

The role of microRNAs in gallbladder cancer (Review)

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Abstract. MicroRNAs (also referred to as miRNAs or miRs) play a crucial role in post-transcriptional gene regulation and serve as negative gene regulators by controlling a variety of target genes and regulating diverse biological processes, such as cell proliferation, invasion, migration and apoptosis. Aberrant expression of miRNAs is associated with the development and progression of cancer. Recent studies have reported that miRNAs may repress or promote the expression of cancer-related genes via several different signaling pathways in gallbladder cancer (GBC) patients and may function as tumor suppressors or oncogenes, thus providing a promising tool for the diagnosis and therapeutics of GBCs. In this review, we summarize the role of dysregulated miRNA expression in the signaling pathways implicated in GBC and discuss the significant role of circulating miRNAs in GBC. Therefore, miRNAs may serve as novel therapeutic targets as well as diagnostic or prognostic markers in GBC.

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1. Introduction

Gallbladder cancer (GBC) is the fifth most common gastrointestinal malignant neoplasm, representing 80-95% of biliary tract cancers, as well as the leading cause of biliary tract malignancy-related mortality worldwide (1,2). Approximately 10,650 cases of GBC and other biliary cancers were diag-

nosed in the United States in 2014 (3). Despite advances in the treatment of GBC in recent years, the majority of patients eventually develop local recurrent or distant metastatic disease, which is associated with a poor prognosis and an overall 5-year survival rate of <10% (4-7). Early diagnosis, which is crucial for long-term survival of GBC patients, unfortunately occurs only accidentally in patients undergoing cholecystectomy for gallstones or cholecystitis, while the majority of the patients present with advanced metastatic disease due to the absence of specific symptoms and efficient biomarkers (8-12). Additionally, coadjuvant therapy consisting of chemotherapy and/or radiotherapy has not yet proved beneficial in terms of patient survival (13-16). Future research should be focused on developing more effective biomarkers for early diagnosis, therapeutic strategies and prognosis. MicroRNAs (also referred to as miRNAs or miRs) have been widely reported to play a crucial role in the development, metastasis and prognosis of various types of cancer (17-20). In particular, the expression levels of circulating miRNAs differ significantly between cancer patients and healthy volunteers (21-26), which may provide a non-invasive method for early detection.

MicroRNAs are single-stranded RNA molecules that constitute a class of small (~18-25 nucleotides) non-coding RNAs that negatively regulate target genes through transcript degradation and translational inhibition by binding to the 3'-untranslated regions (3'-UTRs) of the target messenger RNAs (27-29). Since their initial discovery in 1993, hundreds of similar miRNAs have been discovered in various types of species (30-32). Accumulating evidence indicates that several diseases, such as cardiovascular, liver, kidney and neurodegenerative diseases, as well as cancer, are initiated or sustained by miRNA dysregulation (28,33-37). Of note, recent studies have demonstrated that miRNAs play vital roles in modulating cell proliferation, invasion, migration and apoptosis. Additionally, miRNAs that act as tumor suppressors and oncogenes have been identified in several cancers (38-41). These exciting results have also been reported in GBC, since the potential association between various miRNAs and GBC was first reported in 2010 (42). Previous studies have established that aberrant expression of miRNAs exerts a significant effect on cancer-related processes by targeting specific genetic alterations, which provide effective biomarkers for diagnosis, therapeutics and prognosis of GBC (43-45). In this study, we describe miRNAs in GBC, particularly their roles as oncogenes and tumor suppressors, their value in diagnosis and prognosis and their potential in providing novel therapeutic strategies for disease management.

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2. MicroRNAs and GBCs

miRNA expression profiling has been performed by large-scale microarray analysis in GBC tissues or cells in several studies (26,46-48). Numerous miRNAs exhibit expression changes, with some miRNAs being upregulated, but most being downregulated in GBC cells and tissues. Subsequently, further studies confirmed the function of these miRNAs as either oncogenes or tumor suppressors. In addition, the underlying target genes or mechanisms of miRNA-mediated development and progression of GBC have also been proposed. Furthermore, recent research has demonstrated that circulating miRNA levels were significantly different between GBC patients and healthy volunteers, and were associated with tumor pathological characteristics (26), potentially providing a feasible method for early diagnosis of GBC. A better understanding of the underlying molecular mechanisms of miRNAs may present valuable diagnostic and therapeutic strategies for GBCs (Table I).

