microRNAs in esophageal cancer (Review)

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Abstract. Esophageal cancer (EC) is one of the most common malignant tumors worldwide. EC is usually diagnosed at a locally advanced stage or at a stage with involvement of lymph nodes. Despite aggressive treatment, the overall five-year survival rate remains poor. microRNAs (miRNAs) are small, non-coding endogenous RNAs that negatively regulate gene expression at the post-transcriptional and/or translational level. Accumulating evidence suggests that the deregulation of miRNAs not only results in cancer progression, but also directly promotes tumor initiation. Previous studies found that miRNAs are frequently deregulated in EC, indicating that miRNAs are important in tumorigenesis. In this review, we summarize ther-ecently recognized miRNA expression and its impact on the biology of EC and the potential applications for EC.

Contents

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1. Introduction

The incidence and mortality of esophageal cancer (EC) are high, and rank eighth and sixth, respectively, out of all types of cancer, affecting more males than females (1). Esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (EAC) are the two main subtypes of EC in terms of pathological characteristics. ESCC remains the dominant subtype of EC. EC is usually diagnosed at an advanced stage or with lymph node metastases. Due to the potential characteristics of invasion and metastasis in esophageal carcinoma cells, which are important prognostic factors, the overall 5-year survival rate is poor despite advanced treatment (1,2). Improved understanding of the biological behavior in EC is important for the development of new therapeutic strategies and diagnostic methods.

microRNAs (miRNAs) are non-coding single-stranded, endogenous RNA molecules, approximately 21-25 ribonucleotides in length and highly conserved in evolution, which negatively regulate gene expression at the post-transcriptional and/or translational level. miRNAs are important in diverse biological processes including development, cell differentiation, proliferation, apoptosis, hormone secretion, tumor formation and drug resistance (3-5). Experimental evidence revealed that the majority of human miRNA genes are located at fragile sites and genomic regions involved in cancer, functioning as oncogenes or tumor suppressor genes (6-8). In this review, we aim to summarize the recognized miRNAs that play roles in the development of EC and the mechanism by which miRNAs are involved in EC.

2. miRNA synthesis and biological properties

miRNAs form clusters transcribed as polycistronic transcripts by RNA polymerase II and/or III, which undergo sequential steps of maturation. Clustered miRNAs may be transcribed from a single transcription unit as polycistronic primary-miRNAs (pri-miRNAs), which have the cap structure (7MGpppG) and polyadenylation tail (polyA tail). The pri-miRNAs are processed in the nucleus into pre-miRNAs by the microprocessor complex, which consists of the RNase III endonuclease Drosha and its cofactor, the double-stranded RNA binding protein Pasha/DGCR8. The pre-miRNAs are approximately 70 nucleotides in length, and are then transported into the cytoplasm by Ran-GTP and Exportin 5. The cytoplasmic RNase III Dicer cleaves pre-miRNA into double-stranded RNA, which is 21-25 nucleotides in length.

The double-stranded RNA is unwound to produce single-stranded miRNA in the RNA-induced silencing complex (RISC), where the mature miRNAs and its target mRNA interact (9-17). The miRNA gene mutations, translocations or impaired biosynthesis processes lead to changes in the miRNA expression level.

In 1993, the first miRNA was discovered through the study of the heterochronic gene lin-14 in worms, which was designated as lin-4 (18). Over 1000 miRNAs have been identified in animal genomes through cloning and bioinformatics techniques, approaching approximately 3% of all human protein-coding genes and controlling a wide range of gene regulatory networks (19). Therefore, the dysregulation of miRNAs may contribute to the initiation and development of cancer.

