

Vitamin D modulation and microRNAs in gastric cancer: prognostic and therapeutic role

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Abstract: Gastric adenocarcinoma arises after a complex interaction between the host and environmental factors. Tumor location and TNM are the tools that currently guide treatment decisions. Surgery is the only curative treatment, but relapse is common. After relapse or advanced staged disease survival is poor and systemic treatment has modestly improved survival. An association between sun exposure, vitamin D status and gastric cancer (GC) incidence and mortality has been reported. The molecular differences of the histological subtypes and the new molecular classifications account for the great heterogeneity of this disease and are the basis for the discovery of new therapeutic targets. New prognostic and predictive factors are essential and microRNAs (miRNAs) are endogenous small non-coding RNA molecules with a great potential for diagnosis, prognosis and treatment of cancer. There are hundreds of miRNAs with altered expression in tumor gastric tissue when compared to normal gastric tissue. Many of these miRNAs are associated with clinicopathological variables and survival in patients with GC. Furthermore, the expression of some of these miRNAs with prognostic importance in GC is influenced by vitamin D and others are mediators of some of the actions of this vitamin. This review aims to update the evidence on several miRNAs with prognostic value and therapeutic potential in GC, whose expression may be influenced by vitamin D or may regulate vitamin D signaling.

Keywords: Vitamin D; gastric cancer (GC); microRNAs

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Introduction

Gastric cancer (GC) is the fourth most common cancer and the third cause of cancer mortality (1), although its incidence and mortality rates have been declining steadily since last century, in part due to a change of dietary habits and lifestyle, better conditions in food conservation and eradication of *Helicobacter pylori* (*H. pylori*) (1-4). However, this decrease in GC incidence and mortality is explained by a lower incidence of distal tumors, finding that proximal and junctional tumors have increased their incidence (5).

Data from many ecologic studies support a strong

association between sun exposure and GC incidence and mortality (6). However, studies on vitamin D intake and its serum status and their association with risk and mortality of GC, show more variable data (6).

Adenocarcinoma is the most common histology and this review focuses on this subtype (7). Also tumor location (proximal vs distal tumors; cardia vs no cardia) should be considered in treatment decisions.

Although surgical resection with lymphadenectomy is considered the only curative therapy for GC, multimodality treatment has become the standard of care for GC staged as

IB-III (8,9). Adjuvant chemotherapy and chemoradiation, as well as perioperative chemotherapy are treatment approaches that have reduced recurrence rate and increased survival (10-16). After curative treatment, relapse is the most dreadful event, which rarely has a curative option (17,18). Five year survival rate in metastatic GC is only 4%. In this setting, chemotherapy based on Cisplatin (CDDP), 5-Fluorouracil (5FU), Taxanes, Irinotecan, Trastuzumab (antiHER2 therapy) and in subsequent lines, Ramucirumab and immune checkpoint inhibitors, has improved median overall survival achieving 11–13 months (19).

miRNAs are endogenous small non-coding RNA molecules of 18–25 nucleotides that function at posttranscriptional level, inhibiting translation or promoting degradation of messenger RNA (mRNA) with complementary sequences. Thus, miRNAs regulate the production of hundreds of different proteins, including transcription factors (20-22).

This is not the first review attempting to collect current evidence on Vitamin D modulation of miRNAs expression in cancer (23,24). But in this review we have tried to update the evidence on several miRNAs with prognostic value and therapeutic potential in GC, whose expression may be influenced by vitamin D or may regulate vitamin D signaling.

Prognostic factors in GC. Histology and molecular classifications as prognostic and predictive factors in GC

The TNM staging system is the most relevant prognostic factor and a tool commonly used in clinical practice for setting cancer stage-specific therapeutic strategy (25).

Most existing data on prognostic factors are based on treatment with surgery alone. There are several prognostic indices for GC and nomograms for survival after R0 resection (R0 indicates a microscopically margin-negative resection) (26). More recent nomograms include factors as age at diagnosis, sex, tumor location, depth of invasion, number of metastatic lymph nodes, number of examined lymph nodes, lymphatic/venous invasion and Lauren classification as covariates associated with prognosis (17,27).

In a recent systematic review and meta-analysis, van den Ende *et al.* identified 23 potentially relevant prognostic and 15 predictive factors for the curative treatment of GC (28).

According to Lauren classification, gastric adenocarcinomas are subdivided into intestinal and diffuse subtypes (29).

Furthermore, a genomic classification identified molecular types of GC, intestinal (G-INT) and diffuse (G-DIF), stratified into prognostic groups, with a worse prognosis for the diffuse subtype and associated with worse response to chemotherapy (30).

The molecular definition of the diffuse subtype is loss of E-cadherin expression (31). In contrast, carcinogenesis of the intestinal type follows a multi-step process, generally initiated by *H. pylori* infection and sequential phases of non-atrophic active chronic gastritis, multifocal atrophic gastritis, intestinal metaplasia, dysplasia and invasive carcinoma (32).

The role of histology as a prognostic and predictive factor of response to chemotherapy is a topic of interest. A meta-analysis showed that histology, according to Lauren classification, might be a useful prognostic marker for both early and advanced GC patients, with intestinal-type associated with better outcome (33). More evidence from observations of real-world data supports that tumor subtypes in Lauren classification can predict survival and different responses to chemotherapy (34).