3. Oncogenic microRNAs

miR-21. Since the aberrant expression of miR-21 was first reported in human glioblastoma by miRNA profiling (49), there have been several studies indicating that miR-21 exhibited an oncogenic capacity and played an important role in carcinogenesis and progression of various types of cancer (50-52). miR-21 was one of the first aberrant miRNAs identified in GBC by miRNA expression profiling of BK5.erbB2 transgenic mice (53). In this study, 9 miRNAs were found to be significantly upregulated and 13 miRNAs were found to be significantly downregulated in transgenic GBC tissue compared with wild-type tissue. Treatment with the histone deacetylase inhibitor, PCI-24781, significantly decreased the expression of miR-21, as well as miR-142-3p, miR-142-5p and miR-223, which were all upregulated in GBC. In addition, PCI-24781 also induced a significant upregulation in the expression of miR-122, which was downregulated in GBC, highlighting the potential therapeutic value of reversing aberrant miRNA expression by histone deacetylase inhibition in GBCs. Furthermore, research by Sekine *et al* established that miR-21 displayed oncogenic abilities that repress the phosphatase and tensin homolog and may contribute to the role of aquaporins, which are involved in the proliferation, migration, invasion and prognosis of GBC patients (54). Research on targeted therapies against miR-21 may prove to be promising in the suppression of GBC development.

miR-20a. miR-20a, a member of the oncogenic miR-17-92 cluster, was shown to behave as an oncogene by directly or indirectly regulating several target genes or signaling pathways in different types of tumors (55-59). It was previously established that miR-20a plays a central role in the pathogenesis and poor survival of GBC by targeting the mothers against decapentaplegic homolog 7 (Smad7)/ β -catenin axis (60). The elevated expression of miR-20a was closely correlated with local invasion, distant metastasis and poor prognosis of GBC patients. Additionally, the aberrant expression of miR-20a induced epithelial-to-mesenchymal transition and enhanced the metastatic potential of GBC cells *in vitro* and *in vivo*.

Downregulation of miR-20a by a specific antagomir effectively restored the expression of Smad7 and weakened transforming growth factor (TGF)- β -induced cell metastasis, which may provide a novel therapeutic strategy for GBC patients.

miR-155. Elevated expression of miR-155 has been described in multiple cancers, reflecting tumor staging, progression and treatment outcomes (61-64). In accordance with expectations, miR-155 acts as a vital oncogene in GBCs, as was reported by Kono *et al* (43). Although miR-155 was not upregulated in GBCs compared with pancreaticobiliary maljunction, the expression level of miR-155 was significantly higher in GBCs compared with normal gallbladders. In addition, the overexpression of miR-155 in GBCs was significantly associated with lymph node metastasis and vascular invasion. More importantly, the disease-specific survival rate was significantly lower in GBC patients with high miR-155 expression, compared with that in those with low miR-155 expression. In addition, ectopic expression of miR-155 by transfection significantly enhanced the proliferation and invasion of GBC cells *in vitro*. This provides strong proof that miR-155 may be a useful prognostic factor or tumor marker for therapeutic targeting. However, further research is required in order to identify the underlying mechanisms and target genes downstream of miR-155 in the development and progression of GBC.

miR-182. miR-182 has been reported to significantly regulate cancer progression. Increased expression of miR-182 was associated with poor survival in several types of cancer (65-68). In a study by Qiu *et al*, the expression level of miR-182 was significantly upregulated in GBC compared with that in normal control tissues. Additionally, the expression of miR-182 was significantly higher in gallbladder tumors that eventually metastasized, when compared with primary non-metastatic tumors. Specifically, TGF- β -induced overexpression of miR-182 promoted the migration and invasion of GBC cells, whereas tumor progression was eliminated by miR-182 inhibition (69). In addition, the incidence of pulmonary metastases was inhibited by downregulating the expression of miR-182 using a specific inhibitor *in vivo*. More importantly, the study identified cell adhesion molecule 1 (CADM1) as a novel target gene of miR-182 *in vitro* and *in vivo*, and demonstrated that the ectopic expression of CADM1 in GBC cells partially abrogates miR-182-induced cell invasion.

