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MicroRNAs and Gastroenterological Cancers

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Abstract

MicroRNAs are small noncoding RNAs that control gene expression. In doing so, they functionally contribute to the maintenance of cellular processes as well as several important features related to cancer development and progression such as cell growth control, differentiation and apoptosis. In fact, recent studies have shown that microRNAs are suitable and effective cancer-related biomarkers since they display altered expression profiles in cancers versus normal tissue. In addition, microRNAs have been associated with cancer progression and outcome. In this review, the current state of knowledge microRNA expression and function in relation to gastroenterological cancers will be addressed. Moreover, the mechanisms to alter their expression and the potential application of microRNAs in clinical settings will also be highlighted. Finally, the challenges involved in translating microRNA research to the clinic will be discussed.

Introduction

Since their initial discovery during developmental timing experiments in nematodes, microRNAs, small noncoding functional RNAs that are approximately 22 nucleotides in length, have proved to be important regulators of gene expression [1–3]. New insight into their expression patterns, function and regulation in normal and neoplastic human cells along with emerging data concerning their frequent location at fragile sites, common break-points or regions of amplification or loss of heterozygosity reveal that they play significant roles in human carcinogenesis. Possible mechanisms of altered microRNA expression include defective microRNA processing or post-transcriptional regulation, germ-line or somatic mutation and epigenetic changes such as methylation [4–7]. The abnormal expression of several microRNAs have been observed in Burkitt's lymphomas, B cell chronic lymphocytic leukemia (CLL) and in many solid cancer types, including breast, cervical, colorectal, liver, lung, ovarian, and prostate [8–19]. MicroRNAs can bind to many target mRNA sequences in genes belonging to several signaling pathways. This ability gives microRNAs a large number of biologically diverse functions such as the control of cell growth, differentiation, mobility and apoptosis. Functional analysis has revealed the tumor suppressor function of the let-7 family and the oncogenic function of miR-21 and the miR17-92 cluster [20–22]. The biological and clinical relevance of microRNA expression

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patterns have been established in human B cell CLL and several solid tumors [9,13,19]. Many techniques have been developed to examine microRNA expression. Various techniques have enabled researchers to measure microRNA levels and determine how alterations in their expression level are associated with particular phenotypes and how they can be clinically utilized. These aspects of microRNA expression levels are discussed in the remainder of this review with an emphasis on their roles in cancers of the gastroenterological organs which include the esophagus, stomach, small bowel, colon, gallbladder, liver and pancreas.

The Role of MicroRNAs in Gastroenterological Cancers

Recent evidence indicates that microRNAs play an important role in gastroenterological cancers. These studies have shown that tumor-based microRNA signatures have can identify tissue origins of cancer and could specifically classify gastroenterological cancers derived from the stomach, liver, esophagus, colon and pancreas [18,23]. In fact, gastroenterological cancers seem to have distinct profiles when compared to non-gastroenterological cancers as reflected in a large-scale genomics study by Volinia et al showing that stomach, pancreas and colon cancers have similar microRNA profiles, while lung and breast cancers had a different signature [19].

Each microRNA has the distinct capability of regulating the expression of hundreds of coding genes and can thereby modulate several cellular pathways in the gastroenterological milieu, including proliferation, apoptosis and stress response [24]. Whether their altered expression in cancer is a causative factor or rather, a consequence of the disease state is currently unknown. Dependent upon the nature of their target gene(s), microRNAs in gastroenterological organs may function as tumor suppressors by downregulating target oncogenes (e.g. let-7g and miR-34) or as oncogenes by negatively controlling genes that regulate tumor cell differentiation and apoptosis (e.g. miR-17-92 and miR-21) [25]. On the other hand, changes in microRNA expression may be a downstream effect of potent oncogenes or tumor suppressors in the carcinogenesis process, such as the modulation of miR-34 by p53 in pancreatic cancer [26]. In addition to their capability of distinguishing tumor from nontumor samples, microRNAs have also been shown to play a role in cancer progression through their modulation of cellular adhesion, cell matrix and signaling activities hypoxia-related genes, vascular endothelial growth factors [27–34].

