Epithelial-mesenchymal transition-associated microRNAs in colorectal cancer and drug-targeted therapies (Review)

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Abstract. Colorectal cancer (CRC) is one of the most common malignancies, and the third leading cause of cancer-associated mortality worldwide. Therefore, the identification of effective targets at the early stages of cancer invasion and metastasis, and new tumor markers for early diagnosis and individualized treatment is imperative for CRC. Epithelial-mesenchymal transition (EMT) refers to the phenomenon of the transformation of epithelial cells to mesenchymal cells in specific physiological and/or pathological circumstances. Evidence suggests that EMT plays an important role in *in situ* infiltration and distant metastasis of many types of cancer, including CRC. Recent findings showed that microRNA expression is important in regulating the EMT process. This review aimed to summarize EMT-associated specific miRNA molecules in CRC, with particular emphasis on clinical targets for effective treatment of this lethal disease.

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1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies, and the third leading cause of cancer-associated

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mortality worldwide (1). Although a decrease in CRC mortality has been observed, its incidence continues to rise. Therapeutic approaches such as conventional surgery, chemotherapy and radiation therapy are insufficient with regard to reversal of advanced CRC. Thus, identification of new tumor markers for early diagnosis and therapy, is the key to reducing the incidence of cancer, directly affecting the prevention and treatment of CRC.

Epithelial integrity is crucial during epithelial tumor development. Eradication of the original epithelial structure and obtaining the peculiarity of mesenchymal cells is the first step in tumor invasion and metastasis. This procedure is known as epithelial-mesenchymal transformation (EMT), which plays an important role in *in situ* infiltration and distant metastasis in various types of cancer, including CRC (2).

MicroRNAs (miRNAs) are small non-coding RNA molecules, consisting of 22-25 nucleotides, that regulate the expression of target genes through base-pairing interactions. These molecules are important in tumorigenesis including angiogenesis, circulation, extravasation, intravasation, invasion and metastatic colonization (3). Studies have focused on tumor-associated miRNAs with regard to the expression and function of miRNAs.

This review aimed to summarize EMT-associated miRNA molecules in CRC to determine new types of treatment, particularly drug-targeted therapy.

2. EMT and cancer

EMT-associated concepts. Epithelial and mesenchymal cell types are recognized by their unique cell morphology and peculiarity in tissues. Epithelial cells belong to a group of polarized cells that are connected laterally via adhesion molecules and cellular junctions, including adherent junctions, desmosomes, and tight junctions. By contrast, mesenchymal cells enable themselves to move freely in the extracellular matrix (ECM), without adhesion molecules or junctions (4). In many environments, cells change their own characteristics from epithelial-like to mesenchymal-like and vice versa, particularly in the period of embryonic morphogenesis processes. EMT is a biological process in which epithelial cells lose their polarities, intercellular junctions and epithelial-like characteristics, acquiring a less-differentiated phenotype with motile behavior. The entire process of EMT includes two aspects of cell morphology and genotype changes. It involves molecular

reprogramming of the cell, including loss of cell adhesion molecules such as E-cadherin; changing shape of cells from epithelial morphology to the spindle shape of fiber cells and cell keratin structure; and acquiring some characteristics of mesenchymal cells or fibroblasts, such as overexpression of vimentin, N-cadherin, osteopontin, Snail, slug and other interstitial proteins (5-7). Thus, cells obtain higher abilities with regard to migration, invasion, antiapoptosis and the degradation of extracellular matrix. For these reasons, EMT is crucial during the early stages of invasion and metastasis of epithelial tumors (8-9).

Molecular markers in EMT. EMT is a dynamic process comprising a number of steps and complex regulatory mechanisms, including loss of adhesion between cells, destruction of the basement membrane (BM) and ECM, reconstruction of the cytoskeleton and enhancement of migration and motility (10). Thus, the molecular mechanisms and signaling pathways involved in EMT are mainly associated with the above aspects.

E-cadherin/β-catenin complex. In the process of embryonic development, cellular adhesive molecular regulation follows the principle of specific time and space, of which cadherin is the most important. E-cadherin is a calcium-dependent transmembrane glycoprotein on the cell surface that mediates cell-cell adhesion, which can form the E-/β-/α-catenin complex by binding to β-catenin inside the cytoplasm. The complex connects directly to the actin cytoskeleton to maintain the stability of intercellular adhesion and polarity, maintaining epithelial cell integrity and normal function (11). Thus, loss of E-cadherin expression or conversion between different cadherin proteins may induce EMT. At the same time, decreasing E-cadherin expression can increase β-catenin in the cytoplasm, which binds to transcription factors TCF/LEF. Loss of E-cadherin expression in human tumors is most commonly caused by methylation of its promoter (12), phosphorylation and degradation of protein (13), and upregulation of the transcriptional repressors Snail (SNAI1), Slug (SNAI2), Sip1 (GEMIN2) and Zeb1, targeting the E-cadherin promoter (14-15). In the epithelial tumors, EMT cleaves cancer cells from the primary focal, invades the blood vessels or lymph nodes and migrates. Thus, E-cadherin is an important factor in tumor malignant transformation, invasion and metastasis.

