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Folate and microRNA: bidirectional interactions

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Abbreviations: 1CM: One-carbon metabolism, 5-MTHF: methyltetrahydrofolate, A β : amyloid- β protein, APP: amyloid precursor protein, DHFR: dihydrofolate reductase, MTHFR: methylenetetrahydrofolate reductase, MTR: methionine synthase, NAFLD: Non-alcoholic fatty liver disease, pre-miRNA: precursor miRNA, pri-miRNA: primary precursor miRNA, RISC: RNA-induced silencing complex, SHMT: serine hydroxymethyltransferase,

Abstract

Low folate status is linked to increased risk of a number of conditions, including developmental disorders, some cancers, neurodegenerative and cardiovascular diseases. Some of the mechanisms of these associations are known, but much remains to be elucidated. Aberrant microRNA (miRNA) profiles are also signatures of these conditions, and as such, the association between folate status and miRNA are now being investigated. Potential associations are bidirectional, with miRNA linked to regulation of folate-mediated pathways, and folate linked to modulation of miRNA expression. miRNA are short non-coding RNA, involved in post-transcriptional regulation of gene expression via complementary binding to mRNA. Evidence is emerging that links folate levels to the regulation of miRNA levels, and miRNA to the regulation of the expression of enzymes involved in folate mediated one carbon metabolism. One carbon metabolism is the source of methyl groups for methylation reactions, including DNA methylation and is important in DNA synthesis and repair. miRNA may be modulated by DNA methylation and other epigenetic mechanisms directly, or indirectly via modulation of upstream signalling pathways. As such, there may be bi-directional associations between folate status and miRNA profiles. miRNA may also act as biomarkers for diagnosis or prognosis of conditions associated with folate status.

1. Introduction

Folate (as the 5-methyltetrahydrofolate vitamers; 5-MTHF) is critical in one-carbon metabolism (1CM). As such, folate underpins a number of essential processes including DNA synthesis and repair, methylation of DNA and other biomolecules, cell proliferation and the synthesis of amino acids [1]. Low folate consumption and altered folate metabolism due to polymorphisms in the 1CM pathways have been associated with increased risk of birth defects, some cancers, and neurodegenerative and cardiovascular diseases [1, 2]. For each of these conditions, it is also thought that microRNA (miRNA) modulation may be involved in disease pathogenesis. This review aims to summarise and highlight the potential relationships between miRNA and folate, and the consequences for health and disease. Folate status may modulate miRNA profiles, and miRNA may modulate the activity of folate dependent pathways. A better understanding of these associations may shed light on disease aetiology, identify biomarkers for these diseases and help identify novel therapeutic pathways for complex diseases.

miRNA are a family of short non-coding RNA, involved in the post-transcriptional regulation of gene expression. miRNA were first identified in *Caenorhabditis elegans* by Lee *et al.* in 1993 [3, 4]. To date, over 1800 precursors and 2500 mature miRNA have been identified in humans (MiRBase, <http://www.mirbase.org>; Release 21, August 2017). Due to their small size (~18-23 nucleotides), each miRNA may target hundreds of mRNA [5], and each mRNA can be targeted by multiple miRNA. This allows for complex and multifaceted regulatory feedback and feedforward networks [6]. miRNA are implicated in the regulation of a wide array of basic cellular processes, and dysregulation of miRNA expression or activity can underlie complex disease aetiology. It has been estimated that miRNA are involved in the regulation of 30-80% of human genes [7].

miRNA (as part of the RNA-induced silencing complex; RISC) act on mRNA via complementary base pairing, modulating translation of mRNA (figure 1). In cases of near complete miRNA-mRNA complementarity, the target mRNA may be directly cleaved by argonaute proteins [8, 9]. More commonly, partial complementarity results in modifications that repress translation or reduce the stability of the target mRNA [10-12]. The capability of miRNA to activate target translation under specific conditions has also been demonstrated [13-16].