Although expression of miRNAs related to histological subtypes of GC has not been widely analyzed, Yepes *et al.* showed that miR-100, miR-125b, miR-99a and let-7c were associated with diffuse subtype (35).

Among different attempts, two molecular classifications are worth mentioning. The Cancer Genome Atlas (TCGA) investigators published one comprehensive study on molecular GC classification (36) and four molecular subtypes were identified: EBV (Epstein Barr virus)-related in 9% of cases, MSI-H (microsatellite instability-high) in 22% of cases, GS (Genomically Stable) in 20% of cases and CIN (Chromosomal Instability) in 50% of cases.

Based on this classification, a prediction model was reported by Sohn *et al.* (37), showing best prognosis for EBV subtype, an intermediate prognostic group integrated by MSI-H and CIN subtypes, and the GS as the subtype with the worst prognosis. Also, they reported an association between CIN subtype and benefit from adjuvant chemotherapy, with the limitations of retrospective analysis (37).

The Asian Cancer Research Group (ACRG) provided another GC classification and identified also four subtypes (38). The MSI (Microsatellite unstable) subtype, in 23% of cases, associated with the best overall prognosis, antrum, hyper-mutated intestinal subtype, and early stage. The MSS/EMT (Epithelial to mesenchymal transition) group, in 15% of cases, associated with the worst prognosis,

diffuse histology and advanced stage. The MSS/TP53 active subtype, in 26% of cases, showed EBV positivity and preserved activity of TP53. After MSI subtype, had the best overall prognosis. And MSS/TP53 inactive subtype, in 36% of cases, with the highest prevalence of *TP53* mutations, amplifications of tyrosine kinase receptors and an intermediate prognosis (38,39).

Recently, miR-338/CCL21/NF- κ B signaling, miR-146b/PSMD3/proteasome, miR-34a/VCL/focal adhesion and miR-34c/VCL/focal adhesion subtype-specific subpaths were identified and might have a role in GC development and classification, although data on their prognostic value have not been published (40).

Vitamin D and cancer

Approximately 3 percent of the human genome is under control of vitamin D (41).

Vitamin D is obtained from foods and ultraviolet-B radiation exposure in the skin, through conversion of 7-dehydrocholesterol that is a prohormone that needs two hydroxylation steps, through two enzymes of cytochrome P450, to be active. Firstly, vitamin D is converted to 25-hydroxyvitaminD₃ (25(OH)D) by 25-hydroxylase (CYP27A1) in the liver. Then, in the kidney, is converted to 1- α ,25 dihydroxyvitaminD₃ (Calcitriol), its active form, by 1- α -hydroxylase (CYP27B1) (42). To facilitate reading, we will continue to use the term vitamin D as an equivalent among its metabolites.

There is some evidence that several tissues, outside the kidney, can generate this active form, in an paracrine (or autocrine) way, by expressing 1- α -hydroxylase (41,43).

The vitamin D receptor (VDR) belongs to the superfamily of steroid/thyroid hormone receptor and is nearly universally expressed in nucleated cells (41). In the absence of ligand, some basal level of receptor remains in the nucleus associated with co-repressor complex and leads to silencing of transcription (44).

The union of calcitriol with VDR induce a dimerization with the retinoid X receptor (RXR) and then the complex calcitriol-VDR-RXR translocates into the nucleus. This complex attaches to the vitamin D response elements (VDREs) in the promoters of target genes determinants in the pathways of proliferation, apoptosis and angiogenesis (42). This signal is limited in time, conditioned by the rapid metabolism of the ligand (CYP24A1) and the proteasome mediated receptor degradation (41,44).

The role of vitamin D and its receptor (VDR) in cancer incidence and mortality have been studied for long time (6,45-48). It is suggested that vitamin D deficiency might increased both (49).

In cancer prevention, the majority of vitamin D intervention trials do not show a reduction in cancer risk, however most of them have been carried out in adults without vitamin D deficiency (50-55). Furthermore, it has been reported that common polymorphisms in the VDR modified the association of serum vitamin D and that the relationship of low serum vitamin D levels with major health outcomes varied based on these common genetic differences (56,57).

In a recent randomized trial of postoperative vitamin D₃ supplementation, double-blind placebo control carried out in Japan, in patients with digestive tract cancers, staged I-III, 417 patients were recruited, 42% with GC. Subgroups were made based on baseline serum vitamin D levels (0-20; 20-40; >40 ng/mL) and they reported no significant improvement in relapse free survival (RFS) at 5 years (58). Nevertheless, the authors, in a post hoc analysis, found that the use of bioavailable 25(OH)D, rather than total 25(OH)D, as a biomarker of vitamin D deficiency, indicated a significant difference in RFS at 5 years in the subgroup of patients with low bioavailable 25(OH)D levels (77% in the vitamin D group versus 58% in the placebo group) and no differences in the subgroup with high bioavailable levels of 25(OH)D, suggesting a role for supplementation in patients with bioavailable 25(OH)D deficiency (59).