3. miRNAs as tumor suppressor genes

miRNAs functioning as tumor suppressor genes often have low expression in tumors, whereas target genes are highly expressed. For example, the miR-15a and miR-16a clusters at chromosome 13ql4 were deleted or downregulated in many adult B-cell chronic lymphocytic leukemia patients. The anti-apoptotic factor BCL2, which is target of miR-15a and miR-16a, was negatively regulated by these two miRNAs; therefore, miR-15a and miR-16a downregulation could result in higher BCL2 protein levels and anti-apoptotic activity (20,21). Another important suppressive miR is let-7, which was generally downregulated in lung cancer tissue while the let-7 expression level was positively correlated with the prognosis of patients. However, let-7 overexpression significantly inhibited tumor cell growth in the lung cancer cell line A549, indicating that let-7 plays a role in the development of lung cancer (22,23). Downregulation of miR-29b was also reported in the KMCH cell line of bile duct cell carcinoma and 1/3 of human cholangiocarcinoma, while myeloid leukemia-1 (Mcl-1), its target gene, was overexpressed, ultimately resulting in suppressed apoptosis of cholangiocarcinoma cells (24). miR-122 was reported to have suppressive roles by negatively regulating cyclin G1 and reducing proliferation and inducing apoptosis *in vitro* (25). The genetic region containing miR-34a is often deleted in many tumors, and other studies have found that the p53-dependent inhibition of miR-34a induced the overexpression of BCL2, resulting in the inhibition of apoptosis and cell proliferation (26,27). A similar situation was applied to miR-205, which was reported to directly target ErbB3 and vascular endothelial growth factor A (VEGF-A), which reduced the level of ErbB3 protein, acting as a potential tumor suppressor by inhibiting proliferation (28).

4. miRNAs as oncogenes

As miRNAs negatively regulate gene expression at the post-transcriptional and/or translational level, changes of the expression level of these miRNAs can be tumorigenic, as well as oncogenic. Typical examples are miR-17-92, which is located at 13q31-32, a region commonly amplified in B-cell lymphoma, and usually upregulated. This gene contributes to the development of the tumor by affecting the cell cycle, apoptosis, angiogenesis and metastasis, which suggests that miR-17-92

may act as an oncogene (oncomiRNA) by regulating the transcription factor E2F1 protein and myc protein (29-34). The dysregulation of miRNAs may be involved in cancer progression. Overexpression of miR-21 was found to be involved in cell differentiation, proliferation and apoptosis in various tumor types, and therefore miR-21 functions as an oncogene by directly downregulating the tumor suppressor gene tropomyosin 1 (TPM1) (35). The role of miR-10b as an oncogene in lung cancer was supported by experiments showing that the expression of miR-10b was significantly higher following induction of the transcription factor Twist, and this resulted in the target gene HOXDIO of miR-10b being downregulated and the expression of transfer-related protein RHOC being upregulated (36). With the rapid development of miRNA detection technology, a better understanding of the roles of miRNAs in the progression of cancer is likely to improve our knowledge of the pathogenesis of cancer, serving in molecular diagnosis, risk assessment and therapeutic approaches for cancer in the future.

5. miRNAs in EC

Esophageal carcinogenesis is a multi-stage process, involving a variety of changes in gene expression and DNA structure. miRNAs are efficient regulators of gene expression that are important in the initiation and progression of a variety of tumors, including EC. miRNA expression profiles performed by miRNA array are capable of distinguishing esophageal tumor histologies and discriminating normal tissue from tumor tissue. miRNA expression is also useful for identifying Barrett's esophagus (BE) patients at high risk of progression to adenocarcinoma (AC) (37).

6. miRNAs serving as diagnostic and prognostic biomarkers in EC

The overall prognosis of EC is poor despite advanced treatment. The identification of new diagnostic and prognostic biomarkers are crucial in the clinic and for treatment of the disease. Due to the important regulatory roles of miRNAs in the initiation and progression of cancer and their high tissue specificity, miRNAs may be potential biomarkers for diagnosis, prognosis and prediction of response to therapy in EC.

