Review Article

MicroRNA-21: Mechanisms of Oncogenesis and its Application in Diagnosis and Prognosis of Gastric Cancer

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Abstract

Gastric cancer is a silent killer, claiming more than seven hundred thousand lives every year. This heavy burden creates an irrefutable need for accurate, noninvasive methods of population screening and early detection as well as disease monitoring and management. Gastric cancer is a multi-factorial disease with an uneven geographic distribution, mostly affecting the developing countries and Southeast Asia. The multi-dimensional roles of microRNAs in gene regulation and tumorigenesis have prompted investigators to explore their potentials in diagnosis and treatment of various cancers, including gastric cancer. In this respect, miR-21 has attracted much attention as well as generating some controversies. Here, we aim to describe, in a chronological order, the numerous studies which have explored 1) the interactions of this oncomir with *Helicobacter pylori* infection, as a class I gastric carcinogen, 2) its potential mechanisms of oncogenicity, by various induction/inhibition assays, and 3) its application as a diagnostic/prognostic invasive (tissue) and non-invasive (circulating) biomarker.

Keywords: *Helicobacter pylori*, tumor differentiation, tumor stage, gene regulation, proliferation

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Gastric cancer

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer death worldwide. The Globocan 2012 report[1] has documented 952,000 stomach cancer incident cases around the globe, which claims more than 700,000 lives every year. The regional variations in gastric cancer incidence are due to differences in host susceptibility factors, prevalence of *Helicobacter pylori* (Hp) infection and its antigenic composition,[2–5] as well as environmental inputs, in particular, dietary patterns and smoking habits.[6–8] GC incidence and mortality rates have been declining during the past decades. However, the disease is typically diagnosed at late stages and remains a major clinical challenge in view of timely detection and monitoring. Hp eradication strategies are rapidly becoming the focus of attention in an attempt for global reduction of gastric cancer risk.[9,10] Nevertheless, there is an urgent need for screening and early detection strategies, making use of appropriate biomarkers, in order to increase survival rates.

MicroRNAs

MicroRNAs (miRNAs) are members of small non-coding RNAs which play a crucial role in post transcriptional gene regulation of virtually 30%–60% of all human genes.[11] They are believed to act through degradation of coding RNA and/or inhibition of translation initiation.[12,13] As a single miRNA can target hundreds of different genes, its dysregulation may lead to cancerous transformation of an otherwise normal cell.[14] It has been demonstrated that miRNAs are involved in many biological processes such as proliferation, differentiation, and apoptosis.[15] Alterations in miRNA expression are nominated as potential biomarkers for disease diagnosis and prognosis, particularly in various cancers.[16–22] There is a growing number of studies which have evaluated the role of various miRNA as potential tissue-based as well as circulating biomarkers in gastric cancer.[17,23–27] Among these well studied miRNAs, overexpression of miR-21 has been frequently reported in gastric cancer, as well as a multitude of other cancers.[28–36] Increasing evidence suggests that miR-21 is to be considered as an irrefutable hallmark of gastric cancer. This paper reviews the current literature on the carcinogenic role of miR-21 and its potential applications in GC diagnosis and prognosis.

MiR-21

The mature form of miR-21, also known as hsa-miR-21 or miRNA21, is a conserved mammalian miRNAs, which is encoded by the *MIR21* gene.[37,38] This relatively well characterized miRNA is transcribed from the plus strand of chromosome 17q23.2 (55273409–55273480), where it overlaps with the protein-coding gene *TMEM49* (also called vacuole membrane protein).[39,40] The transcription of *MIR21* is activated by AP-1 (activation protein-1) in conjugation with the SWI/SNF (switch/sucrose non-fermentable) complex through the conserved AP-1 and PU.1 (transcription factor) binding sites in its promoter region.[39] An estimated 3433-nt long primary transcript of miR-21 (pri-miR-21) is transcribed in an intron region of a coding gene *TMEM49*, just downstream from the TATA box of the promoter. This implies that each miRNA could have their own promoter even if overlapping with other genes.[39] Up-regulation of miR-21 in various human cancers, its potential function in targeting a variety of important tumor suppressor genes and association with the progression of cancer offer

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convincing evidence for the use of miR-21 as a diagnostic and prognostic biomarker.[38]

MiR-21 and *Helicobacter pylori* infection

As concretely established, Hp infection is considered a class I gastric carcinogen.[41] Accordingly in 2008, Zhang, *et al.*[42] used TaqMan quantitative real-time PCR to demonstrate overexpression of miR-21 in Hp-infected gastric tissue. This encouraged Shiotani, *et al.*[43] to evaluate their findings by investigating the consequences of Hp eradication on miR-21 expression, in addition to other miRNA, in subjects with and without gastric cancer. They found that Hp eradication caused a significant down-regulation of miR-21, exclusively in cancer-free subjects. Shiotani and colleagues[44] continued, a year later, by investigating serum levels of miRNAs as well as pepsinogen in patients with early gastric cancer, before and after Hp eradication. Their findings confirmed that the relative levels of miR-21 remain elevated in GC patients, regardless of Hp eradication. The stability of serum miR-21 following Hp eradication and its superiority to serum pepsinogen in detection of GC, nominates it as a preferable marker for GC screening. The impact of Hp infection in miR-21 regulation, however, suffers a controversy, as Li and colleagues[45] found no significant differences in its plasma levels in both gastric cancer patients as well as normal controls, with or without Hp infection. Nevertheless, the interaction between Hp infection and miRNA dysregulation remains an understudied area, which calls for further exploration.[46,47]

MiR-21 and gastric cancer

MiR-21 and its targets genes in gastric cancer cell lines

The expression of miR-21 has been studied, as well as manipulated, in various gastric cancer (AGS, SGC7901, MKN1, MKN7, MKN28, MKN45, MKN74, NUGC3, NUGC4, AZ521, KATOIII, NCI-N87, BGC-823, HTB-103, CRL-5974, CRL-5971) and non-cancer (GES-1) cell lines (Table-1). As a result, a number of target genes (RECK, PDCD4, PTEN, Serpini1, FASLG, PTG2) have been identified. Furthermore, the role of miR-21 in mediating resistance to certain chemotherapeutic agents (i.e., trastuzumab, cisplatin) has been investigated (Table 1).