The function of microRNAs altered in gastroenterological cancers

Although much work still needs to be done with regard to our understanding of microRNA function in general, research to date has shown that microRNAs affect many factors related to cancer. Several microRNAs modulate cell cycle progression, proliferation and apoptosis. In esophageal squamous cancer for example, elevated miR-296 levels compared to normal tissue seems to inhibit cancer cell growth via regulation of cyclin D1 and p27 [35]. Recent reports have shown that loss p53 can lead to reduced expression of miR-143 and miR-145, a signaling network that targets the oncogene c-Myc and affect colon cancer growth [36,37]. In addition, Let-7 has been implicated in the modulation of colon cancer cell growth through regulation of KRAS [38]. Meanwhile, miR-21, highly up-regulated in many gastroenterological cancers, and miR-375, highly down-regulated in gastric cancer, directly targets PTEN or regulates PI3K/Akt activity, respectively, to reduce cell viability through the induction of apoptosis [39,40]

Many microRNAs also play important roles in cancer progression including metastasis and stemness. For example, high miR-21 levels is associated with liver metastasis and a high proliferation index in pancreatic cancer [41–43] while let-7g has been shown to target collagen type 1 alpha 2 and inhibit cell migration in hepatocellular carcinoma, thus

suppressing metastasis [44]. In our laboratory, we have found that miR-181 could directly target hepatic transcriptional regulators of differentiation (e.g. caudal type homeobox transcription factor 2 [CDX2] and GATA binding protein 6 [GATA6]) and an inhibitor of Wnt/beta-catenin signaling (nemo-like kinase [NLK]), defining a novel regulatory link between miR-181s and liver cancer stem/progenitor cells [45].

Regulation of the immune system, transcription and viral replication have also been shown to be candidates for microRNA function. Several microRNAs, such as miR-21, can also have altered levels in the tumor-surrounding microenvironment. As such, they have been implicated in affecting immune responses and pathways in cancers an example of which occurs in squamous cell esophageal cancer [23]. MicroRNAs also significantly affect the transcription machinery, such as let-7g, whose reduced expression in gastric cancer tissues contributes to HMGA2 overexpression, a protein which promotes the assembly of regulatory protein complexes at transcription sites [46]. Recent studies have shown that microRNAs also play a significant role in the viral life cycle. Specifically, miR-122, the most abundant microRNA in the liver, has been shown to target and allow replication of the hepatitis C virus [47]. As described above, microRNAs seem to function in every facet of cell biology and thus our understanding of these intricate mechanisms may allow us to more discretely target these molecules which contribute to the promotion and progression of gastroenterological cancers.

Gastroenterological microRNAs as oncogenes or tumor suppressors

Many studies have defined oncogenic microRNAs in gastroenterological cancers (Table 1). In colorectal and gastric cancers, for example, the miR-17-92 cluster is elevated [48–50]. This cluster is positively regulated by c-Myc and E2F3 and repressed by p53 and thus, the intricate balance between the expression of these upstream genes and pathways regulates the capacity of miR-17-92 to affect proliferation, angiogenesis and cell survival. miR-21 is also significantly up-regulated in many gastrointestinal cancers, including colon, gastric and esophageal cancers [23,50–52]. miR-21 is associated with clinical stage, distant metastasis and lymph node positivity as well as poor survival and poor therapeutic outcome in colon cancer. It has been suggested that loss of p53 and inflammatory stimuli induce miR-21. Methods aimed at reducing the levels of these and other oncogenic microRNAs are currently being developed to help thwart gastroenterological cancer progression.

