World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i19.2348

World J Gastroenterol 2010 May 21; 16(19): 2348-2354
ISSN 1007-9327 (print)

REVIEW

Circulating microRNAs: Novel biomarkers for esophageal cancer

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Received: February 7, 2010 Revised: March 19, 2010
Accepted: March 26, 2010
Published online: May 21, 2010

Key words: Esophageal cancer; Circulating microRNA

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Zhou SL, Wang LD. Circulating microRNAs: Novel biomarkers for esophageal cancer. *World J Gastroenterol* 2010; 16(19): 2348-2354 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i19/2348.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i19.2348

Abstract

Esophageal carcinogenesis is a multi-stage process, involving a variety of changes in gene expression and physiological structure change. MicroRNAs (miRNAs) are a class of small non-coding endogenous RNA molecules. Recent innovation in miRNAs profiling technology have shed new light on the pathology of esophageal carcinoma (EC), and also heralded great potential for exploring novel biomarkers for both EC diagnosis and treatment. Frequent dysregulation of miRNA in malignancy highlights the study of molecular factors upstream of gene expression following the extensive investigation on elucidating the important role of miRNA in carcinogenesis. We herein present a thorough review of the role of miRNAs in EC, addressing miRNA functions, their putative role as oncogenes or tumor suppressors and their potential target genes. The recent progresses in discovering the quantifiable circulating cancer-associated miRNAs indicate the potential clinical use of miRNAs as novel minimally invasive biomarkers for EC and other cancers. We also discuss the potential role of miRNAs in detection, screening and surveillance of EC as miRNAs can be a potential target in personalized treatment of EC.

INTRODUCTION

Esophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of cancer death worldwide, affecting more male than female[1]. The dramatic geographic difference in incidence is the striking characteristic for EC. The EC incidence in the high-risk northern Chinese exceeds 100/100000, while that in the low-risk western Africa can be 20-fold lower[2]. EC remains the leading cause of cancer-related death in the high-risk areas, especially in Henan Province, northern China[3]. Esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (EAC) are the two main forms of EC; each of them has different etiologic and pathologic characteristics. ESCC remains the predominant subtype of EC, especially in China; in contrast, EAC is the most common type in Western countries.

The survival and prognosis of EC patients depend on the stage of the tumor at the time of detection. Despite significant investment and advances in the treatment of cancer, the overall survival for advanced and metastatic cancer is still dismal, with a 5-year survival rate of less than 15% for advanced tumors[1,4,5]. The concept of early detection of esophageal cancer has been established

among clinicians and scientists for years. The 5-year survival rate was more than 90% for early EC[1,4,5], about 50% of cancers extend beyond the primary local region at the time of diagnosis and almost 75% of surgically treated patients have proximal lymph node metastasis[6]. At present, gastrointestinal endoscopy remains the primary screening tool, by which the suspected lesions can be biopsied for histopathological analysis. This invasive test, even though it is proved to increase the detection of early tumor and therefore can prolong the survival of the patient, is generally considered to be inconvenient and painful. Because of its limitation, there is an urgent request for discovery of novel predictive markers for EC.

Micro ribonucleic acids (miRNAs) are a class of single-stranded, evolutionarily conserved non-coding RNAs, only 17-25 ribonucleotides long[7]. The first known microRNA (miRNA), the lin-4, was discovered in 1993 through the study of heterochronic gene lin-14 in worms[8]. Since then, miRNA has shown the great potential regulating roles in many aspects of biological and pathological processes. The miRBase, which is the international registries for miRNAs, clearly described their nomenclature, targets and functions and implications in different diseases[9]. The biogenesis of miRNAs is a multi-step process involving a complex protein system, which includes members of the Argonaute family, Pol Ⅱ-dependent transcription and the RNase Ⅲs Drosha and Dicer[10]. MiRNAs are involved in crucial biological processes, including development, differentiation, apoptosis and proliferation[11,12]. The mechanism is still under debate, but probably related to inhibition of translation and messenger RNA degradation[13]. These small RNAs regulate gene expression at the level of translation through imperfect pairing with target messenger RNAs (mRNAs) of protein-coding genes[10]. The specific region in miRNA, which is important for messenger RNA target recognition, is referred to as the "seed sequence", and located at the 5- end of the mature miRNA sequence, from bases 2 to 8[11]. With the seed sequence, we can search for the complementary sequences in the 3'-untranslated regions (3'-UTRs) of known genes that exhibit conservation across species[11]. In animals, most miRNAs are thought to form imperfect base pairs with their target mRNA(s) and these interaction sites are enriched in 3'-UTRs[11]. To date, in the human genome, over 700 mature miRNAs have been identified, and the number is still increasing with the future studies. Up to 1000 miRNAs have been predicated by the bioinformatics studies of the human genome[14].