The up-regulation of miR-21 in gastric cancer (AGS, SGC7901, MKN28, MKN45) vs. non-cancer (GES-1) cell lines was primarily discovered by Zhang, *et al.*[42] These investigators demonstrated alterations in cellular behaviors following forced expression of miR-21 in AGS cell line. These alterations included enhanced cell proliferation, invasion and migration. Conversely, miR-21 gene-knockdown abrogated these behaviors and augmented apoptosis. This sequence of events was shown to be mediated by RECK (reversion-inducing-cysteine-rich protein with kazal motifs), a known tumor suppressor gene and a bona fide target of miR-21.

Another study by Motoyama, *et al.*[48] investigated the association between the expression levels of miR-21 and PDCD4 (programmed cell death protein 4) mRNA, a tumor suppressor gene, in eight (MKN1, MKN7, MKN45, MKN74, NUGC3, NUGC4, AZ521 and KATOIII) human gastric carcinoma cell lines. This study found an inverse correlation between the expression of PDCD4 mRNA and miR-21 in these cell lines. PDCD4 was identified as another putative target gene for the oncogenic effect of miR-21. Two years later, Cao, *et al.*[49] confirmed the inverse correlation between miR-21 and PDCD4 protein expression, following the treatment of AGS cells with increasing doses of resveratrol (an inhibitor of miR-21 expression). However, this phenomenon was not observed at the mRNA level.

Zhang and colleagues[50] examined the stimulatory and inhibitory effects of pre-miR-21 and miR-21 inhibitor in a gastric cancer cell line (BGC-823), respectively. Application of the scratch-healing assay revealed that pre-miR-21-transfected cells healed more rapidly as compared to controls, whereas the down-regulation of miR-21 led to the inhibition of cell migration in a transwell migration assay. The results of western blotting and luciferase reporter assays demonstrated that expression of PTEN (phosphatase and tensin homolog), a tumor suppressor gene, increased remarkably after miR-21 inhibition. These results further supported the involvement of miR-21 in suppressing PTEN in the initiation and development of gastric cancer. In 2013, Yang and colleagues[51] explored the role of miR-21 in gastric cancer chemo-resistance. Their study demonstrated that the expression of miR-21 was up-regulated in the cisplatin-resistant (SGC7901/DDP) compared to its parental (SGC7901) cell line. Over-expression of miR-21 led to a decline in the rate of apoptosis and also the anti-proliferative effects of cisplatin. On the other hand, knockdown of miR-21 enhanced its effects. Additionally, they demonstrated that miR-21 performs its action through down-regulating the expression of PTEN and activation of Akt (protein kinase B) pathway. In 2014, Eto and colleagues[52] provided further evidence that transfection of miR-21-mimic into NCI-N87, a HER2-positive cell line with low expression of miR-21, resulted in down-regulation of PTEN and increased phosphorylation of Akt (proto-oncogene), which in turn caused a significant suppression of trastuzumab-induced apoptosis. The opposite pattern was observed in NUGC4, a HER2-positive cell line with high expression of miR-21, which was transfected with miR-21 inhibitor. Further support was also provided by Li, *et al.*,[53] who confirmed the pivotal role of miR-21 in modulating the expression of these tumor suppressor genes (PTEN and PDCD4) in gastric cancer cell lines. They demonstrated that overexpression of miR-21 by the transfection of miR-21-mimic into two different gastric adenocarcinoma (SGC7901, MKN45) cell lines leads to enhancement of cell invasion and migration. In contrast, miR-21 inhibitor significantly reduces cell proliferation, migration and invasion. Recently, Sha and colleagues[54] evaluated the anticancer effect of celastrol, a plant triterpene, in three gastric cancer cells. They showed that the expression of miR-21 is significantly suppressed in gastric cancer cells treated with celastrol, in a dose-dependent manner, which resulted in diminished phospho-Akt expression and NF-κB activity.

In 2012, Yamanaka and colleagues[55] identified a binding site for miR-21 on the 3'-UTR of Serpini 1 (serpin peptidase inhibitor), a gene with novel tumor suppressive effects in gastric cancer. They showed an inverse correlation between the expression of miR-21 and Serpini 1. Accordingly, the down-regulation of miR-21 in a gastric cancer (MKN28) cell line caused a significant up-regulation of Serpini 1, which in turn led to vigorous G1/S arrest, with the ultimate suppression of tumor growth.

The tumorigenic role of miR-21 was also explored by the treatment of gastric cancer cell line with carcinogenic agents such as Nicotin,[56] which proved to upregulate miR-21 expression in a time- and dose-dependent manner. For this purpose, Shin, *et al.*[56] examined the expression profile of miRNA using a microarray platform covering a panel of 95 human miRNAs in a nicotine-treated gastric cancer (AGS) cell line. They demonstrated that miR-21 was upregulated upon nicotine stimulation via binding of

Table 1. Expression of MiR-21 and its target genes in gastric cancer cell lines.