In a retrospective case-control study, a higher prevalence of vitamin D insufficiency and deficiency was found in GC patients compared to controls without known malignancy (60). In another case-control study designed to evaluate the risk of GC development related to VDR and VDBP (VD binding protein) polymorphisms and Vitamin D serum levels, the authors noted that GC patients had lower vitamin D levels than controls, and found a fourfold higher risk of GC development in cases with severe vitamin D deficiency (<10 ng/mL) (61). Furthermore, in GC clinical stage and lymph node metastasis were found to be inversely correlated with vitamin D levels. Therefore, vitamin D level was proposed as an independent prognostic factor, with a worse prognosis associated with vitamin D deficiency (62).

In vitro, Bao *et al.* reported that direct administration of vitamin D in GC cells induced apoptosis and enhanced VDR, CYP24A1 and p21 expression levels (63).

In a subsequent study, they treated GC cells with vitamin

D or CDDP alone or a combination of both and showed that vitamin D potentiated the effects of CDDP, showing upregulation of BAX, a decrease in levels of phosphorylation of ERK and AKT and a positive regulation of p21 and p27, which induced the cells cycle arrest in G0/G1 phase (64).

Furthermore, it has been suggested that vitamin D acts through the Hedgehog signaling pathway and regulates the expression of PTEN (65) and promotes apoptosis in undifferentiated GC cells (62).

Interestingly, Han *et al.* conducted a multicenter prospective cohort study to determine whether serum vitamin D level had an impact on *H. pylori* infection (Main causal agent of GC) and eradication, showing that serum vitamin D level of ≥ 10 ng/mL was an independent risk factor for a successful *H. pylori* eradication (66).

MicroRNAs

Bioinformatic analysis estimated that more than 60% in mammalian genome could be targeted by single miRNA (67).

In a must-read article, Bartel explained that in the canonical biogenesis of miRNAs, in animals, miRNAs are transcribed by RNA polymerase II (Pol II) as a longer precursor “pri-miRNA”, which is processed by a complex formed by Drosha endonuclease and DGCR8, known as Microprocessor (68). Drosha cut one strand of the stem of the pri-miRNA hairpin and liberates a 60 nucleotides stem-loop called “pre-miRNA” that is exported to the cytoplasm through the action of Exportin 5 and RAN-GTP. In the cytoplasm, the pre-miRNA is processed by Dicer, another endonuclease also associated with TRBP. Dicer cuts both strands near the loop and generates a miRNA duplex. The miRNA and its passenger strand, as a duplex is loaded into an Argonaute protein (Ago). After the expulsion of the passenger strand, the mature silencing complex is formed (RISC) (68). Once loaded into the silencing complex, most miRNAs are very stable, with half-lives of days (68). The passenger strand is thought to be degraded, although there is evidence that mature miRNA and its passenger strand can work in a coordinated manner, improving their repressive effect by binding to the same target (69).

In humans, the repression mode acts without slicing the mRNA. Ago recruits TNRC6 and interacts with the mRNA and causes its destabilization (68).

miRNAs are grouped into families based on their targeting properties, which depend primarily on the identity of their extended seed region (miRNA nucleotides 2–8) (68).

Prognostic and potential therapeutic role of miRNAs in GC

In a systematic review of the GC miRNA expression profile, 352 miRNAs were found differentially expressed when tumor tissue was compared to normal gastric tissue, highlighting the upregulation of miR-21, as the most consistent finding (70).

In another review, a comparison of 14 miRNAs in blood and 36 in tissues of GC patients was made according to their association with clinicopathological characteristics and survival (71). They showed that 42.86% of blood miRNAs have a significant association with one or two clinicopathological characteristics, although around 60% of miRNAs in tissues were significantly correlated to more than three clinicopathological features (71).

Furthermore, upregulation of Drosha, Dicer and DGCR8 was observed in GC tissues compared to normal marginal gastric tissues, although no correlation to clinicopathological characteristics was found, but probably influencing in deregulation of other miRNAs (72).

miRNAs are frequently localized to cancer-linked genomic regions, as well as deregulated in tumors versus normal tissues (73,74). Moreover, miRNAs can regulate oncogenes and tumor suppressors, even acting as such themselves. All this, added to their stability in many body fluids (67) and their role as diagnostic and prognostic biomarkers, give miRNAs great therapeutic potential.

miRNAs could be therapeutic targets, therapeutic weapons, and response prediction tools for other treatments. As pointed out by Tsai *et al.* methods currently under development are anti-miRNA oligonucleotides, miRNA sponges, miR-Mask, antagomiRs, and miRNA inhibitors (75).

Some oligonucleotide drugs approved induce cleavage of a target mRNA or modulate the splicing pattern (56). Some miRNA inhibitors also are in clinical development (76,77), but safety and delivery of these drugs remains a challenge.

Otherwise, chemotherapy and targeted therapy resistance are challenging in GC. Several miRNAs have been implicated in multidrug resistance (MDR) (78). Moreover, it has been shown that vitamin D can potentiate the effects of chemotherapy and contribute to overcome the molecular mechanisms of drug resistance (79).

In our review line, we will discuss of each proposed miRNA the way in which vitamin D modulates it, as well as the data in favour of its prognostic and therapeutic role in GC (Table 1).