N-cadherin. N-cadherin is s skeleton protein present in mesenchymal cells, which is expressed in nerve, musculoskeletal and hematopoietic tissue, but not in normal epithelial tissue (16). Its main function involves the induction of fibers of cell dynamic adhesion, leading to mesenchymal cell migration. Hazan *et al* suggested that conversion occurs between cadherins. Expression of E-cadherin was reduced while that of N-cadherin was increased, an important mechanism of EMT (17). Epithelial tumor cells therefore cleave from the primary focal, invade surrounding tissues, and migrate through lymphatic and blood circulation when E-cadherin expression is reduced while N-cadherin expression is increased (18).

Vimentin. Vimentin is an intermediate filament protein derived from mesenchymal cells, that is hardly expressed in normal epithelial tissue. Abnormal expression of vimentin results in the reconstruction of cytoskeletal proteins, leading to epithelial cells acquiring some characteristics of spindle-like fibroblasts, which migrate more readily. Findings of previous studies have shown the abnormal expression of vimentin in a variety of epithelial tumors such as ovarian, breast, colonic and prostate cancer, which is closely associated with cancer invasion and metastasis (19-22).

Fibronectin. Fibronectin is an extracellular matrix glycoprotein, consisting of two disulfide-bound polypeptide molecular chains, that is mainly present in mesenchymal cells of normal tissue. Fibronectin contains a different functional and structural domain, that binds to the cell surface. In previous studies it was shown that, fibronectin increases migration *in vivo* and enhances the effect on cell adhesion and spreading. Fibronectin has been shown to play a role in cell morphology, cytoskeletal organization, phagocytosis, embryonic differentiation and wound repair (23-24), which are associated with tumor invasion and metastasis.

Transcription factors. Three core groups of transcriptional factors have been consistently identified to be critical during various EMT events, and are considered the core EMT regulators. The first group is the Snail zinc-finger family, including Snail1 and Snail2, both of which directly bind to the E-boxes of the E-cadherin promoter to repress its transcription (25-26). The second group is the distantly associated zinc-finger E-box-binding homeobox family of proteins Zeb1 and Zeb2, which are also able to suppress E-cadherin transcription via a double-negative feedback loop controlling Zeb1/Zeb2 and miRNA-200 family expression (27-28). The third group is the basic helix-loop-helix (bHLH) family, including Twist1 and Twist2. Twist1 can repress E-cadherin through the induction of Snail transcription factors (29-31).

Signaling pathways and mechanisms involved in CRC EMT. EMT is a dynamic process, involving the phenotypic changes in protein, as an external adjustment. It is also associated with a variety of signal transduction pathways. Signal codes specifically bind to cell membrane surface receptors, activating transcription factors in the nucleus through a variety of signal transduction pathways (Fig. 1).

Wnt/β-catenin/LEF-1 signaling pathway. The *Wnt* gene was first identified and reported in 1982 (32). This gene forms a complex signaling pathway in tumor cells, including at least three branches of the Wnt/β-catenin/LEF-1 pathway, Wnt/ the planar cell polarity (PCP) pathway and the Wnt/ca(2+)-mediated pathway (32-34). The Wnt/β-catenin pathway, known as the 'classic Wnt pathway', plays a role in the process of EMT in CRC (35). The Wnt/β-catenin/LEF-1 signaling pathway is closely associated with EMT, which comprises the Wnt protein, frizzled protein, dishevelled protein, APC compounds, GSK-3β, axin protein, β-catenin and TCF/LEF family of transcription factors. In tumors, due to abnormal activation, the *Wnt* gene binds to the frizzled protein through the Wnt ligands, forming a receptor complex with LRP5/6, inducing GSK-3β inactivation. APC, an axin protein is activated in succession, forming the GSK3β/APC/axin complex, and reducing the phosphorylation degradation of β-catenin. Thus, large amounts of β-catenin gather in the cytoplasm and nucleus, interacting with TCF/LEF transcription factors, and inducing the transcription of target genes of Wnt, such as TNC. Phosphorylation of tyrosine in the β-catenin extremity can lead to E-cadherin/β-catenin complex decomposition, activating the Wnt signaling pathway (36-40). Thus, interaction between
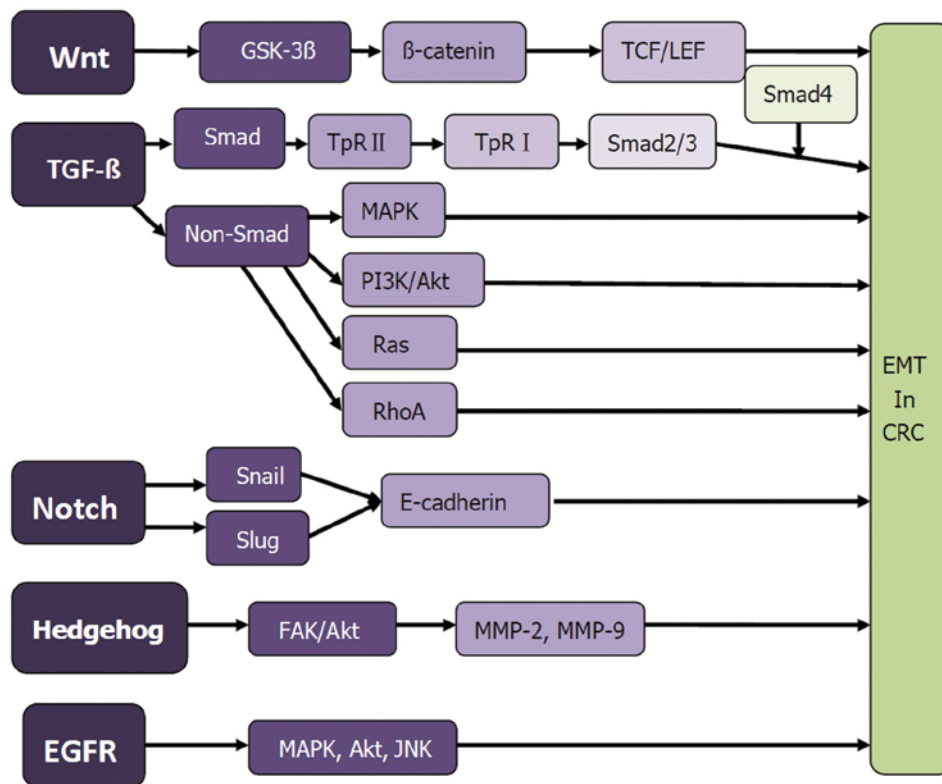
Figure 1. Signaling pathways involved in the epithelial-mesenchymal transition of colorectal cancer.