Several tumor suppressive miRNAs have been identified in gastroenterological cancers (Table 1). MiR-34, for example, plays tumor suppressive roles including the induction of senescence, apoptosis and cell cycle arrest and is frequently downregulated in gastroenterological cancers through transcriptional deregulation and chromosomal instability. In fact, the expression of mir-34b and mir-34c is downregulated through promoter methylation in colon cancer [53]. Another microRNA with tumor suppressor roles, let-7, shows reduced expression in colon and liver cancer with various roles in inhibiting cell proliferation and cell invasion [44,54,55]. We and others have described the tumor suppressive role of miR-26 in liver cancer and its relation to nuclear factor kappa b and interleukin-6 signaling as well as to proteins involved in cell cycle transition [56,57]. Currently, researchers are employing techniques to elevate the levels of these and other tumor suppressive microRNAs in gastroenterological cancers to restore the normal balance of signaling pathways to reduce cancer aggressiveness [8,9,18].

The Clinical Applications of Gastroenterological MicroRNAs

Since microRNAs are altered in gastroenterological cancers, they may function as suitable biomarkers for disease state and progression. Recent studies indicate that expression profiling of microRNAs is a superior method for cancer subtype classification and

prognostication [58,59]. The application of microRNA screening to diagnosis and prognosis are briefly described below.

Diagnostic MicroRNAs

Multiple reports have noted the utility of microRNAs for cancer diagnosis [60]. MicroRNA expression profiles have been used to distinguish tumor from normal samples, identify tissue of origin and to distinguish different tumor subtypes. In addition, several studies have shown the utility of microRNA quantification as a means to identify patients at an early stage of cancer and thus may be quite useful for early detection. In colon cancer Michael et al report that miR-143 and miR145 are reduced in the adenomatous and cancer stages of this disease [61,62]. These two microRNAs were also shown to be reduced in separate studies comparing colon cancer and normal samples [63]. Meanwhile, miR 31 levels are overexpressed in colonic tumors at higher stages [64]. In esophageal cancer, it was shown that a microRNA profile could differentiate dysplastic grades of Barrett's esophagus [65]. Ueda also showed that a miRNA signature could distinguish gastric cancer samples from benign and could also differentiate histological subtypes of gastric cancer tissues [45]. Subtype classification by microRNA signatures have also been defined in liver cancer [66]. In particular, elevation of miR-181 family members is associated with a stem cell-like hepatocellular carcinoma cancer subtype and is functionally related to cancer stemness. In pancreatic cancer, elevated miR-103 and miR-107 and reduced miR-155 were related to tumor status [67] while elevated mir-196a levels were higher in cancer when compared to chronic pancreatitis and normal tissues [68]. Thus important definitions of cancer status and stage can be achieved through the interrogation of microRNA levels in gastroenterological cancers.

Large tissue specimens are not needed for accurate microRNA detection since their expression can be easily measured in biopsy specimens. Although the majority of the studies described above have used tissue to assess microRNA levels, recent studies have shown that microRNAs can be measured in formalin fixed paraffin embedded (FFPE) tissues [69–71]. Given the invasive nature of fresh/frozen tissue collection and the availability of FFPE tissues, this allows for feasible measurement of microRNA levels for diagnostic purposes. Recent studies have also shown that microRNAs can be detected blood products such as serum and plasma, offering the promise of utilizing microRNA screening via less invasive methods. In fact, miR-21 and the miR-17-92 cluster have been detected in the plasma of patients with gastroenterological cancers and their levels were significantly reduced following surgery [8,72]. Along with the apparent ease of microRNA quantitation, mature microRNAs are relatively stable. These phenomena make microRNAs superior molecular markers and targets for interrogation and as such, microRNA expression profiling can be utilized as a tool for cancer diagnosis [8,52,72–76].