miRNA are transcribed from genomic DNA in an RNA polymerase dependent manner [17, 18]. After transcription, Drosha and the cofactor DGCR8 are required to convert the immature primary precursors (pri-miRNA) into precursor miRNA (pre-miRNA). Pre-miRNA are then exported to the cytoplasm [19, 20] where it is cleaved by the enzyme Dicer leaving a miRNA duplex ~22 nucleotides in length [21, 22]. Following further processing by Argonaute proteins, one strand is incorporated into the RNA-induced silencing complex (RISC), and the other strand is discarded or incorporated into another RISC [23, 24]. This process may result in two unique mature miRNA with unique expression levels being generated from the same precursor, depending on which strand of the duplex is selected [25, 26]. Conversely, identical mature miRNA may be generated from different original pri-miRNA [27].

miRNAs are deeply involved in the epigenome. The expression of miRNA can be regulated by epigenetic mechanisms, such as DNA methylation or histone modifications, and miRNA have been identified that directly influence the function of the other components of the epigenetic machinery [28] which in turn, indirectly influences the epigenetic modulation of other genes. Conversely, miRNA expression can also be regulated by classical epigenetic mechanisms [29], and expression of one miRNA may also regulate expression of another miRNA [30]. The interaction and overlapping of these two mechanisms allows for regulation of miRNA levels and thus their genetic targets via complex feedback loops.

As a consequence of this interdependence, folate status may influence miRNA profiles via alteration of DNA methylation of genes coding for miRNA, or via modulation of expression of genes upstream of miRNA signalling pathways (Figure 2). Folate status may also impact miRNA expression levels through modulation of other methylation reactions or via alterations in cellular homeostasis. This modulation may result in miRNA involvement in disease pathogenesis or may allow miRNA to be used as biomarkers for disease processes. miRNA may also act to regulate the expression of genes coding for enzymes in the folate mediated 1CM pathway, altering folate status and availability of substrates for different folate dependent biochemical reactions (Figure 3).

Correlations between miRNA levels and folate status have been observed in human cohorts (Table 1). However, the number, size, and diversity of these studies to date are limited, as is direct evidence of causation. There is evidence modulation of miRNA expression in several cell culture model (Table 2) and animal models (Table 3). Conversely, there is also emerging evidence of the modulation of folate-related pathways by miRNA (Table 4) and relationships between polymorphisms in folate-related genes, miRNA coding genes, and risk for folate related diseases (Table 5). These relationships are summarised by related disease state below.

2. Embryonic Development

Folate plays an important role in the development of the central nervous system (CNS). Maintenance of adequate levels of maternal folate protects against neural tube defects and other developmental disorders [1]. The mechanisms of this prevention are not yet fully elucidated. However they are likely to involve both the methylation and DNA synthesis and repair function of folate [31]. The evidence is emerging the miRNA may play a role in both mediating and modulating the effects of folate deficiency in the growing mammalian embryo. A majority of studies investigating these interactions have been conducted in animal and cell culture models, due to tissue accessibility.

In a rat model of maternal folate deficiency upregulation of let-7a and miR-34a, and subsequent downregulation of their regulatory targets, such as *trim71* and *notch*, which are involved in embryogenesis and neurogenesis, occurs in the brain. This is accompanied by cerebellar and interhemispheric suture defects, and atrophy of selective cerebral layers occurs. Late maternal folate supplementation restores these pathways and reduces the deficiency-associated defects [32]. Maternal methyl donor deficiency in rats also leads to decreased mRNA and protein levels of the transcription factor Stat3 in fetal brains. Conversely, deficiency conditions led to the upregulation of miR-124, a Stat3 regulator. siRNA silencing of miR-124 partly restored Stat3 signalling, and improved the phenotype of folate deficient cells [33].

Spitch embryos (*Sp*^{-/-}) are a murine model of NTD [34]. A feature of this model is decreased expression of the histone demethylase KDM6B, and this is thought to occur at least in part due to miRNA targeting. Relative to wild-type mice, multiple miRNA are upregulated in the caudal neural tubes of *Sp*^{-/-} embryos four of which (miR-138, -148a, -185, and -339-5p) have binding sites in the 3' UTR of *KDM6B*. Administration of folic acid reverses this upregulation, and rescues KDM6B expression, indicating that these miRNA may be important in neural tube development [35]. This suggests that miRNA are involved in neural developmental processes modulated by folate.