Guo *et al* found aberrant expression of 46 miRNAs in EC tissues, of which 7 miRNAs (upregulation of miR-25, miR-424 and miR-151; downregulation of miR-100, miR-99a, miR-29c and miR-140) can be used as biomarkers to distinguish malignant EC lesions from adjacent normal tissue. Moreover, miR-335, miR-181d, miR-25, miR-7 and miR-495 were associated with the general pathological type of EC (fungating vs. medullary); miR-25 and miR-130b were associated with the degree of differentiation of EC and the expression level of miR-103/107 was negatively correlated with the survival rate of EC patients and may be used for early diagnosis as well as gene therapy targets for EC (38). The expression of certain miRNAs showed pathological cell type specificity. By analyzing the expression of miRNAs in EC, Feber *et al* found that miR-203 and miR-205 were downregulated compared with normal epithelium in EC, while miR-21 was overexpressed in the two types of EC. miR-200c, miR-194 and miR-192 were shown to be downregulated in ESCC, but were significantly

upregulated in EAC (37). Mathé *et al* (39) have demonstrated that the overexpression of miR-21 in non-cancerous tissue of ESCC and downregulation of miR-375 in cancerous tissue of EAC with BE were markedly associated with worse prognosis. miR-196a was highly expressed in EAC, BE, benign and malignant junctions and highly malignant tissue and may be used as a biomarker for screening EC (39-41). Among others, the overexpression of miR-129 was identified as a significant and independent prognostic factor in surgically treated ESCC patients (42). The expression level of miR-1322 was higher in ESCC tissue and was able to distinguish ESCC samples from healthy samples (43). miR-31 and miR-142-3p expression were correlated with histological differentiation, and a high miR-142-3p expression was associated with a poor prognosis and may be identified as a potential independent prognostic factor in ESCC (44). The expression profiles of miRNA were altered in progressive stages of neoplastic development and the expression level of miR-31 and -31* was frequently downregulated in HGD and EAC, suggesting miR-31 and -375 as novel biomarkers for cancer progression, in BE (45). Other significant miRNAs with dysregulated expression are miR-16-2 and miR-30e, which were associated with shorter overall and disease-free survival in all EC patients and may also be potential prognostic biomarkers (46).

Several studies have demonstrated that miRNAs are consistently detectable in the circulation, as miRNAs have the ability to resist endogenous ribonuclease activity. Moreover, the expression level of serum miRNAs is reproducible and consistent among individuals, thus circulating miRNAs may be used as potential biomarkers for the identification of EC patients. To demonstrate this point, the expression level of miR-21 was upregulated and miR-375 was downregulated in the plasma of ESCC patients compared with healthy controls and the value of the area under the receiver-operating characteristic curve (AUC) was 0.816; patients with a high plasma level of miR-21 have greater vascular invasion and showed a high correlation with recurrence, indicating that circulating miRNAs may be useful as tumor markers for ESCC (47). A panel of 7 serum miRNAs (miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a and miR-127-3p) were upregulated in ESCC and the area under the ROC curve for the selected miRNAs ranged from 0.817 to 0.949 and could clearly distinguish stage I/II ESCC patients from controls (48). Supporting the role of miRNAs in the circulation, Zhang *et al* have found that miR-31 levels were significantly higher than controls in 523 serum ESCC samples. Additionally, patients with higher levels of serum miR-31 had a poorer prognosis for relapse-free survival. Those authors concluded that miR-31 is capable of serving as a potential diagnostic and prognostic biomarker for ESCC (49). miR-1322 was significantly highly expressed in ESCC serum samples and can be used to distinguish ESCC from healthy patients (43). Thus, the circulating miRNAs may be used as potential biomarkers not only for diagnostic, but also for prognostic and predictive markers in EC.

7. The role of miRNAs in invasion and metastasis of EC

Invasion and metastasis are the two main reasons responsible for cancer-related mortality. The molecular mechanisms of invasion and metastasis are associated with cell-cell and cell-matrix adhesion, with the degradation of the extracellular matrix, and with the initiation and maintenance of early cell growth at the new site. Recently, miRNAs have emerged as post-transcriptional regulators of cancer invasion and metastasis by acting on multiple signaling pathways involved in invasion and metastasis. Analyzing the correlation between miRNA and cell motility and invasiveness in EC is now essential in order to understand the molecular mechanism of cancer invasion and metastasis.