Prognostic MicroRNAs

The potential clinical utility of microRNA extends beyond the diagnostic realm to other important clinical measures such as prognosis and treatment response. A series of publications has shown that microRNAs are useful indicators of clinical outcome in several cancer types [63]. In colon cancer, miR-200c overexpression is associated with patient survival [52] while miR-21 has been shown to be a useful predictor of colon cancer metastasis and response to 5-FU treatment [65]. In gastric cancer, patients with lower let7-g, miR-433 or higher miR-214 had poor survival [35,77,78]. Elevated miR-103, miR-107, miR-129 and miR-296 have also been shown to be prognostic in esophageal cancer patients [67]. In pancreatic cancer, high mir-196a levels could predict poor survival [79,80] while altered levels of miR-21 and miR-122 are associated with liver cancer patient outcome [81–85].

MicroRNAs have also been shown to play a predictive role in determining the tendency for recurrence and metastasis [86]. In gastric cancer patients, reduced miR-451 was related to poor disease-free survival [87]. Another study found that miR-320 and miR-496 could predict recurrence of stage II colon cancer [81]. A series of microRNAs forming a signature were capable of predicting liver cancer metastasis [76,88,89]. These microRNA alterations have not only been found in tumor specimens, but have also been observed in surrounding non-cancerous tissue, suggesting that microRNAs may also serve to detect alterations in the cancer microenvironment [56]. MicroRNAs have also been shown as useful indicators of which patients may favorably respond to a specific treatment regimen. An example of this has recently been shown for liver cancer patients, whereby miR-26 expression could be used to stratify patients who would respond better to IFN treatment [35]. In another study, the reduction of miR-296 could increase the sensitivity of esophageal cancer cells to P-glycoprotein drugs [52,90]. Furthermore, let7-g, miR-181b and miR-21 levels in colon tumors are associated with therapeutic response [91]. The full potential of microRNAs as prognostic factors awaits the results of larger prospective studies.

The Therapeutic Application of Gastroenterological MicroRNAs

As noted above, several microRNAs have been shown to be altered in disease states when compared to normal specimens. In addition, changes in microRNA expression has also been observed as gastroenterological cancers progress. Moreover, microRNA status may reflect patient outcome and capacity to respond to treatment. Since microRNAs are deregulated in cancer, it is thought that normalization of their expression could be a potential method of intervention. In this vein, several therapeutic mechanisms have been put forth and are described below (Table 2).

Strategies for microRNA reduction

MicroRNAs bind to their target sites via the rules of Watson and Crick base-pairing guide. In order to thwart this interaction, anti-microRNA oligonucleotides (AMOs) have been generated to directly compete with endogenous microRNAs [92]. However, AMOs are rather inefficient at specifically inactivating endogenous targets. Therefore, several modifications of AMOs, such as the addition of 2'-O-methyl and 2'-O-methoxyethyl groups to the 5' end of the structure, have been generated to improve their effectiveness and stability [93]. Studies have shown that targeting miR-21, a microRNA that is overexpressed in many gastroenterological cancer types, by such methods could effectively reduce tumor size in a xenograft mouse model [94]. AMOs conjugated to cholesterol (antagomirs) have been also been generated and have been shown to efficiently inhibit microRNA activity in-vivo [95]. In addition, locked-nucleic-acid antisense oligonucleotides (LNAs) have been designed to increase stability and have the benefits of being highly aqueous with reduced toxicity in-vivo [96]. This method has been effectively used to completely eradicate miR-21 [97]. This strategy is also being employed to reduce miR-122 levels to combat hepatitis C virus replication in liver cancer [98]. Another method for reducing the interaction between microRNAs and their targets is the use of microRNA sponges, synthetic mRNAs that contain multiple binding sites for an endogenous microRNA. Sponges designed with multimeric seed sequences have been shown to effectively repress microRNA families sharing the same seed sequence [99,100]. Although microRNA sponges perform equally to chemically modified AMOs in-vitro, their efficacy in-vivo remains to be determined.