the Wnt pathway and the E-cadherin/β-catenin complex potentially promotes tumor development.

TGF-β signaling pathway. The transforming growth factor-β (TGF-β) signaling pathway appears to be a primary inducer of EMT (41). Classic SMAD and other non-SMAD pathways participate in the EMT process induced by TGF-β (41,42). In the early stage of tumor growth, TGF-β induces growth arrest and apoptosis as a tumor suppressor. In advanced stages of tumor progression, TGF-β regulates transcription through SMAD-dependent/-independent TGF-β-receptor signaling pathways, initiating cancer growth and metastasis.

SMAD pathway. SMAD and signal-regulating proteins downstream of TGF-β receptor complexes, are present in the cytoplasm, conducting signals from the cell membrane to the nucleus, and regulating gene transcription. A typical SMAD-dependent pathway involved in the EMT process is described below. TGF-β ligand binds to the type 2 TGF-β receptor, which recruits the type 1 TGF-β receptor. This receptor dimerization and phosphorylation of serine/threonine residues allows the phosphorylation of SMAD2 and SMAD3. Activated SMAD proteins dissociate from the SMAD anchor for receptor activation (SARA) protein, hetero-oligomerize with SMAD4, and translocate to the nucleus, combining with the SMAD binding element located in EMT-associated gene promoter regions. This process mediates target gene expression or repression, such as MMP-2, SPI-1 and α-SMA. As a tumor-suppressor gene, SMAD4 increased epithelial marker expression of E-cadherin, strengthening the connection between cells in CRC and SW480 (7,43-45).

Non-SMAD pathway. TGF-β signaling occurs through a number of non-SMAD pathways, including branches of MAPK, RAS and PI3K/Akt. The main MAPK signaling pathways involved in EMT include the JNK signaling, p38 MAPK and Ras/Raf/MEK/ERK pathways. MAPK is mainly involved in the regulation of cell proliferation, secretion and neuronal differentiation. In the PI3K/Akt pathway, PI3K is activated by Ras combining to P110 or interacting with growth-factor receptors with phosphorylated tyrosine residues or junction protein. Akt activates or inhibits downstream target proteins by phosphorylation, regulating cell proliferation, differentiation, apoptosis and migration. In addition to PI3K/Akt, RAS, and MAPK, TGF-β activates RhoA, c-Src, m-TOR, and protein phosphatase 2A (PP2A)/p70s6K, inducing EMT occurrence (46-51).

Notch signaling pathway. The Notch signaling pathway has been considered a traditional pathway regulating embryogenesis and tissue formation. However, it is involved in the occurrence of EMT. Notch can induce tumorigenesis, causing epithelial cells to lose their polarity, mobility and invasive ability (52). In this process, Notch regulates the expression of E-cadherin by inducing Slug activation or regulating Snail expression, thus enhancing the ability of tumor cell invasion and migration (53,54). Notch signaling pathway does not function independently, but may combine its action with other signaling pathways to induce EMT (55).

Other signaling pathways. Studies have also shown that the Hedgehog (Hh) signaling pathway and epidermal growth factor receptor (EGFR) signaling cascades are involved in the EMT process. The Hh signaling pathway is crucial in embryonic development, formation and maintaining of cancer stem cells (CSCs) and EMT. Binding Hh ligands, such as Snoic, Desert, and Indian to Patched, results in de-repression

of smoothened (SMO) and its activation, internalization and translocation to the primary cilium. The transcription of GLI target genes is followed by the activation of zinc-finger transcription factors GLI-1, GLI-2 and GLI-3 (56). The Hh signaling pathway induces cancer-cell invasion by regulating FAK/AKT signaling pathway-mediated activation, expressing MMP-2 and MMP-9 and inducing the expression of E-cadherin, Snail (57). As mentioned above, the EMT process is associated with ECM, otherwise, EGFR expression or activity may lead to cancer (58). Several auto-phosphorylation tyrosine residues in the C-terminal domain of EGFR elicits are activated by other proteins, principally the MAPK, Akt and JNK pathways (59). This stimulation promotes EMT and modulates phenotypes such as cell proliferation, migration and adhesion.