Year	Authors	Ref	Cell Line (s)	Expression (Fold change)	MiR-21 Target gene(s)	Method	MiR-21 Function
2008	Zhang, *et al.*	42	AGS, SGC-7901, MKN-45, MKN-28 *vs.* GES-1	↑	↓RECK	TaqMan RT-PCR	**Anti:** Apoptosis **Pro:** Proliferation, invasion and migration
2010	Motoyama, *et al.*	48	MKN-1, MKN-7, MKN-45, MKN-74, NUGC-3, NUGC-4, AZ-521, KATOIII	↑	↓PDCD4	TaqMan RT-PCR	—
2011	Shin, *et al.*	56	AGS (nicotine treated *vs.* untreated)	↑	ND*	miRNA microarray	Pro: Proliferation
2012	Cao, *et al.*	49	AGS (dose-dependent resveratrol treatment)	↓	↑PDCD4 protein $PDCD_4$ mRNA(unchanged)	SYBR Green RT-PCR	—
2012	Zhang, *et al.*	50	SGC7-901, MKN-28, MKN-45, AGS, CRL, BGC-823, HTB-103, CRL-5974, CRL-5971 *vs.* GES-1 BGC-823 (pre-miR-21/miR-21 inhibitor)	↑ ↑	— ↓PTEN	TaqMan RT-PCR	Pro: Cell growth, invasion and migration
2012	Golestaneh, *et al.*	58	MKN-45 ($CD44^+$ vs. $CD44^-$)	↑ (30.7)	ND*	SYBR Green RT-PCR	—
2012	Yamanaka, *et al.*	55	MKN28	↑	↓Serpini1	TaqMan RT-PCR	Pro: Cell growth
2013	Yang, *et al.*	51	SGC7901 (cisplatin-resistant *vs.*sensitive)	↑	↓PTEN ↑Akt pathway	SYBR Green RT-PCR	Anti: Apoptosis Pro: Proliferation
2014	Eto, *et al.*	52	HER2-positive GC cell lines [NUGC4 ($miR\text{-}21_{Hi}$) *vs.*NCI-N87 ($miR\text{-}21_{Lo}$)]	↑	↓PTEN ↑Akt pathway	TaqMan RT-PCR	Anti: Apoptosis
2014	Li, *et al.*	28	SGC7901, MKN45	↑	↓PTEN, ↓PDCD4	TaqMan RT-PCR	Anti: Apoptosis Pro: Cell growth, invasion and migration
2014	Yang, *et al.*	57	AGS, SGC-7901, MKN-28, HGC-27, BGC-823 *vs.* GES-1 GES-1 (MNNG treated vs. untreated)	↑ ↑	— ↓FASLG, ↓BTG2	TaqMan RT-PCR	Pro: Malignant transformation and tumorigenesis
2014	Sha, *et al.*	103	Gastric cancer cells (celastrol treated *vs.* untreated)	↓	↓Akt pathway ↓NF-kB activity	qRT-PCR	Anti: Apoptosis

**ND= Not Determined*

NF-κB to mir-21 promoter. Furthermore, their functional study exhibited that ectopic expression of miR-21 in this gastric cancer cell line contributed to the enhanced cell proliferation, while the transfection of anti-miR-21 significantly abrogated this phenomenon. On the other hand, Yang, *et al.*[57] investigated the regulatory role of miR-21 in gastric tumorigenesis following exposure of a non-cancer (GES-1) cell line to MNNG (N-nitroso carcinogen N-methyl-N-nitro-N'-nitrosoguanidine) and detected an elevated dose- and time-dependent gene expression. The MNNG-induced overexpression of miR-21 enhanced the transformation and cell growth of GES-1 cells, through down-regulation of both FASLG (Fas ligand) and BTG2 (B cell translocation gene 2). Their study shed light on the involvement of miR-21 in the process of chemical carcinogenesis. These investigators also confirmed the overexpression of miR-21 in several gastric cancer (AGS, SGC-7901, MKN-28, HGC-27, and BGC-823) relative to non-cancer (GES-1) cell line(s).

In a different study, Golestaneh, *et al.*[58] explored the differential expression of selected miRNAs in $CD44^{+}$ (CSC, cancer stem cells) vs. $CD44^{-}$ gastric cancer (MKN45) cells. Their analysis demonstrated a drastic (30.7 fold) enhancement of miR-21 expression in the former vs. latter cells. Based on the fact that cancer stem cells are thought to be responsible for tumor metastasis and relapse as well as the previously mentioned findings, the authors have further nominated this miRNA as a potential candidate for cancer therapy.

On the other hand, Xu, *et al.*[59] were able to exhibit the suppressing effects of single-(AMOs) and multi-(MTg-AMOs), anti-microRNA antisense oligonucleotides, on proliferation and migration of human gastric cancer (SGC7901) cell line. In this study, suppression of miR-21 significantly decreased the proliferative and migration activity of cells as manifested by cell proliferation and transwell migration assay.

The various roles of miR-21 in cellular events, its putative target genes as well as interaction of carcinogenic vs. chemotherapeutic agents are summarized in Figure 1.

MiR-21 as a diagnostic biomarker for gastric cancer

i. Tissue MiR-21

Several investigators have explored the differential expression of miR-21 in gastric tumors in comparison to its adjacent normal tissue or that obtained from cancer-free subjects (Table 2A). Primarily, using a large-scale miRnome analysis, Volinia, *et al.* studied miRNA profiles in several human cancers, including gastric, lung, breast, prostate, colon, and pancreatic cancer.[60] They identified miR-21 as an overexpressed miRNA in solid tumors relative to their adjacent normal tissue, thereby nominating it as a cancer pathogenesis marker deregulating the natural tumor suppressor vs. oncogenic balance. Thereafter, high-throughput screening of miRNA expression in gastric tumor tissue versus its neighboring normal tissue has been thoroughly performed by a number of investigators. These investigations have unanimously confirmed the overexpression of miR-21, amongst other miRNA,