Table 1 miRNA expression and its possible role in GC

miRNAs related to vitamin D	Potential role in GC	Expression in GC	Therapeutic potential in GC	Reference
miR-125	Oncogene	Upregulated	Possible role in CDDP sensitivity and Trastuzumab resistance in GC cells	(80-83)
	TS	Downregulated		
miR-145	TS	Downregulated	Possible role improving chemosensitivity to CDDP and 5FU in GC cells	(84,85)
miR-99	Oncogene	Upregulated	Associated with CDDP resistance in GC cells	(86,87)
	TS	Downregulated		
miR-498	TS	Downregulated	Possible role in overcoming CDDP resistance in GC cells	(88,89)
miR-22	TS	Downregulated	Possible role in CDDP resistance in GC cells	(90-92)
miR-181	Oncogene	Upregulated	-	(93-95)
	TS	Downregulated		
miR-106	Oncogene	Upregulated	-	(96,97)
miR-532	Oncogene	Upregulated	-	(98,99)
	TS	Downregulated		

GC, gastric cancer; miR, microRNA; TS, tumor suppressor; CDDP, cisplatin; 5FU, 5-fluorouracil.

Regulatory micromas of vitamin d signalling linked to GC

miR-125b

miRNA-125b (miR-125b) is an ubiquitous miRNA and is deregulated in different tumors, although is upregulated in colon and hematopoietic tumors and downregulated in others (Hepatocellular) (100). miR-125a and miR-125b share the same seed region, suggesting they regulate the same target mRNAs (100). Theoretically, it has been hypothesized that its targets are tissue and tumor specific, suggesting that its function depends on the levels of target gene expression (100).

Mohri *et al.* showed that miR-125b prevents the antiproliferative effects of 1,25 (OH)₂D₃, through posttranscriptional regulation of VDR (101). In cancer cells, they showed that endogenous VDR level was repressed by miR-125b and identified a potential miR-125b recognition element (MRE125b) on VDR mRNA (101).

Additionally, a new study indicated that miR-125b regulates the CYP24 mRNA, recognizing the MRE125b on the CYP24 mRNA (102).

The level of VDR expression has been reported to be a good prognostic factor in breast cancer (103) and esophageal adenocarcinoma (104), as well as the lowest levels of VDR expression occurred in tumor tissue

compared to premalignant lesions of GC (105) and of cholangiocarcinomas (106).

In parallel, a progressive downregulation of miR-125a-5p and miR-125b was observed in gastric samples from normal mucosa, to premalignant lesions and finally to intestinal-type adenocarcinoma (80). It has been reported that miR-125b was frequently downregulated in GC tissues (81,82,107) as well as in GC cell lines (82,107). The qRT-PCR levels of miR-125a-5p and miR-125b were inversely correlated with HER2 expression (80). Also, HER2 amplification significantly increased from premalignant lesions to carcinoma and the expression levels of miR-125a-5p and miR-125b were significantly lower in HER2-positive GC than in HER2-negative ones (80). HER2 status is a predictive biomarker commonly used in clinical practice to determine benefit of trastuzumab in combination with first line chemotherapy for metastatic GC (108).

Moreover, HER2 was indicated as a direct target of miR-125b in GC cell lines and miR-125b overexpression prevented proliferation, migration and invasion, *in vitro and in vivo* and enhanced CDDP sensitivity in GC cells (82). Clinical features associated with miR-125b were gender, lymph node metastasis and HER2 status. Patients with high expression of miR-125b had better progression free and overall survival (82). In another study, miR-125b-2 mimic transfection in GC cells reduced cell viability, migration

and invasion and increased cell death, targeting PIK3 (107). In contrast, it has been suggested a possible role of miR-125b in trastuzumab resistance mechanisms, what is of tremendous interest in clinical practice (83). GC patients HER2 positive treated with trastuzumab, showed worse overall survival when they had high miR-125b expression (83).

Wu *et al.* found in GC patients that miR-125b levels were inversely correlated with invasion depth, clinical stage, and lymph node metastasis. Moreover, primary tumors with low expression of miR-125b had a shorter overall and disease free survival (81). miR-125b targeted MCL1 (myeloid cell leukemia 1), thereby suppressing proliferation and favouring apoptosis. Additionally, they transfected GC cells with miR-125b and showed sensitization to 5FU-induced apoptosis (81).

In contrast, miR-125b overexpression was observed in GC tissues compared to matched normal tissues (83, 109-111) and associated with T stage, lymph node metastasis, TNM stage and worse overall survival (83,110,111). miR-125b has been linked to enhanced cellular proliferation and decreased apoptosis (109).

In the same way, it was suggested that miR-125b could act as an oncogene by targeting PPP1CA and up-regulating Rb phosphorylation in GC (110) and that miR-125b overexpression induced cell migration and invasion, through targeting STARD 13 and NEU1 (111). Additionally, miR-125b is upregulated by KDMB4, which has been linked to gastric carcinogenesis and mediate KDM4B induced activation of Wnt signaling (112).

MicroRNAs regulated by vitamin D and linked to GC

miR-145

Downregulation of miR-145 has been reported in GC and associated with unfavourable prognosis in various tumors, including GC (84,113-116). Recently, miR-145 has been suggested as a marker of cancer migration and invasion in different malignant tumors (117).

miR-145 expression level has been found downregulated in GC tissues and cell compared to normal (85,115,118) and associated with tumor depth, lymph node metastasis, lymphatic invasion and TNM stage (85).