3. EMT-associated microRNA in colorectal cancer

MicroRNAs (miRNAs) are small non-coding RNAs regulating target mRNAs through post-transcription. Acting as endogenous suppressors, miRNAs inhibit gene expression through the imperfect binding of RISC subsequent to transcription. The main aim of miRNA is the degradation or translational inhibition of target mRNA (60). Therefore, miRNA affects cell processes, including cell differentiation, proliferation, metabolism and apoptosis by regulating one or two key target genes (61). miRNAs participate in tumor progression by acting as promoters or suppressors. miRNAs are also important in the regulation of cancer EMT, including CRC.

miRNA and cancer EMT

miRNA. miRNA was initially identified lin-4 in *caenorhabditis elegans* (62). However, extensive studies on miRNA were initiated following the identification of Let-7 (63). miRNAs are highly conserved, with tissue- and sequence-specific expression (64-66). The formation of mature miRNA binds to the imperfect complementary site of target mRNA via base pairing. When miRNA is fully complementary to the target site, the combination of these miRNAs often leads to the degradation of target mRNA through a RISC complex. When the miRNA and target mRNA sequences degree of matching is low, the translation process is inhibited (67,68). miRNAs have been associated with various types of cancer. Approximately 50% of miRNAs in the genome were found to bind to fragile sites in tumors (69), suggesting that miRNAs play a critical role in tumorigenesis and metastasis, including the process of EMT.

miRNAs in cancer EMT

miRNAs act as tumor suppressors in EMT. In 2007, Hurteau *et al* found that the expression of miRNA-200c was negatively associated with E-cadherin expression in breast cells (70). miR-200 family and miR-205 were found to regulate EMT through inhibition of Zeb1 and Zeb2 E-cadherin (71,72). In another study, TGFβ2 and Zeb1 were identified as the main targets of miR-200 family and miR-205. These findings indicated a feed-forward loop showing Zeb1 and miR-200 members to be involved in EMT and cancer cell invasion (73). Findings of recent studies have shown that Let-7 is associated with cancer EMT and metastasis. Let-7 was identified as targeting HMGA2, indicating there was a relationship between miRNA Let-7 and EMT (74). Downregulation of miRNA-138

is associated with interstitial cell-like transformation, and can enhance the metastasis and invasion of tumor cells including EMT with a decreased expression of E-cadherin, and enhanced expression of vimentin (75). Newly identified miRNAs, including miR-23b, miR-29c, miR-203, miR-448 and miR-194, similarly inhibited the EMT process (76-78).

miRNAs act as oncogenes in EMT. Overexpression of miR-10b, upregulated by EMT transcription factor Twist, located in the HOX gene cluster, is associated with invasiveness and metastatic potential in breast cancer (79). Results of studies, showed that miR-155 expression was increased in TGF-induced EMT in tumors, by SMAD4-regulated transcription activity. Breast cancer cells with miR-155 downregulation did not effectively induce EMT (80). In addition, miR-21 can promote the breast cancer EMT through overexpression of the SMAD-dependent transcription regulation matrix. By suppressing TIAM1 expression, miR-31 significantly enhanced CRC cell migration. It also induced cancer cell EMT process by activating transcription factors AP1 and Zeb1, located downstream of the TGF-β signaling pathway (81-83). miR-9, miR-661, miR-369-5p, miR-370, miR450a and miR-542-5p also promoted EMT tumor (84-86).

miRNA regulation of EMT in CRC. miRNAs are involved in the CRC EMT, partly by regulating the expression of oncogenes and tumor suppressors and partly by functioning as oncogenes or tumor suppressors themselves (87). These important regulatory functions of miRNA-activated upstream factors, promoted the CRC EMT process. (Fig. 2)