EXPERIMENTAL TECHNIQUES FOR MIRNA ANALYSIS

It is necessary to explore effective tools for detection, quantification, and functional analysis of miRNAs. Oligonucleotide miRNA microarray analysis has been extensively used as high-throughput technique for the assessment of cancer-specific expression levels in a large number of samples[10,13,15]. Another method to gauge miRNA expression level includes utilizing a bead-based flow-cytometric technique by which hybridization occurs in solution and provides a high specificity for closely related miRNAs[16]. The miRNA microarray platform with locked nucleic acid-modified capture probes can improve probe thermostability and increase specificity, so that it can discriminate miRNAs with even single nucleotide differences[17]. Quantitative real-time polymerase chain reaction (PCR) have also been widely applied to miRNA research because of its cost-effectiveness, high throughput and superior detection of low-abundance species[18]. All of these technologies facilitating miRNAs expression profiling are essential for validation of microarray data.

It is also necessary to develop methods of manipulating miRNA expression for elucidating biological significance and manipulating miRNA expression. MiRNA inhibitors such as 2-O-Methyl antisense single-strand oligonucleotides and locked nucleic acid modified oligonucleotides, made the suppression of endogenous miRNA activity and its downstream effect on messenger RNA expression possible[19]. The latest improvement of miRNA inhibition could control miRNA expression *in vivo* by expressing decoy miRNA targets *via* lentiviral vectors[20], with it we can inhibit specific miRNA by expressing various miRNA inhibitors. MiRNA mimicry could be used to identify the cellular processes and phenotypic changes through transfecting specific miRNAs into cell lines[21]. Although these techniques are of different strength and weakness, the large size studies have shown common characteristics of miRNA deregulation in human cancers[13] and shed light on the application of miRNAs as novel biomarkers finally.

MIRNA AND EC

Early studies demonstrated the aberrant expression of miRNA in cancers and the fact that approximately half of miRNA genes are localized in cancer-associated genomic regions or in fragile sites[22] indicates the potential role of miRNAs as a promising class of oncogenes or tumor suppressor gene in human carcinogenesis. MiRNAs still largely intact in conventionally collected, formalin-fixed and paraffin-embedded tissues and even a modest number of miRNAs are adequate to discriminate human tumors according to the developmental lineage and differentiation state of the tumors[16]. Furthermore, compared with messenger RNA (mRNA) profiles, it is easier to use these miRNA expression profiles to classify poorly differentiated tumors[23]. Here, we will focus on the miRNA expression profiles of EC, especially ESCC, to reveal the oncogenic mechanism by miRNA mediated post-transcriptional pathway. We will first discuss the identification of EC specific miRNA signatures for a new class of screening, diagnostic and prognostic biomarkers through miRNA expression profiling of EC; then we will concentrate on how to clarify the functional roles of miRNA in EC and the potential clinical valuation of the circulating miRNAs in EC. We believe that the study of miRNA's role in EC will provide new sight into this disease.

Table 1 Differential expression of miRNAs in EC tissues compared with normal tissue as identified in seven studies

Techniques	Pathology	Population	Overexpressed miRNAs			Underexpressed miRNAs		Ref.
Array profiling	ESCC	Chinese	hsa-miR-25	hsa-miR-424	has-miR-151	hsa-miR-100 hsa-miR-29c	hsa-miR-99a mmu-miR-140	[6]
mirVana miRNA Bioarrays (Ambion)	EAC	Japanese	miR-192 miR-21	miR-194 miR-93	miR-200c	miR-205	miR-203	[25]
	ESCC	Japanese	miR-342	miR-21	miR-93	miR-205	miR-203	
qRT-PCR	ESCC	Cell lines, Chinese	miR let-7d miR-373	miR-330	miR-340			[26]
qRT-PCR	ESCC	Japanese	miR-9 miR-17-5p miR-21 miR-34c miR-107 miR-130a miR-134 miR-151	miR-15b miR-20a miR-25 miR-103 miR-127 miR-130b miR-137	miR-16 miR-20b miR-34b miR-106a miR-129 miR-132 miR-138	miR-133a miR-139	miR-133b miR-145	[27]
Agilent Human miRNA microarray kit version 1.0	EAC	Caucasian	hsa-miR-126 hsa-miR-146a hsa-miR-195 hsa-miR-199a hsa-miR-424	hsa-miR-143 hsa-miR-181a hsa-miR-28 hsa-miR-30a-5p	hsa-miR-145 hsa-miR-181b hsa-miR-199b hsa-miR-29c	hsa-miR-149 hsa-miR-205 hsa-miR-221 hsa-miR494 hsamiR-617	hsa-miR-203 hsa-miR-210 hsa-miR-27b hsa-miR-513 hsa-miR-99a	[28]
miRNA microarray chips version 3	EAC	Caucasian	miR-21 miR-194	miR-192 miR-223		miR-203		[29]
	ESCC	Japanese	miR-21			miR-375		
qRT-PCR	ESCC	Cell lines, Chinese	miR-10					[30]