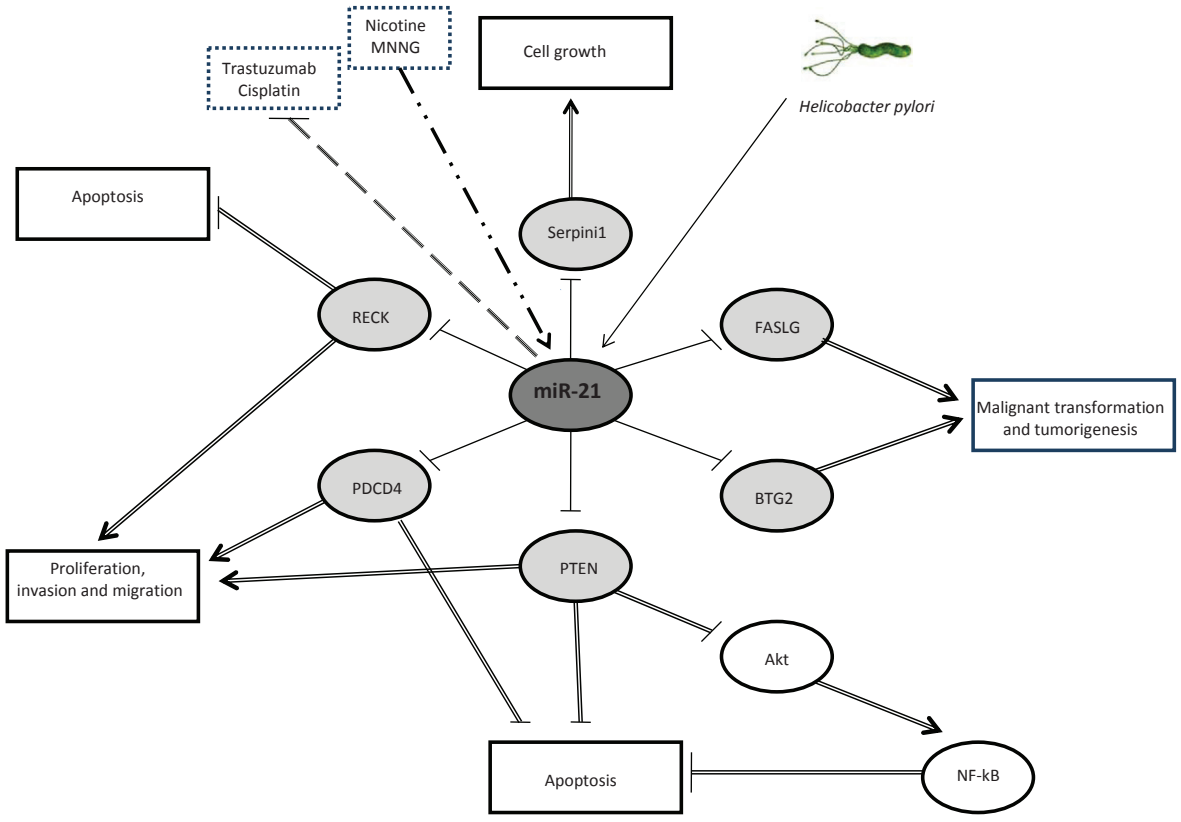

Figure 1. MiR-21 targets and its effector functions. Abbreviations: PDCD4 (programmed cell death protein 4); PTEN (phosphatase and tensin homolog); RECK (reversion-inducing cysteine-rich protein with Kazal motifs); Serpini1 (serpin peptidase inhibitor); FASLG (Fas ligand); BTG2 (B cell translocation gene 2); Akt (protein kinase B); NF-kB (nuclear factor kappa B); MNNG (N-methyl-N'-nitro-N-nitrosoguanidine).

Table 2. MiR-21 as a diagnostic biomarker for gastric cancer.

A. Tissue MiR-21									
Year	Authors	Ref	Sample	Sample Size	TNM Stage	Method	Reference gene Array/qRT-PCR	AUC	Expression (fold change)
2006	Volinia, *et al.*	60	Fresh Tissue	Tumor = 20 Adjacent = 21	—	MiRNA microarray	Global Median	—	↑
2008	Petrocca, *et al.*	63	Fresh Tissue	Tumor = 20 Adjacent = 20	—	MiRNA microarray TaqMan RT-PCR	Global Median U6	—	↑(4.5)
2008	Chan, *et al.*	73	Fresh Tissue	Tumor = 37 Adjacent = 37	Early (I-II) = 13 Late (III-IV) = 24	TaqMan RT-PCR	U6	—	↑
2009	Guo, *et al.*	64	Fresh Tissue	Tumor = 3 Adjacent = 3	Late (III) = 3	MiRNA microarray	Global Median	—	↑
2010	Ueda, *et al.*	67	Fresh Tissue	Tumor = 160 Adjacent = 160	—	MiRNA microarray/	Global Median	—	↑(2.0)
				Tumor = 24 Adjacent = 24		TaqMan RT-PCR	RNU49		↑
2010	Tsukamoto, *et al.*	66	Fresh Tissue	Tumor = 22 Adjacent = 5	—	MiRNA microarray	Global Median	—	↑(4.05)
2010	Motoyama, *et al.*	48	Fresh Tissue	Tumor = 52 Adjacent = 52	—	TaqMan RT-PCR	U6	—	↑
2010	Ding, *et al.*	65	Fresh Tissue	Tumor = 48 Adjacent = 48	Early (I-II) = 13 Late (III-IV) = 35	MiRNA microarray	Global Median	—	↑(1.61)
2010	Tchernista, *et al.*	62	Fresh Tissue	Test (Tumor = 6, Adjacent = 6)	—	MiRNA microarray	Global Median	—	↑(4.62)
				Validation (Tumor = 20, Adjacent = 20)		SYBR green RT-PCR	U6		↑
2010	Liu, *et al.*	74	FFPE Tissue	Tumor = 20 Adjacent = 21	Early (I-II) = 6 Late (III-IV) = 14	TaqMan RT-PCR	U6	—	↑
2011	Oh, *et al.*	71	Fresh Tissue	Tumor = 40 Adjacent = 40	—	MiRNA microarray	Global Median	—	↑
2011	Li, *et al.*	69	FFPE Tissue	Tumor = 30 Adjacent = 30	—	TaqMan RT-PCR	U6	—	↑(2.0)
2011	Li, *et al.*	70	Fresh Tissue	Tumor = 6 Adjacent = 6	Late (III-IV) = 6	MiRNA microarray	Global Median	—	↑(10.44)
2011	Jiang, *et al.*	75	FFPE Tissue	Tumor = 55 Normal = 5	Late (III-IV) = 55	TaqMan RT-PCR	RNU44	—	↑
2011	Kim *et al.*	68	Fresh Tissue	Tumor = 90 Normal = 34	—	MiRNA microarray	Global Median	—	↑(1.49)
2011	Osawa *et al.*	77	FFPE Tissue	Tumor = 37 Adjacent = 37	Early (I-II) = 16 Late (III-IV) = 21	MiRNA microarray TaqMan RT-PCR	Global Median Human gastric reference RNA	—	↑(2.73)
2012	Cao *et al.*	49	FFPE Tissue	Tumor = 46 Adjacent = 46	—	SYBR green RT-PCR	U6	—	↑
2012	Zhang *et al.*	50	Fresh Tissue	Tumor = 30 Adjacent = 30	Early (I-II) = 7 Late (III-IV) = 23	TaqMan RT-PCR	U6	—	↑
2012	Inoue *et al.*	72	Fresh Tissue	Tumor = 5 Adjacent = 5	—	Real-Time PCR based MiRNA array	Global Median	—	↑(4.11)
2012	Yamanaka *et al.*	55	Fresh Tissue	Tumor=7, Normal = 3	—	MiRNA microarray	Global Median	—	↑
				Tumor = 39, Normal = 40		TaqMan RT-PCR	U6		↑(1.8)