Using a gain-of-function assay, Kano *et al* (50) revealed that miR-143, miR-145 and miR-133a/b significantly inhibited cell growth and invasion by targeting the FSCN1 gene (51). Matsushima *et al* proved that miR-10a was downregulated in EC and affected cell migration and invasion by targeting homeobox genes (52). A significant correlation of miR-10b overexpression with strong cell motility and invasiveness by suppression of the tumor suppressor gene KLF4 was observed (53). miR-92a was overexpressed in tumor tissues, which was significantly correlated with the status of lymph node metastasis and TNM stage, and miR-92a enhanced cell migration and invasion at least partially by suppression of CDH1 expression (54). ARTN, a tumor metastasis-related neurotrophic factor, is a direct target of miR-223, and it was overexpressed in EC and negatively associated with miR-223. On further study, it was demonstrated that the overexpression of miR-223 decreased the expression of ARTN and decreased cell migration and invasion (55).

Several studies analyzed the correlation between the expression levels of miRNAs and the clinicopathological parameters of ESCC patients and found that the expression level of miRNAs were correlated with tumor location and lymph node status and recurrence of metastasis, including miR-21, miR-145, miR-205, miR-99b, miR-199a-3p, miR-199a-5p, miR-126 and miR-16-2 (46,56-60). Zinc finger E-box-binding (ZEB) proteins ZEB1 and ZEB2 are transcription factors essential in TGF-β-mediated senescence, epithelial-to-mesenchymal transition (EMT) and cancer stem cell functions, whereas the miR-200 family are negative regulators of ZEBs, and the downregulation of the miR-200 family negatively regulated ZEBs, which were involved in anchorage-independent colony formation, invasion and TGF-β-mediated EMT in ESCC (61). Transfection with a precursor or inhibitor of miR-205 was found to inhibit cell invasion and migration, whereas knockdown of miR-205 expression in ESCC cells substantially enhanced the expression of zinc finger E-box binding homeobox 2, accompanied by the reduction of E-cadherin (62). Kong *et al* demonstrated that miR-375 was capable of inhibiting clonogenicity, cell motility and metastasis in ESCC (63). miR-31 was upregulated in ESCC and was shown to promote ESCC migration and invasion, by a mechanism in which miR-31 may interact with tumor suppressor genes of epithelial membrane protein 1 (EMP1), kinase suppressor of ras 2 (KSR2) and regulator of G-protein signaling 4 (RGS4) (49).

8. The role of miRNAs in the growth of EC

Tissue homeostasis relies on an intricate balance between cell proliferation and cell death, the latter of which usually occurs in the form of apoptosis. Uncontrolled cell proliferation or

evasion of apoptosis are common features of malignancy, which are likely to induce unlimited cell growth. Dysregulation of miRNA has been shown to affect tumor growth by influencing the biological processes of cell proliferation and apoptosis in the tumorigenesis of EC.

F-box and WD repeat domain-containing 7 (FBXW7) is a cell cycle regulatory gene whose protein product ubiquitinates positive cell cycle regulators such as c-Myc, cyclin E and c-Jun, and acts as a tumor-suppressor gene. miR-223 expression was significantly higher in cancerous tissues and had an inverse relationship with FBXW7 protein in ESCC (64). miR-375 was able to inhibit clonogenicity, cell proliferation and tumor formation in mice via the downregulation of IGF1R (63). miR-31 was upregulated in ESCC and promoted colony formation through the EMP1, KSR2 and RGS4 genes (49). Ding *et al* have demonstrated that miR-29c is frequently downregulated in ESCC tissues and cells and it is capable of inhibiting proliferation of ESCC cells *in vitro* and *in vivo* by inducing cell cycle G(1)/G(0) arrest, mainly through the modulation of cyclin E expression (65). Kan *et al* found that miR-106b-25 may enhance cell proliferation, inhibit apoptosis, promote cell cycle entry and subsequently promote tumorigenesis in ESCC (66). The mutation of tumor suppressor gene p53 is closely correlated with human cancer. Wild-type p53 is a negative regulator and plays important biological roles in cell cycle regulation, cell differentiation, apoptosis and aging. The p53 network is capable of activating a number of transcriptional targets to inhibit tumor formation. A previous study found that p53 regulated the miR-34 gene family and miR-34 activation is capable of not only inducing cell cycle arrest and cell apoptosis, but also playing a coordinated role in the process of inhibiting cell proliferation (67). p53 is also able to induce the expression of miR-215 and miR-192 and regulate the cell cycle. Downregulation of miR-215 would directly weaken the ability of cell proliferation in EC (68).