miRNAs: Micro ribonucleic acids; EC: Esophageal cancer; ESCC: Esophageal squamous cell carcinoma; EAC: Adenocarcinoma; qRT-PCR: Quantitative real-time polymerase chain reaction.

Role of miRNAs in carcinogenesis, progression, invasion and metastasis of EC

MiRNA acts as oncomiRNA or tumor suppressors to affect the tumorigenesis if their target mRNAs were encoded by tumor suppressor genes or ontogenesis[24]. Aberrant miRNA expression has been identified and confirmed in EC (Table 1). Comparing the miRNAs expression in ESCC with EAC, we can find that only a few miRNAs expressions were the same in these two diseases, each of which has the different miRNAs expression profiling, indicating that both of ESCC and EAC have distinct etiologic and pathologic characteristics. The miRNAs which are highly expressed in tumor can act as tumor promoter by targeting and inhibiting different tumor suppressor genes such as B-cell CLL/lymphoma 2 (BCL2)[31], *tension homologue* gene (PTEN)[32], large tumor suppressor homolog 2 (LAST2)[25], annexin A1 (ANXA1)[33]. Those genes were all studied in EC tissues and it was suggested that all of them were involved in carcinogenesis of esophagus. Those mature miRNAs with relatively lower expression in cancer may act as tumor suppressor miRNAs. The loss of such miRNAs may enhance the activity of oncogenes.

Apart from identifying different cancer-related miRNAs, efforts have been made to identify their target genes, messenger RNAs and receptors, which will lead to further studies about their contribution in cancer treatment. For example, the putatively identified targets[31,32,34] of miR-21 are BCL2, PTEN, tumor suppressor gene tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4) and maspin. Zhu *et al*[34] found that the biological function of miR-21 is probably due to the simultaneous repression of multiple tumor suppressor genes, including PTEN, TPM1, PDCD4, and maspin. PTEN is capable of restricting growth and survival signals by limiting the activity of the phosphoinositide 3-kinase (PI3K)/AKT pathway[35]. A decrease in functional PTEN causes constitutive activation of downstream components of the PI3K/AKT pathway, leading to tumor progression and metastasis[35]. Thus, down-regulation of PTEN by miR-21 may contribute to transformation and increased tumor cell survival[35]. TPM1 is an actin-binding protein which is capable of stabilizing microfilaments, regulating both microfilament organization and anchorage-independent growth, so its importance in cell transformation has been highlighted[36]. Another important mechanism of cancer development is epigenetic silencing of tumor suppressor genes. DNA hypomethylation, CpG island hypermethylation and histone-modification losses represent epigenetic markers of malignant transformation[37]. Calin *et al*[22] showed that some miRNA (e.g. miR-127) are located in a CpG island, a region that is involved in several types of translocations identified in hematological cancers and deleted by loss of heterozygosity in solid tumors. This phenomenon gives us a clue that the expression of tumor suppressor can be altered in epigenetic silencing mechanism through expression of miRNA.

Dysplasia and Barrett's esophagus are precursors of EC and the progression of these lesions to cancer is a multistep process that involves different sequential DNA aberrations and changes in gene expression. MiRNA expression profiles in tissues of Barrett's esophagus with high-grade dysplasia were significantly different from

their corresponding normal tissues/EAC tissues, but, no significant difference was found between low-grade dysplasia and paired normal tissues[28]. These observations were consistent with the finding of Feber *et al*[25], which suggests that low-grade dysplasia was with a lower malignant transformation rate compared with high-grade dysplasia. Yang *et al*[28] found that seven miRNAs (hsa-let-7b, hsa-let-7a, hsalet-7c, hsa-let-7f, hsa-miR-345, hsa-miR-494, and hsa-miR-193a) were potentially important in the progression from high-grade dysplasia to EAC. Hsa-let-7b, hsa-let-7a, hsalet-7c and hsa-let-7f belong to the let-7 miRNA family, most of their members function as tumor suppressors through negative regulation of the *RAS* gene[38]. Accordingly, RAS mutations and amplifications have been frequently identified in precancerous tissues and EC tissues[39,40]. Oncogene RAS may play a role in cell growth and differentiation[41].