Chang *et al.* after treating GC cells with vitamin D3, found a decreased percentage of cells in phase S and promoted apoptosis, as well as an increased in miR-145

expression level (115). They determined that inhibition of miR-145 led to a decreased in the antiproliferative effects of vitamin D. Furthermore, they indicated that vitamin D3-induced increase in miR-145 expression was mediated by VDR. *In vitro*, miR-145 brought about a blockage in S phase/G2 transition in GC cells and suppressed at protein level both E2F3 and CDK6, and downregulated CDK2 and CCNA2 (Figure 1) (115).

Findings in another report suggested that miR-145 might have a role as a tumor suppressor and examined other potential targets whose expression could be modulated by miR-145 in GC cells, such as downregulation of C-MYC, PI3K/AKT, MMP2 and MMP9 proteins and upregulation of p21 (116).

After performing miRNA microarray profiling in GC, precursor lesions and normal tissues, to find differentially expressed miRNAs during early gastric carcinogenesis, Hwang *et al.* found that downregulation of miR-145 might be an early event in the development of premalignant gastric lesions. In comparison to normal gastric mucosa, adenomas showed decreased expression in five miRNA (miR-26a, miR-375, miR-574-3p, miR-145 and miR-15b) (120).

Interestingly, in GC cells, overexpression of miR-145 inhibited migration, invasion and downregulated ZEB2 and N-Cadherin and increased E-cadherin expression, by direct regulation of ZEB2 (85).

Similarly, Gao *et al.* observed that N-cadherin protein expression was elevated in GC tissues samples with low levels of miR-145 (118). Here too, miR-145 suppressed GC cell invasion and migration, *in vitro and in vivo*. *In vitro*, GC cells expressing miR-145 could reduce N-cadherin levels. *In vivo*, miR-145 positive tumors were less locally invasive and miR-145 prevented the spread of lung metastases (118).

In this line of research, Xing *et al.* demonstrated that CTNND1 (Catenin- δ 1) is a direct target of miR-145. CTNND1 is part of the cadherin-catenin complex, bound to the cytoplasmic tail of E-cadherin and associated with cell migration, invasion and cell division (121). *In vitro*, miR-145, through its downregulation, mediated CTNND1 translocation from cytoplasm to membrane, recovering the membranous localization of CTNND1 and E-cadherin (121). CTNND1 promoted GC cell migration and invasion, with no effects on proliferation and apoptosis. They also indicated that cytoplasmic localization of CTNND1 protein was associated with higher clinical stage, positive lymph node metastasis and poorer prognosis in GC patients (121).