miR-200 family. As mentioned earlier, the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) plays a key role in cancer EMT, including CRC. miR-200 family members were found to enhance E-cadherin expression by targeting complementary sites in the 3-UTR of Zeb1 and Zeb2. Loss of the basement membrane (BM), EMT was considered a key step in induction of metastasis. Zeb1 was found to be the key transcriptional repressor of BM components in CRC (88,89). In addition, Zeb1 inhibits cell polarity factors and Lgl2 expression in CRC, which is critical for the epithelial phenotype (90). Transforming growth factor-β (TGF-β), an EMT activator, is produced by tumor cells, triggering the expression of Zeb1/2, an upstream master regulator of EMT progression. Zeb1 directly suppressed the expression of miR-141 and miR-200c (on human chromosome 12), downregulating some essential features of EMT. After knockdown of Zeb1 in CRC, an increased expression of miR-141 and miR-200c can induce the epithelial phenotype, increase cell-cell adhesion, induce E-cadherin expression, and reduce cell migration and invasion (73). In addition, second miRNA clusters (miR-200b, miR-200A, and miR-429) of the miR-200 family also contain highly conserved putative Zeb1 binding sites in the upstream sequence (91). Results of those studies showed that, Zeb1 can trigger a miRNA-mediated feed-forward loop stabilizing the EMT and promoting cancer-cell invasion. Otherwise, strong induction of miR-200 may promote the differentiation and inhibition of epithelial-mesenchymal-specific gene expression, by downregulating Zeb1 and Zeb2 conversely. Presumably, Zeb and the miR-200 family participate in a double-negative feedback loop stabilizing differentiation along the EMT-MET axis (92).
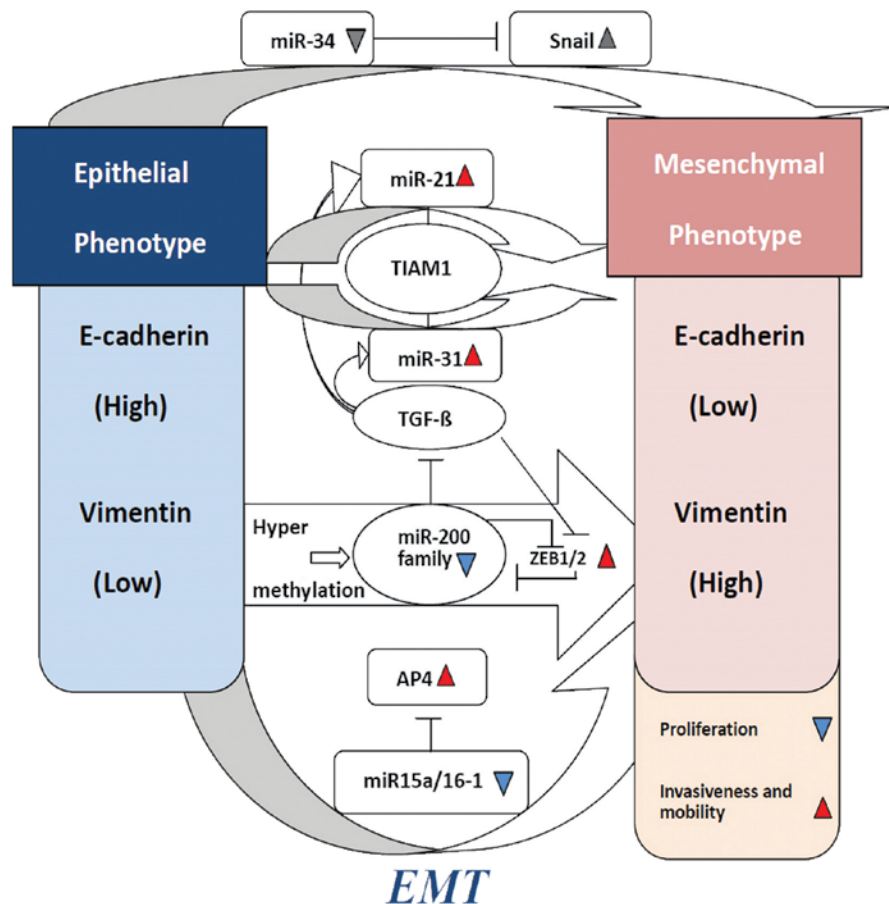
Figure 2. Regulation of epithelial-mesenchymal transition by miRNAs in colorectal cancer. Several miRNAs have been shown to directly or indirectly regulate the activities of cell phenotypic transition.

miR-21 and miR-31. miR-21 is located on human chromosome 17q23.2. In CRC, this genomic region always shows copy number gain, which is frequently observed in metastatic tumors. This observation suggests miR-21 is important in the CRC metastatic pathway. miR-21 encodes a single hairpin and is regulated by its own promoter containing binding sites for transcription factors, such as AP-1 and PU.1 (93). Upregulation of TGF-β1 increased the expression of miR-31, resulting in a higher percentage of cells using a 'spreading' morphology. The levels of miR-21 and miR-31 were markedly elevated under the synergistic actions of TGF-β/TNF-α. The two miRNAs are likely to have a number of different direct targets. They converge on TIAM1, a guanine nucleotide exchange factor (GEF) for the Rac GTPase-regulating migration and invasion of various cancer cells including CRC cells (81). It has been identified that, high miR-21 with low Integrin-β4 (ITGβ4) can be exclusively expressed in polarized epithelial cells, and the level of PDCD4 expression was able to regulate EMT in CRC (94).

miR-34. The miR-34 family includes miR-34a, miR-34b and miR-34c. The miR-34 family participates in cell cycle progression, senescence and cell apoptosis (95-97). The members inhibit EMT by downregulating the expression of Snail, one of the EMT-inducing transcription factors (EMT-TF) (98,99). It has been revealed that IL-6 induces EMT, invasiveness, and the metastatic properties of CRC cells. By directly repressing miR-34a, miR-34a can suppress tumor progression by inhib-

iting the IL-6R/STAT3/miR-34a feedback loop. Thus it can promote and maintain EMT, invasiveness, and metastasis in CRC (100).

miR-15a/16-1. miR-15a and miR-16-1 are located on human chromosome 13q14.3, which is highly conserved (101). Emerging evidence has shown that they target the 3'-untranslated region (3'-UTR) of the transcription factor AP4, which is downregulated by p53. AP4 directly represses E-cadherin via a non-canonical AP4-binding motif and induces N-cadherin, mediating EMT in CRC. This finding suggests that miR-15a/16-1 and AP4 are involved in a double-negative feedback loop, stabilizing epithelial and mesenchymal states. Respectively, they may determine metastatic prowess in CRCs (102,103).