Large tumor suppressor homolog 2 (LATS2) belongs to the LATS tumor suppressor family and is capable of regulating the cell cycle and apoptosis. miR-373 and LATS2 showed a negative correlation in ESCC. miR-373 was an oncogene that inhibited the expression of the tumor suppressor gene LATS2 (69). Annexin A1 (ANXA1) mediated apoptosis and inhibited cell proliferation. miR-196a inhibited ANXA1 expression, thus the cell proliferation and inhibition of apoptosis led to EC (70). PDCD4, a new tumor suppressor gene, not only regulated programmed cell death, but also inhibited tumor cell growth. miR-21 was negatively correlated with the expression of PDCD4 in ESCC and may play a role in abnormal proliferation by inhibiting the tumor suppressor gene PDCD4 (58). Transfection with the precursor of miR-145 and miR-133a/b may inhibit cancer cell proliferation by preventing FSCN1 expression (50).

Yuan *et al* demonstrated that miR-203 was able to inhibit cell proliferation in human ESCC through the ΔNp63-mediated signaling pathway and suggested that miR-203 may be used as a therapeutic agent for ESCC (71). Inhibition of miR-17-92 cluster member miR-19a by antisense oligonucleotides (ONs) induced apoptosis and inhibited tumor growth *in vivo*, probably through targeting tumor necrosis factor-α (TNF-α) (72). A marked decrease in the level of miR-210 was observed, particularly in poorly differentiated EC, and miR-210 inhibited

cancer cell survival and proliferation by inducing cell death and cell cycle arrest in the G(1)/G(0) and G(2)/M stages (73).

9. Single nucleotide polymorphisms of miRNAs in EC

The susceptibility of an individual to environmental risk factors is associated with the incidence and prognosis of the tumor. Single nucleotide polymorphisms (SNPs) are not directly responsible for the incidence of a malignancy but they may make an individual susceptible to particular environmental factors. SNPs of miRNAs could eventually lead to a change in the amount of mature miRNA and new miRNA generation, thus affecting the susceptibility of the tumor (74).

Wang *et al* studied the genetic association between the SNP (rs11614913) in pre-miRNA-196a and ESCC susceptibility, finding that the homozygote CC of this SNP increased the risk of ESCC compared with the homozygote TT, and the risk was more evident in smokers than non-smokers (75). The SNP rs6505162, which is located in the pre-mir423 region, was associated with a reduced risk of EC. Target genes PABPC1 and FGFR2 of miR-423 were closely correlated with EC; downregulation of PABPC1 was associated with the tumor volume, tumor metastasis and low survival; and upregulation of FGFR2 was associated with the degree of tumor differentiation (76). A strong correlation was found between the G/C polymorphism (rs2910164) of miR146a and EC risk in a Han population; the GG genotype may cause a significantly increased risk of EC. In addition, this locus polymorphism was associated with TNM stage in EC patients (77). miR-200b/200c/429 were upregulated in EC and the SNP site rs1045385 (A or C allele) located in the 3' untranslated region (UTR) of the AP-2α gene, a target gene of the miR-200b/200c/429 family, may decrease the binding of miR-200b/200c/429 to the 3' UTR of AP-2α, which in turn upregulated the AP-2α protein expression (78).