4. Tumor suppressor microRNAs

miR-218-5p. miR-218-5p was shown to be downregulated in a variety of carcinomas, including cervical, prostate, bladder, pancreatic and esophageal carcinoma, and to exert tumor-suppressive effects (70-74). Previous research has confirmed the marked downregulation of miR-218-5p in GBC compared with paired adjacent normal gallbladder tissue. In addition, miR-218-5p was shown to inhibit GBC cell invasion, migration and proliferation by targeting the polycomb group gene, B-cell-specific moloney murine leukemia virus integration site 1 (Bmi1), with the effects being abrogated by miR-218-5p inhibition (75). Further research revealed that the oncogenic activity of colon cancer-associated transcript-1, a long non-coding RNA (lncRNA), is in part through negative

Table I. The functions of microRNAs in gallbladder cancer.

| miRNA (Refs.) | Expression | Sample | Target | Role | Functions |
|------------------|------------|------------------|-------------|------------------|--|
| miR-21 (53) | Up | Tissue and cell | PTEN | Oncogene | Proliferation; migration; invasion; apoptosis; prognosis |
| miR-20a (60) | Up | Tissue and cell | Smad7 | Oncogene | Invasion; metastasis; prognosis |
| miR-155 (43) | Up | Tissue and cell | | Oncogene | Invasion; proliferation; lymph node metastasis; prognosis |
| miR-182 (69) | Up | Tissue and cell | CADM1 | Oncogene | Migration; invasion; metastasis |
| miR-218-5p (75) | Down | Tissue and cell | Bmi1 | Tumor suppressor | Invasion; migration; proliferation |
| miR-335 (44) | Down | Tissue | | Tumor suppressor | Histological grade; tumor invasion; lymph node metastasis; pTNM stage; prognosis |
| miR-34a (82) | Down | Tissue and cell | PNUTS | Tumor suppressor | Proliferation; colony formation; prognosis |
| miR-130a (86) | Down | Tissue and cell | HOTAIR | Tumor suppressor | Invasion; proliferation |
| miR-135a-5p (47) | Down | Tissue and cell | VLDLR | Tumor suppressor | pTNM stage; proliferation; colony formation |
| miR-26a (46) | Down | Tissue and cell | HMGA2 | Tumor suppressor | pTNM stage; proliferation |
| miR-146b-5p (96) | Down | Tissue and cell | EGFR | Tumor suppressor | Tumor size; development; proliferation; apoptosis |
| miR-1 (48) | Down | Tissue and cell | VEGF-A; AXL | Tumor suppressor | Proliferation; apoptosis |
| miR-145 (48) | Down | Tissue and cell | AXL | Tumor suppressor | Proliferation; apoptosis |
| miR-143 (26) | Down | Tissue and blood | | Tumor suppressor | Lymph node metastasis; pTNM stage |
| miR-122 (26) | Up | Tissue and blood | | Oncogene | Lymph node metastasis; pTNM stage |
| miR-187 (26) | | | | | |

PTEN, phosphatase and tensin homolog; Smad7, mothers against decapentaplegic homolog 7; CADM1, cell adhesion molecule 1; Bmi1, B-cell-specific moloney murine leukemia virus integration site 1; PNUTS, phosphatase nuclear targeting subunit; HOTAIR, HOX transcript antisense RNA; VLDLR, very low-density lipoprotein receptor; HMGA2, high-mobility group AT-hook 2; EGFR, epidermal growth factor receptor; VEGF-A, vascular endothelial growth factor-A; AXL, AXL receptor tyrosine kinase; pTNM, pathological tumor/node/metastasis.

regulation of miR-218-5p and subsequent modulation of Bmi1 in GBC cells *in vitro* and *in vivo*. Although this provides significant evidence that lncRNAs may function by targeting miRNAs, further investigation is required to identify the association between these two types of RNAs.

miR-335. miR-335 serves as a tumor suppressor miRNA, is transcribed from the genomic region on chromosome 7q32.2 (76) and is downregulated in various human digestive malignancies, such as pancreatic carcinoma, hepatocellular carcinoma, colorectal cancer and gastric cancer (77-80). A similar result was also observed in GBC patients. Previous research has verified that miR-335 is also an important tumor suppressor gene and was significantly downregulated in GBC. The expression level of miR-335 was lower in the majority of GBC tissues compared with that in adjacent normal tissues, as measured by reverse transcription-polymerase chain reaction (RT-PCR). In addition, low expression of miR-335 was correlated with poor histological differentiation, advanced pathological tumor invasion, lymph node metastasis and pathological TNM stage. Importantly, the expression level of miR-335 was an independent prognostic factor for the

overall survival of GBC patients by multivariate analysis (44). Unfortunately, the target genes involved in miR-335-mediated tumor suppression in GBC remain unknown.