4. Strategies for therapy targeting EMT in colorectal cancer

Improved understanding of the pathology of molecules in cancer had led to the development of a number of target drugs, which have demonstrated improved outcome of cancer patients with metastasis. However, the aim of ongoing animal experiments and clinical trials is to identify further drugs (Fig. 3).

Targeting EMT-associated molecules and pathways in CRC. As shown in Fig. 1, EMT-associated pathways provide therapy targets for CRC patients.
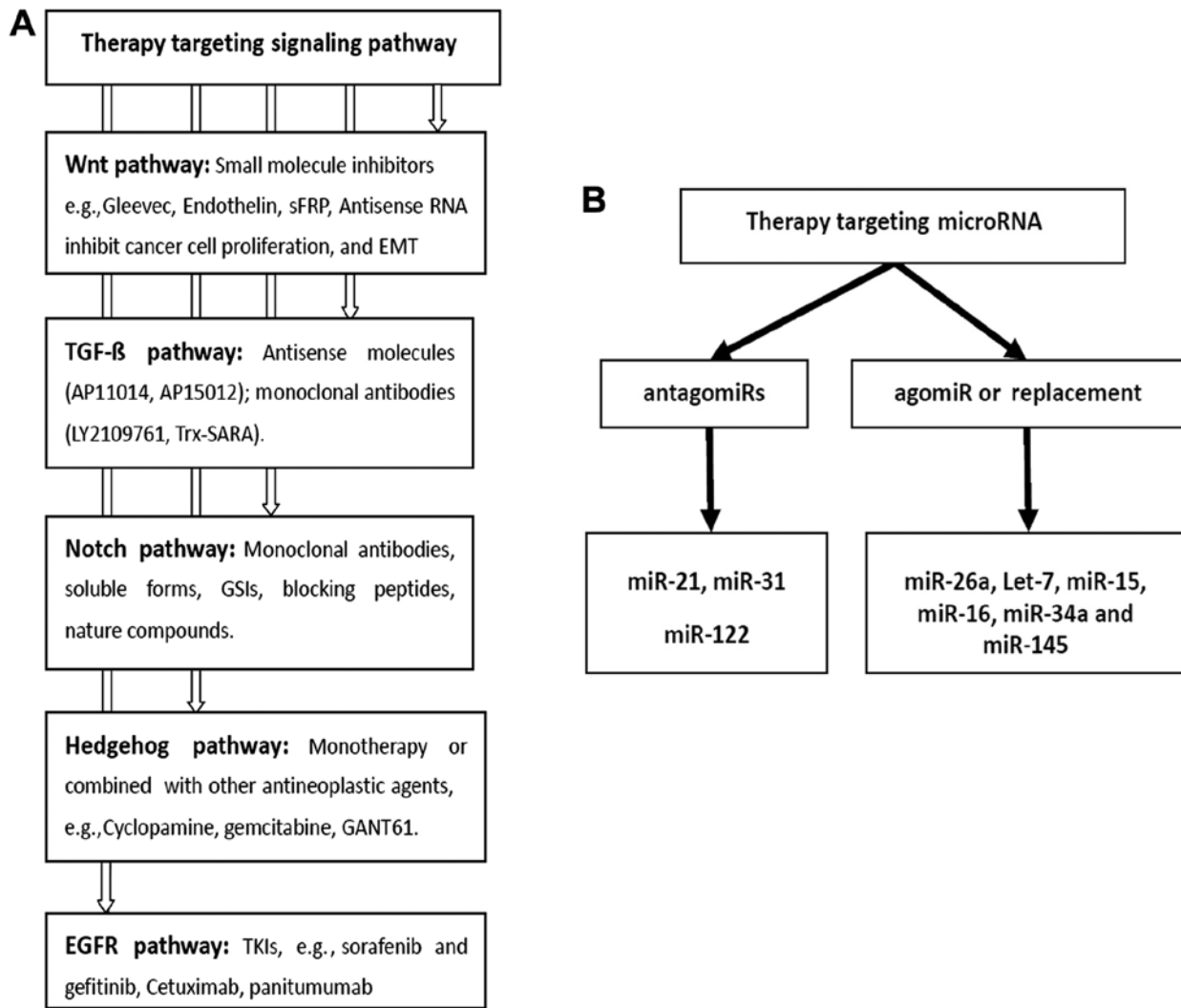
Figure 3. Targeting therapies in colorectal cancer cell lines. (A) Targeting point in the epithelial-mesenchymal transition-associated signaling pathway. (B) Restoration of silenced miRNAs and blocking of oncogenic miRNAs.

Drugs targeting Wnt/β-catenin signaling pathway. The Wnt signaling pathway is closely associated with the occurrence and development of CRC. *In vitro* experiments on drugs targeting the Wnt signaling pathway are currently ongoing. Non-steroidal anti-inflammatory drugs (NSAIDs) including Aspirin (104), Sulindac (105), Celecoxib and rofecoxib (106) downregulate the β-catenin level by inducing β-catenin phosphorylation and degradation or inhibiting the transcription of target genes.