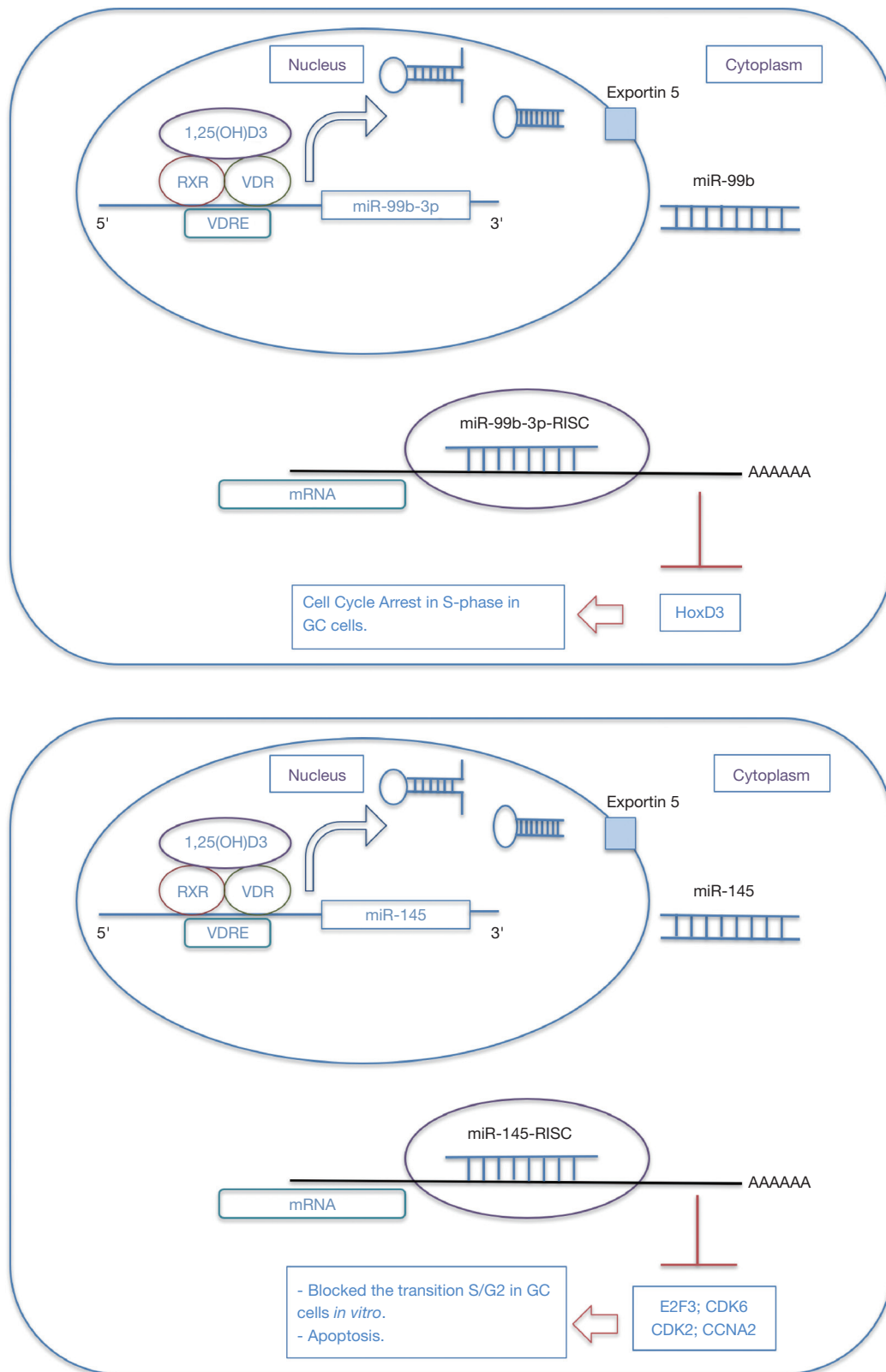


Figure 1 Vitamin D induced miR-145 and miR-99b-3p expression in GC cells (115,119).

Additionally, in GC cells miR-145 directly targeted FSCN1 and an inverse correlation between expression level of miR-145 and FSCN1 in normal/tumor paired samples of GC patients was observed (122). According to this finding, it was possible to distinguish between invasive and expansive GC based on the fact that the former had lower levels of miR-145 and higher levels of FSCN1 (122). Fascins are actin-binding proteins that stimulates cell motility, migration and invasion (122) and FSCN1 is absent in normal epithelia, but overexpressed in several carcinomas (123).

Likewise, downregulation of miR-145 in GC tissues compared to normal gastric mucosa, was inversely correlated with v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1) overexpression in GC tissues and in GC cell lines. Ets1 overexpression was associated with deeper gastric wall invasion, lymph node metastasis, distant metastasis and advanced TNM stage (124).

Ets1 is a transcription factor linked to migration, invasion and angiogenesis of cancer cell and is a direct target of miR-145 in GC cells (124), causing, *in vitro* and *in vivo*, inhibition of invasion, metastasis and angiogenesis.

Furthermore, Zeng *et al.* published interesting results on the prognostic and therapeutic potential of miR-145 (84).

CD44 (Cluster of differentiation 44)-positive GC cells shows self-renewal abilities and chemoresistance, as stem cells do and its expression is associated with aggressive tumor phenotype (125) and it is suggested that miR-145 is a key regulator of stem cell properties in GC cell by targeting the CD44 3'UTR (84). They demonstrated that repression of CD44 is necessary for miR-145 inhibition of self-renewal properties of GC cells (84). After administration of CDDP and 5FU to GC cells, miR-145 improved chemosensitivity and overexpression of CD44 was associated with resistance to these drugs. However, the miR-145-mediated chemosensitivity can be overcome by re-expression of CD44. Thus, they analyzed that miR-145 mimic treatment of GC cells repressed ABCG2 expression [an ABC transporter involved in drug efflux and a marker of GC stem cells (126)] and that CD44 overexpression enhanced ABCG2 expression (84).

Besides, tumor spheres used in their experiments expressed higher levels of SOX2, OCT-4 and Nanog, which demonstrated that spheres enriched the GC stem cells population (84). Interestingly, SOX2, OCT-4 and Nanog are transcription factors responsible of regulation of self-renewal and pluripotency of stem cells (127). Moreover, in human embryonic cells, miR-145 is upregulated during differentiation and represses OCT-4 and SOX2 expression,

inhibits their self-renewal properties, prevents expression of pluripotency genes and induces lineage-restricted differentiation (128) and finally, OCT-4 can repress miR-145 promoter, suggesting a double negative feedback loop. More recently, it has been reported overexpression of SOX2, OCT-4 and NANOG in GC tissues compared to paired normal gastric tissues and association with T size, TNM, tumor grade and worse survival (129).

miR-99 family

Recently, miR-99b-3p was found underexpressed in GC tissues compared to normal and linked to inhibition of cell viability and induction of cell cycle arrest in S-phase in GC cells, by targeting HoxD3 (119). As well, miR-99b-3p was induced by vitamin D through direct VDR binding on its promoter domain (*Figure 1*) (119).

miR-99 family is composed by miR-99a, miR-99b and miR-100. Their target genes include *mTOR*, *Homeobox A1 (HOXA1)*, *CTD small phosphatase-like, N-myristoyltransferase 1 (NMT1)*, *Transmembrane protein 30a (TMEM30A)* and *SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5 (SMARCA5)* (130).

miR-99b-5p and miR-99a were reported underexpressed in GC tissues (86,131) and cells (86). Furthermore, both exert their effects through targeting IGF1R (86,131). miR-99b-5p expression level was associated with suppression of cell growth, colony formation, cell cycle arrest and apoptosis in GC cells (86). miR-99a expression in GC tissues was inversely correlated with IGF1R expression and suppressed invasion and migration in GC cells (131).

Conversely, miR-99b overexpression has previously been reported in *H. Pylori* positive intestinal GC (132). Yang *et al.* suggested a role of miR-99b in *H. Pylori*-induced gastric carcinogenesis after finding higher expression of miR-99b in the *H. Pylori* positive GC samples compared to the negative samples, as well as in the *H. Pylori* infected GC cells (133). miR-99b promoted autophagy by targeting mTOR and thus negatively regulated proliferation of GC cells (133).