miR-34a. The miR-34 family, including miR-34a, miR-34b and miR-34c, is directly regulated by p53 and has been reported to induce apoptosis and cell cycle arrest and, thus, act as a tumor suppressor in cancer cells (81). As was expected, low expression of miR-34a has also been found to be important in GBC (82). Additionally, previous studies have demonstrated that altered telomere length may contribute to cancer development and progression (83). In a study by Jin *et al*, miR-34a levels and telomere length were evaluated in 77 gallbladder adenocarcinomas and 36 peritumoral tissues by RT-PCR (82). The results revealed significantly lower expression of miR-34a and longer telomere length in GBC tissues, and, more importantly, that low miR-34a expression was associated with poor GBC patient survival. Interestingly, forced overexpression of miR-34a by an adenovirus may weaken the colony-forming abilities of CD44⁺ CD133⁺ GBC tumor stem-like cells *in vitro* and inhibit xenograft tumor growth *in vivo*. Additionally, adenovirus-mediated ectopic expression of miR-34a may

downregulate phosphatase nuclear targeting subunit expression and reduce telomere length in xenograft GBC tumor cells, thus identifying an underlying target gene of miR-34a in GBC.

miR-130a. miR-130a has been confirmed to be downregulated in a variety of carcinomas and to exhibit tumor-suppressive activity (84,85). In GBC, it was previously demonstrated that miR-130a was significantly downregulated in cancer tissues compared with adjacent normal tissue. In addition, miR-130a levels were negatively associated with a lncRNA, HOX transcript antisense RNA (HOTAIR), which has been shown to be a poor prognostic factor in several carcinomas, and to be correlated with tumor metastases. Furthermore, loss of HOTAIR has been associated with the inhibition of cancer invasiveness (86). It was previously demonstrated that the expression of HOTAIR was negatively associated with miR-130a in GBC tissues, and that knockdown of HOTAIR may decrease the invasion of GBC cells, a phenotype that may be partially reversed by miR-130a inhibition. In addition, knockdown of HOTAIR *in vitro* reduced the fraction of cancer cells in S-phase, thus suppressing proliferation, while miR-130a inhibition may reverse this effect. These data provide strong evidence of the inverse association between HOTAIR and miR-130a.

miR-135a-5p. It has been confirmed that miR-135a-5p acts as a tumor suppressor, affecting the proliferation of several carcinomas through interactions with various target genes (87-89). In the study of Zhou *et al*, miR-135a-5p was selected for further investigation due to its aberrant expression and tumor-related functions in GBC, based on miRNA chip and Cell Counting Kit-8 assays, respectively. miR-135a-5p levels were significantly downregulated in GBC tissues, and were correlated with the histological grade of the tumors. Additionally, the expression level of miR-135-5p was found to affect GBC cell proliferation. Specifically, the transfection of a miR-135a-5p mimetic may inhibit the proliferative and colony-forming abilities of GBC cells by arresting the cells in the G1/S phase. Lentivirus-mediated overexpression of miR-135a may significantly decrease the proliferation of GBC cells compared with cells infected with lenti-green fluorescent protein (GFP). Additionally, xenografts established in nude mice derived from the miR-135a-infected cells were significantly smaller compared with those derived from the GFP-infected cells. These data provide proof that miR-135a-5p may inhibit the proliferation of GBC cells *in vitro* and *in vivo*. Furthermore, it was demonstrated that miR-135a exhibited this function through directly binding the 3'-UTR of very low-density lipoprotein receptor, thus resulting in activation of the p38 mitogen-activated protein kinase pathway (47).

miR-26a. miR-26a, located on chromosome 3p22, a region characterized by high frequent loss of heterozygosity in cancer, is a tumor suppressor (90,91). miRNA chip was used to functionally screen for miRNAs in 4 paired GBC and paracancerous tissues, and miR-26a was found to be significantly downregulated in GBC (46). Further investigation revealed that the expression of miR-26a was correlated with pathological TNM stage, and contributed to inhibition of GBC cell proliferation; however, this effect could be reversed by

reintroduction of high-mobility group AT-hook 2 (HMGA2), a gene whose expression was negatively associated with miR-26a levels. Thus, miR-26a-induced changes in GBC cell proliferation were mediated by HMGA2.

