs have not been clearly separated. Recent analysis showed that ERK dimers were detected predominantly in the cytoplasm, together with scaffold proteins that serve as ERK-dimerization platforms that allow ERKs to find their cytoplasmic substrates. ERK dimerization is also indispensable for cellular transformation and the transmission of tumorigenic signals by RAS/ERK pathway oncogenes.<sup>85-87</sup> Kang et al showed that miR-326 plays tumor-suppressive roles in melanoma by directly regulating KRAS and indirectly regulating the ERK signaling pathway.<sup>88</sup>

### Effects of miR-326 on Drug Resistance

Chemotherapy is widely used to treat cancer, but various processes can prevent the effective killing of cancer cells by anticancer drugs, such as diversification in absorption, anomalous metabolism, and multidrug resistance (MDR).

MDR presents a significant problem in chemotherapy, especially for patients who cannot sustain surgical resection or radiation therapy.<sup>89,90</sup> Studies have reported that miR-326 is involved in the MDR mechanism of hepatocellular carcinoma and BC, with two genes reported to be involved, ABCC1 and Bcl-xL.<sup>12,91</sup>

ABCC1 is a key efflux transporter and a member of the ABCC family, and can affect the absorption of drugs by cells. Suppression of ABCC1 expression has been linked to reduced tumor progression and chemotherapy resistance.<sup>92,93</sup> Adriamycin (ADM) is an effective and widely used drug for hepatocellular carcinoma and BC chemotherapy.<sup>94,95</sup> Ma et al found that miR-326 regulates the expression of the ABCC1 gene and ABCC1-mediated ADM-resistance in hepatocellular carcinoma. They transfected miR-326 mimic or negative control into HepG2 cells and then determined the cell survival rate by MTT assay. The result showed that transfection of miR-326 mimic significantly reduced cell viability compared with the negative control and mock transfection, and suggesting the underlying mechanism might be the blocking of MDR-related genes.<sup>91</sup> VP-16, a semi-synthetic derivative of podophyllo-toxin, is one of the most effective antineoplastic agents used routinely in first-line combination chemotherapy treatment of small cell lung cancer, testicular cancer, and non-Hodgkin's lymphoma. Liang et al found that miR-326 could attenuate the expression of ABCC1 and sensitize BC cells to ADM and VP-16. They transfected miR-326 mimic into MCF-7/VP-16-resistant (MCF-7/VP) cells and determined the sensitivity of these mimic-transfected cells to VP-16 and ADM. MTT assay was performed with increasing concentrations of VP-16 and ADM with 48h treatment. The IC<sub>50</sub> of MCF-7/VP cells resistant to VP-16 prior to transfection of miR-326 was 15.3 times higher than that of their parental cells, MCF-7. The IC<sub>50</sub> of miR-326-transfected MCF-7/VP cells to VP-16 was 7.1 times lower than MCF-7/VP cells transfected with control ADM, and only 2.1 times higher than the MCF-7 parental cells. Transfection of MCF-7/VP with miR-326 also resulted in decreased resistance to ADM. The resistance of MCF-7/VP prior to transfection of miR-326 to ADM was 20 times higher than the MCF-7 parental cells. After MCF-7/VP cells were transfected with miR-326, their IC<sub>50</sub> to ADM was 10 times lower than that of MCF-7/VP cells transfected with control oligonucleotide and only 1.9 times higher than the MCF-7 parental cells.<sup>12</sup> However, the details of the mechanism have not been determined. Another in vivo study also transfected miR-326 mimic or negative controls into HepG2 cells and similarly found significantly reduced cell viability compared with negative control cells and mock-transfected cells. They



found that miR-326 altered the protein expression of Bcl-xl by luciferase assay, suggesting that miR-326 might sensitize hepatocellular carcinoma cells to 5-Fluorouracil by targeting Bcl-xL, though the detailed mechanism remains unclear.<sup>96</sup> Bcl-xL belongs to the Bcl-2 family, which contains pro-apoptotic and anti-apoptotic (Bcl-2 and Bcl-xL) members.<sup>97,98</sup> An effective and widely used chemotherapeutic agent, 5-Fluorouracil is applied in treatment of colorectal cancer and other tumors including pancreatic cancer, esophageal cancer, gastric cancer, hepatic cancer, and BC. This drug disturbs DNA replication by replacing thymidine with fluorinated nucleotides that are incorporated into DNA, thus causing cell death.<sup>99,100</sup>

## Conclusion

We discussed the potential roles of miR-326 in cell proliferation, apoptosis, migration, invasion, metastasis, and signaling pathways in diverse cancers. There is significant evidence that miR-326 can act as a tumor suppressor gene and is associated with tumor prognosis in many cancer types. We may be able to artificially inhibit tumor growth and metastasis using miR-326 mimics or synthetic agents, or to predict the prognosis of tumor patients by detecting the expression level of miR-326. Overall, we need to further explore the mechanisms by which miR-326 affects tumor suppression and study the molecules and pathways that interact with miR-326 to understand the roles of this RNA in cancer and to develop gene therapy strategies for clinical treatment.

## Abbreviations

Adam17, A disintegrin and metalloprotease 17; ADM, Adriamycin; AKT, Protein kinase B; BC, Breast Cancer; CCND1, CyclinD1; CTTN, Src substrate cortactin; ELK1, ETS domain-containing protein; ERK, Extracellular signal-regulated kinase; FGF, Fibroblast Growth Factor; FGFR, FGF receptor; FSCN, Fascin 1; Hh/Gli, Hedgehog/Gli; JNK, c-Jun amino terminal kinase; MAPK, Mitogen-activated Protein Kinase; MDR, Multidrug Resistance; MEK, MAPK Kinase; MiRNA, MicroRNA; miR-326, miRNA-326; mRNA, Messenger RNA; MUC1, Mucin1; NGF, Nerve growth factor; NOB1, Nin one binding 1; NRAS, Neuroblastoma RAS Viral Oncogene Homolog; NSBP1, Nucleosome-binding protein 1; NSCLC, Non-small Lung Cancer; Phox2a, Paired-like homeobox 2a; PI3K, Phosphatidylinositol 3-kinase; SHH, Sonic Hedgehog; SHH-MB, SHH-driven Medulloblastoma; SOS1, Son of sevenless homolog 1.

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## Disclosure

The authors report no conflicts of interest in this work.

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