Although these oligonucleotide-based methods have succeeded in reducing microRNA levels, they elicit off-target side effects and unwanted toxicity due to the capability of regulating hundreds of genes. The miR-masking method is an alternative strategy designed to combat the off-target effect. By utilizing a sequence with perfect complementarity to the target gene, duplexing occurs with higher affinity than what would naturally occur between the target

gene and its endogenous microRNA, although the choice of target gene must be specific in order to effectively reduce the interaction. This gene-specific, microRNA interfering strategy has been shown to reduce the activities of miR-1, miR-133 and miR-430 in several model systems [101]. Another strategy to increase specificity is the use of small molecule inhibitors against specific microRNAs such as azobenzene, a specific and efficient inhibitor of miR-21 [102]. Another recent approach to silence miRs is through the use of tiny locked nucleic acids (tinyLNAs) [103,104]. This method involves transfection of seed targeting 8-mer LNAs to simultaneously inhibit miRs within the same family and has successfully inhibited let7 members with long term silencing. Although the effectiveness of such inhibitors awaits exploration in-vivo, they are potentially promising tools for cancer therapy.

Strategies to overexpress microRNAs

A strategy one could think of to restore tumor inhibitory functions in the cell is to elevate the expression of microRNAs with tumor suppressive roles. This can be achieved through the use of viral or liposomal delivery mechanisms [16,105–107]. Several microRNAs have been introduced to cells via this methodology, including miR-34, miR-15, miR-16 and let-7 [57]. Systemic administration of miR-26, a tumor suppressive microRNA in liver cancer, using adenovirus-associated virus (AAV) in an animal model of liver cancer, led to inhibition of cell proliferation and tumor-specific apoptosis [108–110]. Since AAV vectors do not integrate into the host genome and are eventually eliminated, toxic effects are reduced. Viral vector-directed methods such as the one described above transfer genes with high efficiency, however they lack tumor targeting and can elicit immunogenic effects due to the presence of residual viral elements. Therefore, non-viral methods of gene transfer such as cationic liposome mediated systems have been developed. Although these lipoplexes are promising, they have relatively low transfection efficiency when compared to viral vectors and lack tumor specificity.

MicroRNA mimics have also been used to increase microRNA expression. These small, chemically modified double-stranded RNA molecules mimic endogenous mature microRNA. These mimics are now commercially available and promising results have been reported with systemic delivery methods using lipid and polymer-based nanoparticles [111]. Since these mimics do not have vector-based toxicity, they are promising tools for therapeutic treatment of tumors.

Summary and Conclusions

As described above, many microRNAs are altered in gastroenterological cancers. The development of strategies to specifically and accurately quantify these changes and place them mechanistically into the context of cancer cell biology is an ongoing task in the field of cancer research. Furthermore, since microRNAs are related to tumor subtypes and patient outcome new technological advances to utilize microRNAs as therapeutic tools are in the pipeline. In order to fully achieve this however, conceptual and technical issues still need to be overcome. Since microRNAs can potentially inhibit many genes, a major hurdle to overcome is achieving specificity. Partial complementarity can lead to off-target gene silencing and undesired biological effects. Given the multi-gene targets of a single microRNA, the magnitude of an off-target association may be quite large. Thus, it remains important to comprehensively evaluate each specific microRNA and determine how its expression or activity can be modulated to achieve the desired effect for the therapeutic benefit of a gastroenterological cancer patient. Conversely, it may be useful to target multiple members of a gene family with a single microRNA. Such strategies are currently underway to design small multiple target artificial (SMART) microRNAs to simultaneously target members of a single gene family, such as E2F [36,37,48,52,60–62,112] or the tinyLNA strategy described earlier. Importantly, we have seen that certain microRNAs such

as miR-21 are abundantly expressed in many cancer types while other microRNAs show a more restricted pattern of expression in specific cancers. This information is quite useful since it may guide whether a patient should receive therapy based on a single or multiple microRNA strategy. The fact that multiple microRNAs can target the same genes or hit multiple genes along different nodes of the same pathway are also important facets to consider in future research design and clinical strategies. A more thorough understanding of microRNA biology and function will allow for the development of more suitable strategies. Another issue that warrants future study is the efficiency of delivery of microRNA to specific sites. One needs to achieve a certain amplitude of target gene modulation and to maximize the number of cells that receive therapeutic microRNA at target sites. This effect also needs to be long-lasting with minimal toxicity to the recipient. Further advances in the area of drug delivery will no doubt improve upon the current tools of the trade.