Diagnostic and prognostic value of miRNA for EC

To date, the available data of miRNA expression profile in cancer suggest that miRNA may have diagnostic and prognostic potential. It is crucial to correlate miRNA expression with tumor subtypes or clinical parameters.

Lu *et al*[16] were the first to systematically demonstrate that the specific miRNA expression can be applied not only for diagnosis but also for classification of different tumors, and the accuracy is 70%. Guo *et al*[6] were the first to investigate the expression of miRNA in EC tissues using miRNA microarray techniques. They found that five miRNAs (miR-335, miR-181d, miR-25, miR-7 and hsa-miR-495) correlate with gross pathologic classification (fungating *vs* medullary) and two miRNAs (miR-25 and miR-130b) correlate with differentiation classification (high *vs* middle *vs* low)[6]. The miRNA expression profiling of ESCC is distinct from EAC[25]. Compared with its expression in normal epithelium (NSE), miR-194, miR-192 and miR200c are significantly up-regulated in EAC but not in ESCC[27]. Oppositely, miR-342 is aberrantly expressed in ESCC but not in EAC[25]. Furthermore, the miRNA expression profiling of NSE and SCC samples are more similar to each other than to the EAC samples[26]. The Barrett's esophagus (BE) samples sit between the EAC and NSE and one high-grade dysplasia specimen has a miRNA expression profile similar to the EAC[24]. This makes good biological sense that miRNA expression profiles can distinguish esophageal tumorigenesis and may be proven useful for identifying BE patients who are at high risk for progression to EAC.

As we mentioned before, the survival and prognosis of EC is dismal. Therefore, the discovery of new prognostic markers could be a prodigious advantage for identification of patients that would benefit from more aggressive therapy. The high expression of both miR-103 and miR-107 has been shown to correlate with poor survival in 53 Chinese patients with ESCC either by univariate analysis or by multivariate analyses[6]. In an independent study of 30 Japanese patients with ESCC, overexpression of miR-129 was found to significantly correlate with a shorter survival and miR-129 was identified as a significant and independent prognostic factor in surgically treated ESCC patients[27]. The largest study so far, found that low expression of miR-375 in EAC patients was strongly associated with worse prognosis, whereas ESCC patients with high expression of miR-21 had poor prognosis[29]. An earlier study conducted by Sugito *et al*[42], measured the expression levels of DICER1, DGCR8, and RNASEN messenger RNA in 73 surgically treated ESCC patients by real-time reverse-transcription PCR, and clarified that high RNASEN expression correlate with poor prognosis in ESCC, indicating that RNASEN may be a good candidate for molecular prognostic marker. Altered expression of these miRNAs is tissue specific, which may provide clues as to the different molecular progression undertaken by these cancers, and opens a potential avenue for targeted therapy to improve prognosis[29].

Circulating microRNAs: Novel minimally invasive biomarkers for EC

Scientists studying on EC are continually dedicated to search for sensitive, and minimally invasive markers which can detect early neoplastic changes, thus identifying EC at an early stage, as well as monitoring the progress of patients with EC and their response to treatment. Existing methods for identification of EC have many inherent deficiencies. Endoscopic biopsy and histopathological examinations are currently the golden standard diagnostic methods for EC. However, it has limitations, including the use of invasive tool and limited number of experienced physicians. Besides, it is difficult to be applied in large-scale studies because of its tedious approaches. To date, there are still no other effective biomarkers established in the routine assessment of EC.

The much desirable biomarker should be sensitive enough and easily accessible to detect the cancer patients at early stage. Recent studies have shown that tumor-derived miRNAs are resistant to endogenous ribonuclease activity so it can be present in human serum in remarkably stable form[43]. Furthermore, the expression level of serum miRNAs is reproducible and consistent among individuals[43,44]. These tumor-derived miRNAs are present in the circulating blood at levels sufficient to be measurable as biomarkers for the detection of tumors and more than 100 circulating miRNAs can be detected in the blood of healthy individuals[43]. Either plasma or serum can be used for identification of these blood-based biomarkers because the miRNAs levels in plasma and serum are strongly correlated[43,45]. Chen *et al*[44] demonstrated that the colorectal cancer patients shared a large number of serum miRNAs with lung cancer patients. Moreover, they identified a unique expression profile of serum miRNAs for colorectal cancers that were not present in lung cancer. Though miRNA research is only emerging recently, it has aroused great interest in clinical and scientific communities. Since the presence of miRNA in serum was first described in patients with diffusive large B-cell lymphoma[46],