miR-146b-5p. miR-146b-5p has been reported to possess critical tumor suppressor properties in recent studies (92-95). The expression level of miR-146b-5p was significantly downregulated in GBC tissue compared with that in adjacent tissues, and was found to be significantly correlated with tumor size and development by Cai *et al* (96). Additionally, increased expression of miR-146b-5p in GBC cells may inhibit cell growth by inducing apoptosis and G1 phase arrest. Furthermore, the results demonstrated that epidermal growth factor receptor (EGFR) mRNA levels and miR-146b-5p levels were negatively correlated. EGFR was a direct target of miR-146b-5p and acted as an essential mediator of the cancer-related functions of miR-146b-5p in GBC. In addition, ectopic expression of EGFR may abrogate the inhibition of proliferation induced by miR-146b-5p. These data indicate that the mechanism of action of miR-146b-5p in GBC involves the regulation of EGFR expression.

miR-1 and miR-145. In a previous study, the expression levels of miR-1 and miR-145 were consistently downregulated in GBC compared with normal gallbladder mucosa, as revealed by microarray hybridization, with similar results also observed in GBC cell lines (48). The ectopic expression of miR-1 and miR-145 by microRNA mimetics significantly reduced growth and promoted apoptosis in NOZ cells. Furthermore, the expression level of VEGF-A and AXL mRNAs were significantly decreased in miR-1-transfected cells compared with control-transfected cells. However, in response to miR-145 transfection, the expression of VEGF-A mRNA was unchanged and AXL mRNA was significantly increased, indicating complicated mechanisms that have not yet been elucidated.

5. Circulating microRNAs in GBC

A recent study discovered that the expression levels of 11 miRNAs were altered at least twofold in GBC tissue compared with neighboring non-cancerous gallbladder tissues, as revealed by miRNA microarray analysis (45). Five of these miRNAs, namely miR-21, miR-370, miR-187, miR-122 and miR-202, were upregulated, while 6 miRNAs, namely let-7a, miR-200b, miR-143, miR-31, miR-335 and miR-551, were downregulated. Using blood samples from 40 GBC patients and healthy volunteers, aberrant expression patterns for 6 of these miRNAs (let-7a, miR-21, miR-187, miR-143, miR-202 and miR-335) were confirmed and found to be in agreement with those measured by microarray; in addition, the total levels of circulating miRNAs in GBC patients were significantly different compared with those in healthy individuals. Of note, further investigation demonstrated that 3 of the miRNAs (miR-187, miR-143 and miR-122), were correlated with lymph node metastasis and pathological TNM stage. In addition, the association between genetic variants of miRNAs and susceptibility to GBC was also analyzed with blood samples; however, the results demonstrated that common miRNA variants did

not contribute to GBC susceptibility (42). Furthermore, it was demonstrated that the combination of miR-27a_{rs895819}, miR-570_{rs4143815} and miR-181a_{rs12537} was the most suitable gene-gene interaction model for predicting susceptibility to GBC and treatment response of GBC patients. Additionally, the interaction of miR-27a_{rs895819} and miR-181a_{rs12537} was correlated with hematological toxicity (neutropenia) in GBC patients undergoing chemoradiotherapy. However, the genetic variants of miRNAs did not affect the response to chemoradiotherapy or the survival outcomes of GBC patients (97). Further research is urgently required to elucidate the important role of circulating miRNAs in GBC.

6. Conclusions

GBC, a common malignant gastrointestinal neoplasm, represents the leading cause of biliary tract malignancy-related mortality, and is currently associated with a significantly lower survival rate compared with a number of other common cancers. MicroRNAs have been widely reported to play crucial roles in cancer development, metastasis and prognosis of GBC patients. In particular, the expression levels of circulating miRNAs differ significantly between cancer patients and healthy volunteers. Elucidating the role of miRNAs in the biology of GBC may provide novel therapeutic strategies for the management of GBC, and identify effective biomarkers for early diagnosis. In this review, we discussed how aberrant miRNA expression has been shown to contribute to the development and progression of GBC, through the upregulation of oncogenic miRNAs and downregulation of tumor-suppressing miRNAs. Additionally, detection of circulating miRNAs may be a non-invasive method, valuable for early diagnosis and prediction of outcome. Importantly, novel, less toxic, miRNA and anti-miRNA therapy has the potential to target multiple genes simultaneously, which provides new tools for the research and development of treatments for GBC.

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