Small molecule inhibitor is a research hotspot on molecular targeting therapy. Gleevec, a type of tyrosine kinase inhibitor, is a small molecular protein, which can significantly inhibit CRC cell proliferation, reduce transcription activity, and induce β-catenin migration from the cell nucleus to the membrane (107). Endothelin (ET) can promote the degradation of β-catenin, inhibit target-gene cyclin D1 promoter activity, and induce cell-cycle arrest, apoptosis and tumor angiogenesis (108). The sFRP Wnt pathway inhibitor can suppress CRC cell proliferation and transformation ability (109). Antisense RNA can silence β-catenin expression or inhibit CRC cell proliferation and differentiation by eradicating the TCF/β-catenin complex in the Wnt pathways (108). Thus, the

Wnt signaling pathway is expected to offer a new effective method for the treatment of CRC.

Drugs targeting the TGF-β signaling pathway. As we described earlier, the TGF-β signaling pathway plays a key role in CRC EMT. Therapeutic strategies against TGF-β are effective methods that are classified into the ligand, ligand-receptor and intracellular levers (110). Powerful anti-TGF-β strategies have been developed and tested in pre-clinical studies as well as clinical trials. Antisense molecules can prevent TGF-β synthesis on the ligand lever by binding to specific mRNA. AP11014 and AP15012 are antisense molecules used in pre-clinical trials for treatment of non-small cell lung cancer, prostate carcinoma, CRC and MM, respectively (111). By blocking downstream signaling, monoclonal-neutralizing antibodies acting as receptor kinase inhibitors were more efficient as compared to ligand traps or antisense molecules (110). LY2109761 is a small molecule inhibiting the kinase activity of TβR I and TβR II. This compound inhibits metastatic formation in mouse models of breast cancer, CRC and pancreatic cancer (112-114). The Trx-SMAD anchor for receptor activation (Trx-SARA) is an example of a peptide aptamer, which reduces the levers of TGF-β-induced SMAD-2/-3 in the complex (115), and inhibits

EMT following TGF-β stimulation in breast cancer epithelial cells (116). Ongoing pre-clinical trials are currently focused on other drugs targeting the TGF-β signaling pathway. Future studies on drug development targeting EMT in CRC remain to be conducted.

Drugs targeting Notch signaling pathway. The Notch pathway has great potential as a new cancer treatment target. Various Notch inhibitors, including monoclonal antibodies against Notch receptors or ligands, soluble forms of the extracellular domain of Notch receptors or ligands, GSIs, blocking peptides, and nature compounds have been identified (117-124). GSIs are the most widely studied Notch receptors, having potential for bio-distribution and pan-Notch inhibition (125). GSIs are utilized in Phase I clinical trials as a monotherapy or in combination with other drugs (126). Therefore Notch is a potential antitumor therapeutic target and its implementation should occur subsequent to in-depth study with a focus on the Notch signaling pathway.

Drugs targeting Hedgehog signaling pathway. Currently, all of the small-molecules Hedgehog (Hh) pathway therapeutics in clinical trials are designed to target Smoothened (SMO). The reagents act as monotherapy or are combined with other antineoplastic agents for the treatement of various tumors including colon cancer. The Hh inhibitor cyclopamine is an antagonist of the Hh signaling pathway component SMO. Studies have shown that cyclopamine can eliminate pancreatic cancer metastasis (127). Cyclopamine inhibits the invasion of pancreatic cancer by suppressing EMT, upregulating E-cadherin and downregulating Snail. In an orthotopic xenograft model, the combination of cyclopamine and gemcitabine can restore metastases (128). GLI was found to be activated by other signaling pathways, such as Ras, TGF-β and Wnt signaling pathways. In human colon carcinoma cells, specific targeting of GLI, which is downstream of SMO, can induce cancer cell death (129). GLI has a broader role, blocking the downstream gene transcription and activation (130-131).

Drugs targeting the EGFR signaling pathway. The epidermal growth factor receptor (EGFR) is known to be overexpressed in CRC EMT, while anti-EGFR therapy is considered effective. Tyrosine kinase inhibitors (TKIs), such as sorafenib and gefitinib, were designed to block the cascade of reactions which are crucial to tumor development and survival (132). Sorafenib is currently being tested in phase II trials of CRC. A recent study has indicated that the ability of TKIs strongly suppress P13K signaling in CRC, suggesting that such treatments have great therapeutic value (133). Cetuximab and panitumumab, monoclonal antibodies to EGFR, were approved by the FDA for the treatment of CRC in 2004 and 2006, respectively. The two antibodies prevent EGFR auto-phosphorylation by binding to the extracellular domain. They also inhibit activation of the MAPK and P13K downstream cell signaling pathways (132). In addition, the two drugs exhibited significant antitumor activity, although limitations and side effects were observed (134-137).

Targeting miRNA in CRC EMT. miRNAs play a key role in proliferation, cell cycle, evasion of apoptosis, invasion, migration and EMT. One of the most appealing properties of miRNA as therapeutic drugs in comparison with targeting single genes is their ability to target multiple molecules in the network. Abnormal expression levels of miRNAs show that, miRNAs can be used as drugs in CRC. Aiming at an abnormally high expression of onco-miR in tumor, small molecule drugs known as 'antagomiRs' have been designed, to knock out abnormal miRNAs or suppress expression of miRNAs. Targeting low or no expression of tumor-supressor miRNAs, corresponding exogenous miRNAs are known as 'agomiRs'. AgomiRs can be guided into the patient's body, restoring or improving the function of miRNAs in tissues (138).