A recent report also showed that miR-99a and miR-491 were associated with resistance to CDDP *in vitro* (87). Upregulation of both were found in CDDP resistant GC cells as Calpain small subunit 1 (CAPNS1) was downregulated. Inhibition of miR-99a and miR-491 or overexpression of CAPNS1 foster CDDP sensitivity on resistant GC cells like the opposite increased CDDP resistance (87).

Zhang *et al.* observed that GC patients with high

expression level of miR-145-3p, miR-125b-5p and miR-99a-5p had a poorer prognosis than those with a low expression level of miR-145-3p, miR-125b-5p and miR-99a-5p (134).

miR-498

A thought-provoking study previously reported on the actions of vitamin D in ovarian cancer cells, showed that vitamin D induced the expression of miR-498 through its binding to the VDRE located in the 5' regulatory region of the miR-498 gene and directly targeted the 3' UTR of hTERT (human telomerase reverse transcriptase) mRNA (135). hTERT downregulation resulted in cell death and growth suppression not only in ovarian cancer cell lines, but also in other cancer cell lines, suggesting that this hTERT downregulation by vitamin D through

miR-498 might be found in other vitamin D sensitive cancer (135).

In two previous publications, miR-498 downregulation in GC cells and tissues has been reported (88,89) and associated with poorer prognosis in GC patients (89).

In GC, miR-498 overexpression inhibited invasion, proliferation and overcome CDDP resistance, *in vitro and in vivo* (88). Also, miR-498 inhibited cell proliferation and metastasis in GC cells through blocking EMT (Epithelial to mesenchymal transition) and AKT pathways (89). The data from these two studies showed that BMI-1 (B lymphoma Mo-MLV insertion region 1 homolog) is overexpressed in GC (89) and a direct target of miR-498 (88,89). Furthermore, BMI-1 overexpression reversed the inhibiting functions of miR-498 in GC cells (88). Previously, BMI-1 has been suggested to play a role in hTERT-induced EMT and cell immortalization (136). Therefore, miR-498 could play a role as a tumor suppressor in GC.

miR-22

Vitamin D induced miR-22 expression in colon cancer cells in a VDR-dependent manner. The actions of vitamin D on cell migration were mediated by miR-22, as well as the down-regulation of known vitamin D target genes in human colon cancer cells. *In vitro*, the application of anti-miR-22 reduced the antiproliferative effects of vitamin D (137).

miR-22 was found downregulated in GC tissues and GC cells compared to normal controls (90,91,138) and is associated with more aggressive phenotype and worse survival (91). *In vitro*, miR-22 suppressed cell growth,

migration and invasion in GC cells and metalloproteinase 14 and Snail were its direct targets. *In vivo*, miR-22 abrogated tumor growth and metastatic dissemination (91).

Also, miR-22 has been linked to the regulation of cell growth and motility through targeting Sp1 (Specificity protein 1 transcription factor) and CD151 (90,138). Transfection of miR-22 expression plasmid inhibited cell migration and invasion in GC cells. Sp1 was directly target of miR-22 and their expression levels in GC tissues were inversely correlated, thus playing a role as a tumor suppressor (90).

Another target of miR-22 is NLRP3 (NLR Family pyrin domain containing 3) that has been found overexpressed in GC. NLRP3 stimulates inflammasome activation and promotes gastric epithelial cell proliferation. Notably, *H. Pylori* infection prevents miR-22 expression and enhances NLRP3 expression, which could play an important role in GC tumorigenesis (139).

Moreover, miR-22 has been implicated in the development of CDDP chemoresistance in GC cells due to the negative regulation it exerts on the expression of ENO1 (Glycolytic enzyme enolase 1), which is overexpressed in GC and associated with poor prognosis and CDDP resistance (92). Restoration of miR-22 could be a potential therapeutic target or a chemoresistance marker.

miR-181 family

In humans, the miR-181 family consists of four distinct 5p mature forms: miR-181a-5p, miR-181b-5p, miR-181c-5p and miR-181d-5p. Their precursors also yield 3p mature forms, in lower levels. All 5p forms share the same seed region and miR-181 members can behave as oncogenes or tumor suppressor (140).

In cultured myeloid leukemia cells, vitamin D downregulated miR-181 family and increased expression of p27kip1 and p21cip1, induced monocytic proliferation and cell cycle arrest in G0/G1. Moreover, p27kip1 is a direct target of miR-181, thereby, the repression of miR-181 by vitamin D appears to mediate its effects on cell differentiation and cell cycle arrest (141). More evidence of Vitamin D repression effect on miR-181 expression comes from a study of chronic myeloid leukemia cells (142). Here, miR-181 downregulation was associated with an increased expression of Mcl-1 (Myeloid cell leukemia-1) a Bcl-2 family protein associated with relapse and survival in AML (Acute myeloid leukemia) and chemotherapy resistance (142).

The role of miR-181 as an oncogene in GC is supported

by different studies.

In a recent study, miR-181a overexpression was found in GC tissues and cell lines and associated with worse overall survival and more advanced clinicopathological factors (larger tumor size, more lymph node and distant metastasis, and higher TNM stages). Caprin-1 was identified as a direct target of miR-181a (143). Both *in vitro* and *in vivo*, ectopic expression of miR-181a enhanced proliferation, migration, invasion and antiapoptotic effects (143).