a number of studies on other solid cancers (ovarian cancer[47,48], lung cancer[49,50], breast cancer[51], colorectal cancer[52,53]) have been reported about the presence of miRNA in circulation and their potential for use as novel biomarkers. Taylor *et al*[48] demonstrated that the expression of 8 miRNAs in the serum of patients with ovarian cancer were significantly distinct from their expression observed in benign diseases, yet could not be detected in the serum of healthy controls. Another study indicated that miRNA levels were significantly different between lung cancer patients and controls[49]. Both of these studies suggested that there was no significant difference in the miRNA expression between peripheral circulation and tumors tissues[48,49], indicating that circulating miRNA could act as surrogate of tissue miRNA for early diagnosis. And this will initiate a new application that will be widespread in clinical treatment and disease prevention.

Blood-based miRNAs provide a novel class of minimal invasive biomarkers for high-risk subject screening and early diagnosis for cancers. At the time of writing, there was no article about circulating miRNAs profile of EC. We focus on the circulating miRNA expression profiles of ESCC which is the main type of EC, especially in Asia. Checking circulating miRNA expression profiles of ESCC can help identify EC specific circulating miRNAs signatures for a new brand of screening, diagnostic and prognostic biomarkers. Studies can also be performed to clarify the functional role(s) of miRNAs in EC.

Potential in screening, surveillance and treatment of EC

So far, endoscopic biopsy and histopathological examinations are the golden standard for high-risk subject screening and early detection for EC worldwide. We can only identify 1%-2% early carcinomas and 15%-20% precancerous lesions in asymptomatic population aged higher than 35 years in high-incidence areas of EC through endoscopic biopsy and histopathological examinations[3]. Yet nearly 80% asymptomatic populations were in the normal range. In other words, there exists over endoscopic application. Besides, as there are no sufficient experienced physicians in the countryside, the misdiagnosis and missed diagnosis would be inevitable. In addition, standard sterilizing procedure also restricts the wide application of endoscopy in asymptomatic population. Furthermore, the projected cost of mass screening in China for detection of cancers is increasing annually. An urgent and important problem to be solved is to diminish blindness in the screening, minify the range of endoscopy and elevate the detection sensitivity of early carcinoma in high risk subjects. Easily accessible and minimal invasive biomarkers for EC and for other diseases are much desirable. The PCR technique for detection and quantification of miRNA[18] in the blood would be one of the promising methods of screening individuals for EC and other cancers because of its universal application, convenient management, low cost and high sensitivity. Rouet *et al*[54] set up a model of qRT-PCR for serum RNA analysis, suggesting that its cost was only less than £1 for each clinical specimen when used in a group of 84 patients. Once the accuracy of detection is established, MiRNAs test should be introduced into the national EC and other disease screening programs. The best option might be that annual miRNA blood test in asymptomatic populations in high risk areas of EC, and then the patients with positive test result can choose endoscopic biopsy and histopathological examinations for final diagnosis.

Given the emerging evidence of miRNAs acting as oncogenic or tumor suppressor activities, it is important to seek approaches to interfere with the miRNA[55], and eventually, develop these as novel biomarkers into EC therapies. Scientists are making efforts to explore miRNA and utilize that in cancer. Meng *et al*[32] showed that miRNA expression profiles changed during treatment with gemcitabine, and the sensitivity of cholangiocarcinoma tumor cells to this chemotherapy *in vitro* are increasing with the modulation of some miRNAs. On the contrary, it could be feasible to introduce tumor suppressor miRNA which are tissue-specific to the target gene, and then it may help prevent further progression, or even shrink EC. Yet, recently identified genomic and proteomic biomarkers, tumor cell mutations and microsatellite instability cannot be recommended for clinical use because of insufficient available data[56]. Though recent studies on miRNA in EC are limited, the potential value of miRNAs as prognostic and predictive biomarkers in EC is elegantly highlighted and will be elucidated eventually. However, experimental miRNA therapy need to be validated comprehensively through studies involving different cohorts of patients before introduced into clinical practice.

CONCLUSION

Although much of the work on miRNA is still in its infancy, the previous studies have demonstrated the significant role(s) of miRNA in the initiation and progression of cancer and other diseases. Further exploration is required for better understanding their role in carcinogenesis of EC and for better application in the future. Notwithstanding there are still many questions to be answered about circulating miRNA, there is no doubt that further studies will lead to great progress in the future therapy of EC. The exploration of miRNA will improve our knowledge of the roles of these novel biomarkers, and further demonstrate their true potential in therapeutic methods.

ACKNOWLEDGMENTS

We are grateful to Dr. Dong Xie from Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China for carefully revising the manuscript.

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