Strategies for antagomiRs

Anti-miRNA oligonucleotides. Anti-miRNA oligonucleotides (AMOs) are single-stranded oligonucleotides consisting of 22-25 nucleotides, inhibiting miRNAs (139,140). Because of not being chemically decorated, antisense oligomeric nucleotide chain is not stable, almost invalid in the body. AMOs are often modified chemically, such as 2'-O-methylation (141), 2'-O-methoxyl ethylation and locked nucleic acids (LNAs) (142,143). The modification of AMOs can suppress miRNAs effectively. Experimental results showed that injecting inhibitors in mice specifically silenced their target miR-122 or miR-16 in liver and other organs, such as kidney, lung, heart and colon etc. (144). LNA-modified anti-miRNA oligonucleotides have been shown to inhibit miR-21 in breast cancer and in glioblastoma cells, resulting in the inhibition of tumor cells *in vitro* and *in vivo*. In xenograft tumor models, 2'-O-methyl-modified oligonucleotides (anti-miR-21) were injected into MCF-7 cells as instantaneous transfection. The results showed that the transfected tumor cells were reduced by ~50%. The study proved that 2'-O-methyl-modified oligonucleotides were a potential cancer treatment candidate (145,146). Of note, inhibition of miR-21 expression in extrahepatic cholangiocarcinoma cells can increase the drug sensitivity of gemcitabine (147). Studies showed that the miRNA-based antitumor treatment combined with chemotherapy can improve treatment efficiency. However, the mechanism of downregulating the miRNA activity through AMOs inhibitors remains to be elucidated. The mechanism may occur by blocking miRNA biosynthesis via interaction with prior pre-miRNAs, or binding to mature miRNA to exert its role in the RISC compounds.

Other novel inhibitions. miRNAs sponges, miRNA-masking antisense oligodeoxynucleotides and small molecule inhibitors also suppress the overexpression of miRNA. Synthetized chemically, miRNAs sponges is a single-stranded RNA (148), blocking interactions between endogenous miRNA and its target mRNA (149). The sponge-mediated inhibition of the anti-metastatic miR-31 has been successfully applied in an orthotopic breast-cancer model. Results of that study demonstrated that a significant induction of lung metastases appeared, even with use of non-aggressive breast cancer cells (138-150). miRNA-masking antisense oligodeoxynucleotides bind perfectly to sites of complementary sequence, forming dimers with higher affinity, closing the sites of miRNAs through prior combining with target mRNA, and inhibiting the combination of miRNA and target mRNA (151). Other miRNA inhibitors, such as azobenzene, can block the transcription of miR-21, providing evidence that small organic molecules can be used in the miRNA context as well (152).

Strategies for agomiR or replacement. Treatment strategies, such as targeting low expression of miRNAs in CRC EMT, replacement therapy or agomiRs to restore physiological

miRNA levels, can be applied. Current replacement therapy includes gene therapy and transfection of miRNA mimics. The approach of gene therapy involves generation of the pre-miRNA hairpin structure, transfecting and ensuring long-term and stable expression of mature miRNA. In addition, miRNA mimics, small double-stranded RNAs synthetized chemically, are widely used. It can stimulate active endogenous miRNA molecular and target-specific mRNA through transfection *in vitro* or in systemic administration *in vivo* by liposomes or nanoparticles polymer. Through virus-mediated miRNA re-introduction of miR-26a in liver cancer mouse, investigators observed the inhibition of cancer cell proliferation and induction of tumor-specific apoptosis (153). *In vitro*, Let-7 inhibits cancer cell growth in lung cancer cells (154). In an autochthonous NSCLC mouse model, the overexpression of Let-7 through lentiviral vector can lead to a significant growth reduction (155). Another example of miRNAs replacement therapy is miR-15 and miR-16, targeting apoptotic inhibition gene *Bcl-2*. The re-introduction of miR-15a/miR-16 into prostate carcinoma xenografts by lentiviruses can also reduce tumor growth (156). In addition, using lipid-based delivery reagents, miR-34 led to the inhibition of non-small lung cancer and reduction of metastatic tumor load in the lung, basing on survivin inhibition (157,158). The expression lever of miR-145, and downregulation in tumor tissues of CRC patients were increased *in vitro*. Thus, cell proliferation was reduced while sensitivity to radiotherapy was increased (159).

miRNAs and antisense miRNA drugs are currently at the pre-clinical stage. Owing to poor stability, low efficiency of transmission and toxic reaction, the clinical application of targeting miRNA requires additional investigation.

5. Conclusion and perspective

In the present review, we attempted to summarize the molecules, mechanisms and corresponding targeted therapies involved in the EMT process. In the past few years, it has become clear that EMT can reprogram normal epithelial cells. The process occurs systematically at the transcriptional, post-transcriptional, translational and post-translational levels. An improved understanding of the EMT process in CRC may lead to the identification of novel drug-targeted therapies and approaches.

Acknowledgements

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