2013	Kim *et al.*	[78]	FFPE Tissue	Tumor = 91 Normal = 26	Early (I-II) = 32 Late (III-IV) = 59	SYBR green RT-PCR	U6	—	↑
2013	Wang *et al.*	[76]	Fresh Tissue	Tumor = 32 Adjacent = 32	—	SYBR green RT-PCR	U6	—	↑
2013	Cui *et al.*	[79]	Gastric juice	Normal mucosa or Minimal gastritis = 47 Atrophic gastritis = 18 Gastric ulcer = 34 Gastric cancer = 42	Early (I-II) = 7 Late (III-IV) = 35	SYBR green RT-PCR	miR-16	0.969	↑

B. Circulating MiR-21

Year	Authors	Ref	Sample	Sample Size	TNM Stage	Method	Reference gene Array/qRT-PCR	AUC	Expression in cancer (elevation/fold change)
2010	Tsujiura *et al.*	[82]	Plasma	GC = 69 Healthy = 30	Early (I-II) = 51 Late (III-IV) = 18	TaqMan RT-PCR	mirVana miRNA Reference Panel	0.673	↑
2011	Zheng *et al.*	[86]	Circulating Tumor Cells	GC=53 Healthy = 20	Early (I-II) = 16 Late (III-IV) = 37	SYBR green RT-PCR	U6	0.853	↑
2012	Song *et al.*	[84]	Serum	GC = 40 Healthy = 20	Early (I-II) = 20 Late (III-IV) = 20	SYBR green RT-PCR	miR-16, miR-93	—	↑(stage IV)
2012	Song *et al.*	[88]	Serum	GC = 14 Controls = 14 CAG*/SG	—	MiRNA microarray	(U6-RNU44-RNU48)	—	↑ (32.07)
				GC = 14 Controls = 14 CAG*/SG**		TaqMan RT-PCR	Spiked-in cel-miR-39		↑ (3.63)
				GC = 68 Controls = 68 CAG*/SG**		TaqMan RT-PCR	Spiked-in cel-miR-39		Not elevated
2012	Wang and Zhang	[85]	Serum	GC = 30 Healthy = 39	Early (I-II) = 11 Late (III-IV) = 19	SYBR green RT-PCR	miR-16	0.81	↑
2012	Li *et al.*	[45]	Plasma	GC = 70 Healthy = 70	Early (I-II) = 23 Late (III-IV) = 47	TaqMan RT-PCR	Spiked-in cel-miR-39	0.794	↑
2013	Cai *et al.*	[89]	Plasma	GC = 30 Healthy = 30	Early (I-II) = 35 Late (III-IV) = 56	SYBR green RT-PCR	Spiked-in cel-miR-39	—	↑(2.0)
				GC = 60 Healthy = 60		SYBR green RT-PCR	Spiked-in cel-miR-39		Not elevated
2014	Karimi Kurdistani *et al.*	[87]	Serum	GC = 30 NUD = 25	Early (I-II) = 15 Late (III-IV) = 15	TaqMan RT-PCR	miR-16	—	↑(5.56)

**CAG= Chronic Active Gastritis, **SG=Superficial Gastritis*

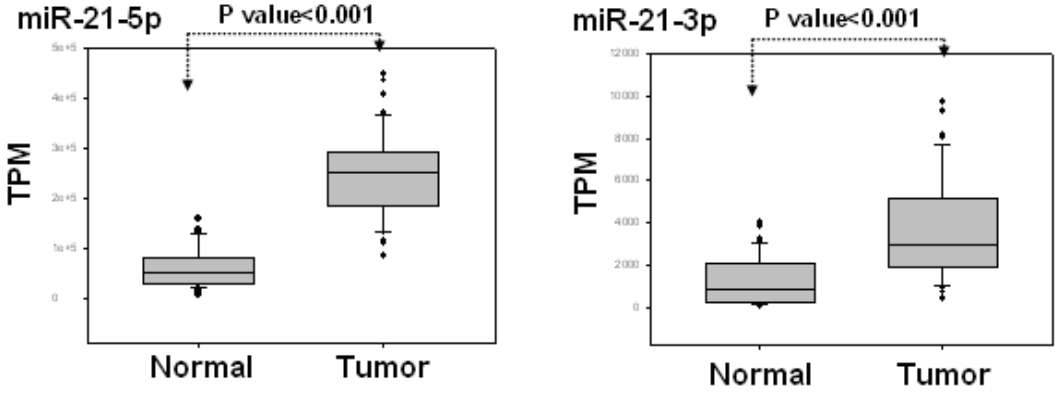

Figure 2. The expression levels of miR-21-5p and miR-21-3p increase in human gastric cancer tissues. We obtained the miRNA expression next-generation sequence (NGS) short-read level-3 data of human gastric cancer (STAD) from TCGA data portal. In this figure, the miRNA expression profiles of gastric cancer tissues and corresponding adjacent normal gastric tissues are generated from 42 patients. The expression levels of the miR-21 were presented in transcripts per million (TPM) and analyzed using the Student *t* test ($P < 0.05$ was considered significant).

by gastric tumor tissue. However, the reported fold changes of its over-expression are quite variable and range from 1.49–10.44.[61–72] To collate these results, we have analyzed the information available at the portal data of the cancer genome atlas[39] and found a statistically significant over-expression of miR-21-5p and -3p in gastric cancer tissue relative to the corresponding adjacent normal tissue (Figure 2).

Chan, *et al.*,[73] however, specifically concentrated on the expression level of miR-21 in gastric tumor tissue using quantitative PCR. They reported that miR-21 was up-regulated in gastric cancer samples compared to its corresponding normal tissues. Other researchers[48–50,55,69,74–78] have, thereafter, successively confirmed the significant up-regulation of miR-21 in gastric cancer tissues *vs.* non-cancer tissues (Table 2A).