MicroRNAs have become a key player in the field of cancer biology and treatment. Research within the last few decades has significantly added to our knowledge of the biogenesis and function of microRNAs. These studies have shown that microRNAs play a comprehensive and intricate role in cancer such that minute changes in their expression and/or activity could profoundly affect cancer phenotypes such as proliferative potential and metastasis. Recent genome-wide surveys have revealed additional novel short and long non-coding RNAs, including lncRNAs, whose regulation, activity and function are currently being explored in the context of cancer and other diseases [113–115]. New technologies such as next generation sequencing may identify additional novel microRNAs that are important regulators of cancer initiation and progression. Additional work aimed at alleviating the methodological difficulties described above, such as off-target effects, and the translation of these findings to in-vivo models and clinical studies will unquestionably lead to the eventual use of microRNAs in the clinical setting. The concept of microRNAs as therapeutic agents is gaining momentum and these small RNA species will play a very large role in advancing research and alleviating the dismal outcome of gastroenterological cancer patients.

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Table 1

MicroRNAs Associated with Gastroenterological Cancers

Cancer Type	Human microRNA	Potential Function	Validated Targets	References
Colon Cancer	miR-21; miR-17-92; <i>miR-34a; miR-143,</i> <i>miR-145</i>	OncomiR; <i>Tumor Suppressor</i>	PTEN, MYC, HRAS, SOX2	[52,87,112,112]
Esophageal Cancer	miR-21; miR-103, miR-107, miR-129 <i>miR-296</i>	OncomiR; <i>Tumor Suppressor</i>	CDK2, CDK6, NOTCH1	[23,35,77]
Gastric Cancer	miR-21, miR-17-92; <i>miR-375, miR-214;</i> <i>miR-451</i>	OncomiR; <i>Tumor Suppressor</i>	YYIAP1, MAPK8, MIF	[39,48,49,51,65,86]
Liver Cancer	miR-21, miR-34a, miR221, miR-222, miR-106, miR-303; <i>miR26a/b, let-7g,</i> <i>miR-122, miR-422b, miR-</i> <i>145, miR-199</i>	OncomiR; <i>Tumor Suppressor;</i> <i>Metastasis</i>	FOXO3, CDKN1B, RB1, KRAS, COL1A2, MET, SMAD1	[44,56,81,89,116]
Pancreatic Cancer	miR-21; miR-196a, miR196b; <i>miR-155</i>	OncomiR; <i>Tumor Suppressor</i>	HOXB7, ANXA1, SMAD5	[65–67,98,117,118]

Table 2

Strategies to Alter MicroRNAs

Strategy	Modulator	Delivery	Clinical Utility	References
Inhibit mature microRNA cluster	microRNA sponge	Sponge plasmid vector	Silence oncomiR cluster	[98]
Inhibit mature microRNA	2' OME-AMOs	RNA-Liposome Complex	Silence OncomiR	[119]
Inhibit mature microRNA	2' MOE AMOs	Oligonucleotide-Liposome Complex	Silence OncomiR	[120,121]
Inhibit pri-microRNA	AMOs	Oligonucleotide-Liposome Complex	Silence miR cluster	[122,123]
Inhibit mature microRNA	LNA-antagomiR	Unconjugated	Silence OncomiR	[65,102,124]
Silence selected target	Synthetic microRNAs	Conjugation	Tumor Suppressor Function	[125,126]