10. The roles of epigenetic regulation of miRNAs in EC

Pri-miRNA has a 7-methylguanosine cap and poly(A) tail, the same as regular protein-coding genes (79), suggesting that miRNAs can be regulated by epigenetic alterations. Saito *et al* found that approximately 5% of human miRNAs were upregulated by the treatment of T24 bladder cancer cells with DNA demethylating agent and histone deacetylase (HDAC) inhibitor (80). DNA hypermethylation in the miRNA 5' regulatory region may be responsible for the downregulation of miRNA in tumors (81).

It was reported that miR-205 was upregulated and miR-10a was downregulated in ESCC cell lines. Further functional analysis revealed that miR-10a was directly regulated by DNA demethylation and the histone deacetylase inhibitor. miR-205 regulated cell invasion and metastasis through the inhibition of E-cadherin expression, in turn affecting epithelial-mesenchymal transition (EMT) (52). Kong *et al* and Li *et al* demonstrated that miR-375 was frequently downregulated, caused by hypermethylation of its promoter. miR-375 plays a role in the progression of ESCC by downregulating the target genes IGF1R and PDK1 (63,82). miR-203, miR-34b/c, miR-424 and miR-129-2 are embedded in CpG islands, as the promoter region of miR-34a. Chen *et al* investigated the methylation status of miR-203, miR-34b/c, miR-424 and

miR-129-2 in ESCC using bisulfite sequencing PCR (BSP) and methylation-specific PCR (MSP) methods, and showed that miR-34a, miR-34b/c and miR-129-2 were frequently down-regulated, which are hypermethylated in ESCC (83).

11. The role of miRNAs in treatment of EC

Hummel *et al* examined the impact of chemotherapy on miRNA expression in EC cells and found that 13 miRNAs (miR-199a-5p, miR-302f, miR-320a, miR-342-3p, miR-425, miR-455-3p, miR-486-3p, miR-519c-5p, miR-548d-5p, miR-617, miR-758, miR-766 and miR-1286) were deregulated following treatment with cisplatin or 5-fluorouracil for 24 or 72 h. Ingenuity Pathway Analysis (IPA) revealed that these miRNAs may target molecular pathways involved in cell survival following chemotherapy (84). miR-141 was the most highly expressed in the cisplatin-resistant ESCC cell lines and the cell viability was significantly increased following cisplatin treatment. The target of miR-141 is YAP1, which is an apoptosis-inducing gene in DNA-damaging agents (85). miR-296 and miR-27a were over-expressed in EC and the knockdown of miR-296 and miR-27a was capable of increasing sensitivity to both P-glycoprotein-related and P-glycoprotein-non-related drugs, in turn promoting ADR-induced apoptosis in EC cells (86,87). Overexpression of miR-200c significantly correlated with the response to chemotherapy and this effect was associated with the Akt pathway (88). miR-148a upregulation significantly increased sensitivity to chemotherapy in the majority of cells, but the exact mechanisms involved require further study (89). miR-200b/200c/429 were upregulated in endometrial cancer and EC, and their over-expression correlated with resistance to cisplatin treatment (78). miRNA expression profiles in an acquired radioresistant EC line revealed that hsa-let-7a, hsa-miR-185, hsa-miR-141, hsa-miR-92b, hsa-miR-22 and hsa-miR-301a were important genes associated with radioresistance, confirming the involvement of miRNAs in radiation resistance in EC (90).

12. Conclusion

Although investigations into miRNAs have just begun and the molecular mechanisms of miRNAs in tumorigenesis are not well understood, more evidence that miRNAs are important in the initiation and development of tumors is emerging. Further exploration is required for a better understanding of their roles in the carcinogenesis of EC and for better application in the future. EC-specific miRNAs may eventually constitute useful biomarkers as well as therapeutic targets. Investigations conducted in the field of miRNA are crucial for the personalized treatment of EC.

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