In another study, although miR-181a was found overexpressed in GC tissues, no association with clinicopathological characteristics was demonstrated (144). Instead, in GC cells miR-181a stimulated cell proliferation, G1/S phase transition and suppression of apoptosis, mainly through downregulation of RASSF1A, but also of Bax protein and upregulation of CDC25A, cyclin A2 and Bcl-2 (144).

As well, high expression of miR-181b in GC tissues has been associated with worse five year overall survival in GC patients with stage II and III and its expression levels were significantly correlated to tumor size, pathological differentiation, depth of infiltration, pT, lymph node, pN, TNM stage and presence of distant metastasis (93). In another study, miR-181b overexpression was reported in GC tissues compared to normal gastric tissues and low expression levels were associated to better survival, even in patients with more advanced GC stages (94).

Conversely, a possible role as a tumor suppressor of miR-181a in GC has been suggested (95). The authors found that miR-181a was downregulated in GC tissues compared to normal samples and that lower levels of miR-181a were associated with pTNM stage. *In vitro*, miR-181a expression blocked GC cell proliferation, migration, and invasion by direct targeting of Prox1 (95).

In this line, miR-181b was observed significantly decreased in GC tissues (145). miR-181b overexpression in GC cells blocked cell viability, proliferation and reduced glucose consumption, lactate production and ATP concentration, suggesting that miR-181b may regulate the switching between glycolysis and oxidative mitochondrial metabolism for ATP production, by direct targeting of Hexokinase 2 (HK2), an enzyme involved in glycolysis (145).

miR-106b

Treatment with vitamin D, *in vivo*, lead to an increased expression of miR-106b in the prostate tissue and contributes to the regulation of p21(waf/cip) (146), a cell

cycle inhibitor.

In GC, miR-106b is overexpressed in tumor tissues and cancer cells compared to non-tumorous and is suggested as a marker for diagnosis (96,147-149). Besides, miR-106b was found at a significantly higher level in the plasma of GC patients than in controls and was reduced in postoperative samples compared to preoperative (150). miR-106b belongs to a highly conserved cluster, form by miR-106b, miR-93 and miR-25, also upregulated in GC (151).

Overexpression of miR-106b in GC downregulates p21 (152,153). *In vitro*, miR-106b expression levels in GC cells increased according to the degree of malignancy and this upregulation shortened the G0/G1 phase and reduced the expression levels of p21 and E2F5, with no effects on migration and invasion (154).

Downregulation of miR-106b promoted apoptosis of GC cells via inhibiting JAK1/STAT3 signaling pathway, *in vitro* and *in vivo* (149). Zhu *et al.* showed that ALEX1, a member of the armadillo family, was a direct target of miR-106 in GC cells and its up-regulation restored cellular apoptosis induced by the miR-106b inhibitor (149).

Moreover, miR-106b is upregulated in cancer associated fibroblasts compared to normal fibroblasts in GC and patients with high miR-106b stromal expression were associated with shorter overall survival (97). Its oncogenic effect was linked to an increase in migration and invasion, by targeting PTEN (97).

miR-532-3p

Recently, it has been explored the effect of vitamin D supplementation versus placebo in healthy subjects on plasma miRNA profile, but no consistent findings were reported, except a significant correlation between serum vitamin D and miR-532-3p expression at baseline and a significant change in miR-221 expression from baseline to 12 months but in placebo group (155).

RAB3A-interacting protein (Rab3IP) is a Rab-specific GEF (Guanine nucleotide exchange factor) and a major activator of Rab proteins. Rab3IP is overexpressed in GC cells and tissues and interacts with SSX2, inducing an aggressive phenotype of GC cells and its expression is correlated with markers involved in the EMT (156). Guo *et al.* reported that miR-532-3p was downregulated in GC samples compared to normal gastric tissues and that RAB3IP is a direct target of miR-532-3p in GC (98). As well, RAB3IP transcript and protein expression levels were higher in GC tissues than in normal gastric mucosa

samples (98). Furthermore, expression level of Rab3IP was associated with tumor size, T stage, differentiation, N-stage of lymph nodes, and serum tumor marker levels CEA and CA-724 (98).

In contrast, Dai *et al.* found that miR-532-3p was overexpressed in GC tissues compared to non-cancerous tissues and might be involved in regulation of APC/B-catenin signaling (99).

Conclusions

We think that there is convincing evidence that vitamin D deficiency plays an important role in the appearance of molecular events that promote gastric carcinogenesis. Also, here we have reviewed that, *in vitro and in vivo*, vitamin D could modulate the expression of some miRNAs that are deregulated in GC tissue with respect to normal and in GC cells. Furthermore, vitamin D and some miRNAs might be capable of modulating the appearance of chemoresistance at the cellular level.

In contrast, vitamin D supplementation studies have not shown a reduction in the risk of GC either in the general population or in relapse in patients with GC operated on, but it is possible that the appropriate variables have not been chosen to demonstrate its benefit.

The low toxicity profile of vitamin D and its analogues, the ubiquity in the body of the VDR, as well as the important role that miRNAs have in protein expression regulation and possible synergies with vitamin D, project new working hypotheses that deserve to be explored.

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