Detection of miR-21 in the gastric juice of gastric cancer patients versus controls introduces the potential for a less invasive manner to conduct gastric tissue assessment. Cui and colleagues[79] primarily confirmed previous findings of miR-21 overexpression in gastric cancer tissue compared to its neighboring normal tissue. They proceeded by detecting cell-free miRNA in the gastric juice samples obtained from endoscopy subjects, which varied from normal to gastritis to gastric ulcer and cancer patients. Consequently, miR-21 was detected at higher levels in the gastric juice of GC patients relative to the rest of the cancer free controls, nearing an excellent (AUC = .969) discrimination power. Furthermore, miR-21 was able to differentiate GC according to subtype and was found significantly elevated in intestinal type GC *vs.* diffuse type.

ii. Circulating MiR-21

MiR-21 detection in the gastric tissue, although attractive as a diagnostic marker, is undesirable due to its invasive nature. Accumulating previous studies showed that miRNAs stably exist in various body fluids and are suitable as diagnostic and prognostic biomarkers for gastric cancers.[80–82] Therefore, attempts in evaluating circulating biomarkers have also concurred this area and an increasing number of investigators are searching for the diagnostic power of miR-21 in blood-derived (serum and plasma) samples, in which gastric cancer patients are assessed in comparison to healthy or cancer-free dyspeptic subjects (Table 2B).

Accumulating evidence for the up-regulation of miR-21 in gastric tumors, prompted Tsujiura and colleagues[82] to explore the likelihood of the circulating miRNAs behaving as a novel non-invasive biomarkers in diagnosing gastric cancer. They were able to demonstrate the moderate elevation of circulating miR-21 in GC patients vs. healthy controls (AUC = 0.673). Of remarkable value is the fact that miR-21 levels declined one month following gastric tumor resection. Post-operative decline of circulating miR-21 was supported by the results of Ma, *et al*.,[83] who employed qRT-PCR to compare the plasma levels of miR-21 between paired pre-operative and post-operative patients with primary GC. They, too, showed that the levels of miR-21 expression in the post-operative plasma samples declined dramatically in all patients compared to their pre-operative state.

In reference to the technical and biological validation of miRNA analysis, Song and colleagues[84] aimed to identify a suitable reference gene for analyzing circulating miRNA in gastric cancer. The serum expression of miR-21 was evaluated using different normalization strategies such as normalization on the basis of serum volume and use of two reference genes (miR-16 and miR-93). Serum volume normalization, without assessing relative expression of miR-21 to a reference gene, exhibited no significant difference between GC patients and healthy controls. However, when miR-16 and/or miR-93 were used as reference genes, the relative expression of miR-21 was found up-regulated solely in the advanced (stage IV) GC patients as compared to healthy subjects. Their results particularly recommended the use of miR-16 and miR-93 as two appropriate reference genes for qPCR analysis in gastric cancer. In addition to providing further support for the elevation of circulating miR-21 in GC patients *vs.* healthy controls, Li, *et al.*[45] were also able to exhibit such differentiation in early stages of GC.

Wang and Zhang[85] were able to demonstrate the up-regulation of circulating miR-21, not only in gastric cancer patients, but also in other solid (breast, esophageal, colorectal and lung) tumors, thereby recommending it as a fairly accurate solid tumor marker (AUC = 0.81). In addition, they were able to implement a cost-saving measure using SYBR green real-time quantitative reverse transcription-PCR instead of Taqman RT-qPCR, which was first introduced by Zheng, *et al.*[86] The latter group of investigators was able to detect the over-expression of miR-21 by circulating tumor cells (CTCs) in the peripheral blood of GC patients compared to

healthy controls (AUC = 0.853).

Due to the high prevalence of GC in East Asian countries, all of these results were collected therefrom. However, our recent preliminary analysis of circulating miR-21 in GC subjects from Iran[87] has provided additional confirmatory evidence from a West Asian country. According to this study, circulating miR-21 exhibited a stepwise upregulation by factors of 3.90 and 5.94 in the early and late stages of gastric cancer, respectively. An added advantage of this study was the use of PML (premalignant lesion)-free endoscopy subjects as controls.

A note of caution, however, is directed toward the studied sample sizes, which should be taken into careful consideration before a firm conclusion is drawn. Two concrete examples are portrayed by Song, *et al.*[88] and Cai, *et al.*[89] who primarily detected circulating miR-21 overexpression in GC patients vs. age- and gender-matched healthy controls in their pilot studies, but failed to validate their results in larger sample sizes. Nevertheless, considering the number of miRNAs studied by these investigators, the authors have not elaborated on the potential reasons behind their observed discrepancies, in the case of circulating miR-21. However, the disconformity observed between their larger studies and previous reports could be partly owing to the differences in the source of samples, quantification methods and screening facilities.[90] In order to address these controversies, further studies with larger sample populations from geographically diverse areas are clearly needed.

MiR-21 as a prognostic biomarker for gastric canser

i. Tissue MiR-21

The application of miR-21 in gastric cancer monitoring and prognosis has been evaluated by several groups in fresh or formalin-fixed paraffin-embedded (FFPE) gastric tissue, as well as serum/plasma, which has resulted in controversial data. The studied prognostic indices include: tumor size, grade of differentiation, Borrmann pTNM staging and survival rates (Table 3A). In 2010, Li and colleagues[91] evaluated the association between a seven-miRNA signature, in which miR-21 was included. In this breakthrough investigation, the tested miRNA signature (miR-10b, miR-21, miR-223, miR-338, let-7a, miR-30a-5p, miR-126) categorized GC patients into low and high-risk groups, the latter manifesting shortened overall and relapse-free survival rates, regardless of tumor stage and histologic differentiation. The observed close association with clinical outcomes recommended this type of miRNA risk profiling for stratification of GC patients as candidates for adjuvant therapy.

A growing body of evidence has produced a controversial collection of information regarding the association of miR-21 and patients' survival rates. For instance, in regards to the overall survival rates, two independent groups of investigators[77,92] demonstrated a significant reverse correlation with miR-21 expression levels, whereas two others did not.[78,93] Jiang and colleagues[75] demonstrated a reverse association between miR-21 expression and overall survival rates, regardless of the choice of the chemotherapeutic regimen. In addition to survival analysis, Xu and colleagues[92] explored the association of miR-21 expression with other clinicopathologic characteristics. They found that the expression level of miR-21 was significantly up-regulated in patients with lymph node metastasis and was associated with the tumor histologic type and pTNM stage of the tumor.

On the other hand, Tchernitsa, *et al.*[62] investigated the differential expression of miRNA using the multi-species miRNA microarray probe set (containing 857 mammalian probes) in primary gastric cancers (with and without lymph node metastases). The results of differential expression were then evaluated by RT-PCR on an independent validation set of patients with gastric cancer. Their findings identified miR-21 among five other miRNAs (miR-103, miR-145, miR-106b, miR-146a, and miR-148a) capable of discriminating node-positive from node-negative gastric cancer patients.

Other investigators have attempted to demonstrate significant correlations between elevated miR-21 expression and tumor size,[48,76] depth,[48] degree of differentiation, local invasion and lymph node metastasis.[50,76] However, the existing reports include controversial data, exemplified by Chan, *et al.*[73] who failed to find any association between overexpression of miR-21 and the clinicopathological features such as tumor size, tumor location, cell differentiation, gross appearance, Lauren's histological type, lymph node metastasis, lymphovascular invasion, peritoneal seeding, depth of cancer invasion or 5-year overall survival rate. Similarly, amongst the eleven selected miRNAs evaluated on FFPE tumor tissues, Kim and colleagues[78] found no association between miR-21 expression and lymph node metastasis, distant metastasis or serosa-penetration of tumors.

More recently, Hirata and colleagues[94] investigated the efficacy of miR-21, its target gene (PDCD4), as well as CD44v9 (CD44 variant 9) expression and the mucin phenotype in predicting tumor recurrence in patients with multiple early gastric cancer (EGCs) following endoscopic submucosal dissection (ESD). In contrast to the remarkable ability of CD44v9 expression in predicting recurrence, the expression of miR-21 or PDCD4 was not informative. A different turn of events occurred when Uozaki and colleagues,[95] in addition to evaluating miR-21 expression in tumoral and non-tumoral tissue, investigated its expression in stromal tissue underlying the two mentioned areas. As a result, they found that unlike the tumoral miR-21 expression, its stromal expression was closely associated with clinic-pathological indications of tumor progression, including tumor stage, size and lymph node metastasis. These authors speculated that the circulating miR-21 may actually originate from the underlying stromal tissue rather than the tumor itself.

ii. Circulating MiR-21

Komatsu, *et al.*[96] investigated the effectiveness of plasma concentrations of miR-21, in addition to other candidate miRNAs (miR-17-5p, miR-106a, miR-106b), in prognostic assessment of gastric cancer. Consequently, poorer cause-specific survival rates plus increased vascular invasion of tumors were observed in patients with higher plasma levels of miR-21. In another study, Kim, *et al.*[97] analyzed the concentrations of a panel of serum miRNAs, including miR-21, in gastric cancer patients to predict lymph node (LN) metastasis. As a result, serum miR-21 was able to highly discriminate between GC patients with and without LN metastasis (AUC = 0.954). Of particular value was the fact that such segregation was possible during early pT (pT1a, pT1b) stages of GC. However, no significant independent correlation was observed between serum levels of miR-21 and pT stage, Lauren's classification, gender, or age.

Another piece of evidence supporting the value of serum miR-21 in predicting GC prognosis and surgery outcomes was provided

Table 3. MiR-21 as a prognostic biomarker in gastric cancer.

A. Tissue MiR-21

Year	Authors	Ref.	Samples	Sample Size	TNM Stage	Method	Reference gene	Prognostic Indices								
								T size	pT	pN	pM	Grade	Recurrence	Survival rate	P value	AUC
2008	Chan *et al.*	[73]	Fresh Tissue	Tumor = 37	Early (I-II) = 13 Late (III-IV) = 24	TaqMan RT-PCR	U6	N	N	N	N	N	—	N	—	—
2010	Tchernista *et al.*	[62]	Fresh Tissue	Tumor = 26	—	MiRNA microarray SYBR green RT-PCR	Global Median U6	—	—	Y	—	—	—	—	—	—
2010	Motoyama *et al.*	[48]	Fresh Tissue	Tumor = 49	Early (I-II) = 26 Late (III-IV) = 23	TaqMan RT-PCR	U6	Y	Y	N	N	N	—	—	—	—
2011	Valladares Ayerbes *et al.*	[93]	FFPE Tissue	Tumor = 38	Early (I-II) = 6 Late (III-IV) = 32	SYBR green RT-PCR	5S rRNA U6	—	N	N	N	N	—	N	NS	—
2011	Jiang *et al.*	[75]	FFPE Tissue	Tumor = 55	Late (III-IV) = 55	TaqMan RT-PCR	RNU44	—	—	—	—	—	—	Y	$P = 0.0004$	—
2011	Osawa *et al.*	[77]	FFPE Tissue	Tumor = 37	Early (I-II) = 16 Late (III-IV) = 21	MiRNA microarray TaqMan RT-PCR	Global Median	—	—	—	—	—	—	Y	$P = 0.024$	—
2012	Xu *et al.*	[92]	Fresh Tissue	Tumor = 86	Early (I-II) = 64 Late (III-IV) = 22	TaqMan RT-PCR	Let-7a	—	Y	Y	N	Y	—	Y	$P < 0.05$	0.790
2012	Zhang *et al.*	[50]	Fresh Tissue	Tumor = 30	Early (I-II) = 7 Late (III-IV) = 23	TaqMan RT-PCR	U6	N	Y	Y	—	Y	—	—	—	—
2013	Kim *et al.*	[78]	FFPE Tissue	Tumor = 91	Early (I-II) = 32 Late (III-IV) = 59	SYBR green RT-PCR	U6	—	N	N	N	—	—	N	NS	—
2013	Hirata *et al.*	[94]	Fresh Tissue	Tumor = 88	—	TaqMan RT-PCR	U6	—	—	—	—	—	N	—	—	—
2013	Wang *et al.*	[76]	Fresh Tissue	Tumor = 32	—	SYBR green RT-PCR	U6	Y	Y	Y	—	Y	—	—	—	—
2014	Uozaki *et al.*	[95]	FFPE Tissue	Tumor = 469	Early (I-II) = 202 Late (III-IV) = 267	ISH/SYBR green RT-PCR	RNU6B	Y	Y	Y	—	—	—	—	—	—

B. Circulating MiR-21

Year	Authors	Ref	Samples	Sample Size	TNM Stage	Method	Reference gene	Prognostic Indices								
								T size	pT	pN	pM	Grade	Recurrence	Survival rate	P value	AUC
2013	Komatsu *et al.*	[96]	Plasma	GC = 69	Early (I-II) = 52 Late (III-IV) = 17	TaqMan RT-PCR	mirVana miRNA Reference Panel	-	N	N	N	-	N	Y	P=0.0133	-
2013	Kim *et al.*	[97]	Serum	GC = 79	Early (I-II) = 48 Late (III-IV) = 31	TaqMan RT-PCR	cel-miR-39	-	N	Y	-	-	-	-	-	0.954
2013	Song *et al.*	[98]	Serum	GC = 103	Early (I-II) = 31 Late (III-IV) = 72	SYBR green RT-PCR	miR-16/miR-93	Y	Y	N	N	N	N	N	NS	-
2013	Ma *et al.*	[83]	Plasma	GC = 42	—	SYBR green RT-PCR	RNU6B	—	—	Y	—	Y	—	—	—	—

NS=Not Significant

Table 4. Systematic reviews of miR-21 as a diagnostic and prognostic biomarker in gastric cancer.

Year	Authors	Ref.	No of studies	Samples	Sample size	AUC (95% CI)	Pooled sensitivity (%)	Pooled specificity (%)	Median fold change (range)	OS HR	Clinical application
2013	Zeng, *et al.*	[99]	5	Plasma/serum	GC = 251 Controls = 184	0.8 (0.76–0.83)	66.5 (55.0–76.3)	83.1 (69.4–91.5)	—	—	Diagnostic
2014	Zhu, *et al.*	[100]	8	Plasma/serum	GC=421 Controls = 317	0.91	78 (71–85)	89 (82–94)	—	—	Diagnostic
2014	Shrestha, *et al.*	[101]	10	Tissue	GC = 404 Controls = 317	—	—	—	4.05 (1.49–10.44)	—	Diagnostic
2014	Wang, *et al.*	[33]	8	Tissue/Serum	GC = 523 Stages = I-IV	—	—	—	—	2.0	Prognostic

by Ma and colleagues,[83] who further manifested the association of its serum level expression with tumor grade of differentiation and lymph node metastasis. On the contrary, Song and colleagues[98] found no association between serum miR-21 levels and lymph node metastasis or other prognostic factors such as postoperative survival rates. The only tumor characteristics which they found to be associated with serum miR-21 were tumor size and advanced pT stage. These data are summarized in Table-3B.

Systematic reviews

A meta-analysis was carried out by Zeng, *et al.*,[99] in 2013, in order to evaluate the diagnostic value of circulating miR-21 for gastric cancer. Their systematic review was restricted to 5 qualified studies comprising of a total of 251 GC patients and 184 controls. The overall diagnostic power (AUC), pooled sensitivity and specificity were determined as 0.80 (95% CI: 0.76–0.83), 66.5% (95% CI: 55.0%–76.3%) and 83.1% (95% CI: 69.4%–91.5%), respectively. The limitations of this analysis included a relatively small sample size, all of which came from East Asian countries. Keeping in mind that larger prospective studies are needed to draw a firm conclusion, the authors recommended circulating miR-21 as a diagnostic biomarker for GC with moderate sensitivity and good specificity.

In 2014, Zhu, *et al.*[100] performed a meta-analysis which included 22 studies on a collection of 35 miRNA, 8 of which evaluated miR-21. They concluded that the up-regulation of circulating miR-21 was most consistent among the 35 studied miRNA for detection of GC, with overall diagnostic power (AUC), pooled sensitivity and specificity of 0.91, 78% (95% CI: 71%–85%), and 89%(95% CI: 82%–94%), respectively. Admitting to the need for larger studies, using high throughput techniques, the consistent elevation of circulating miR-21 in GC patients and its subsequent decline following the resection of tumors, prompted the authors to re-emphasize the diagnostic potential of circulating miR-21 in detecting GC.

The next systematic review, which was carried out the same year by Shrestha and colleagues,[101] complemented that of Zhu, *et al.,*[100] by pooling the available miRNA data obtained from gastric tissue. Similar to the conclusions made on serologic studies, these investigators found miR-21 as the most consistently up regulated miRNA in the gastric tumors vs. normal tissue. Collection of data from the 10 evaluated reports yielded a median fold change of 4.05 for miR-21 overexpression in tumor tissue, ranging from 1.49 to 10.44. This analysis once again highlighted the diagnostic value of miR-21 in diagnosis of GC, but at the tissue level.

The only meta-analysis assessing the value of miR-21 in prognosis of GC was performed by Wang and colleagues[102] who evaluated 8 eligible studies published from 2008 to 2013. These investigators concluded that higher expression of miR-21 is significantly associated with poorer survival, tumor differentiation, lymph node metastasis, and TNM stage. Their analysis, despite a relatively limited sample size with a geographic bias, highlights the application of miR-21 in prognosis of gastric cancer patients.

Concluding remarks

In summary, the indicative role of circulating miR-21, as a non-invasive reflection of GC progression and regression, makes this oncomir a particularly attractive diagnostic and prognostic biomarker. The fact that it can be manipulated and its oncogenicity can be inhibited or reversed, qualifies it as a potential target in devising treatment strategies. Altogether, the state-of-the-art supports its inclusion in the list of high priority GC biomarkers, but calls for additional larger sized validation studies, tracing it back to the pre-neoplastic stages, when detection of GC risk may lead to disease prevention and/or treatment.

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