The relationship between folate and miRNA expression has also been demonstrated in cell culture systems. Mouse embryonic stem cells cultured in folate deficient media fail to proliferate, have higher rates of apoptosis and accumulate in the G0/G1 phase, compared to cells cultured in control media. Array methods demonstrated that 60 miRNA were upregulated and 34 were downregulated under folate deficient conditions relative to cells grown in control media. Functional consequences were demonstrated, with parallel

modulation of thousands of mRNA (upregulation of 2316 and downregulation of 2544). qPCR confirmed that 12 miRNA (let-7a, miR-15a, -15b, -16, -29a, -29b, -34a, -130b, -125a-5p, -124, -290, and -302a) were differentially expressed in folate-deficient cells. Bioinformatics analyses suggested a particularly critical role for miR-302a in mediating the effects of folate on cell cycle progression. This was confirmed *in vitro* as playing a role in regulating proliferation, apoptosis and cell cycle phase by directly targeting the Lats2 gene [36].

Folate receptor alpha, which translocates to the nucleus on stimulation and acts as a transcription factor, is also critical for embryonic development, including neural crest development. Stimulation of folate receptor alpha in cranial neural crest cells (O9-1) leads to a downregulation of miR-138, which targets Oct4, and miR-let-7, which targets Trim71 (an Oct4 downstream effector) [37]. Oct4 is important in the maintenance and regaining of stem cell pluripotency [38]. Co-immunoprecipitation data suggests that folate receptor alpha interacts with Drosha and DGCR8 to alter the processing of pre-miRNA. Transfection of anti-miR-138 or anti-miR-let-7 into non-proliferating *Sp*^{-/-} neural crest cells led to a restoration of proliferation [37].

miRNA may also impact folate dependent pathways important in development via modulation of expression enzymes involved in folate mediated 1CM, with consequences for folate dependent outcomes, independent of a deficiency of intake. Polymorphisms in these genes alter the risk of neural tube defects and other developmental and reproductive abnormalities [39]. These polymorphisms may alter the binding and function of miRNA, modulating their effects. miR-197 and miR-9 have been shown to downregulate the expression of *MTHFD1L* in human embryonic kidney cells (HEK293 cells) and breast cancer cells (MCF-7 cells) via 3'UTR binding. *MTHFD1L* codes for the mitochondrial formyltetrahydrofolate synthase enzyme, which is required to generate tetrahydrofolates for use in *de novo* synthesis of purines and thymidylate and to contribute to methylation pathways. Polymorphisms in *MTHFD1L* are associated with risk for NTD. The rs7646 polymorphism significantly affects miR-197 binding affinity to the *MTHFD1L* 3'UTR, causing more efficient post-transcriptional gene repression in the presence of the allele that is associated with increased risk of NTDs [40]. Similarly, there is a miR-34b binding site in the 3'UTR of the methylenetetrahydrofolate reductase (*MTHFR*) gene, which may be impacted by the rs55763075 polymorphism, which may alter 1CM and has been linked to risk for idiopathic azoospermia [41].

Low maternal folate levels during pregnancy also increases the risk of delivering infants small for gestational age, and placental dysfunction. The mechanisms of this association are not fully elucidated. However, miRNA may play a role. In a human cohort study low folate levels in mothers correlated with upregulated expression of miR-22-3p, -141-3p and -34b-5p in placental tissues. Network prediction models identified that these miRNA are involved in regulating cell turnover, suggesting that miRNA may mechanistically link low maternal folate to placental dysfunction and offspring that are small for gestational age [42].

This current evidence, although limited, suggests that miRNA are a mechanism involved in modulating conditions of folate deficiency or genetic abnormalities in enzymes of folate metabolism. This may be involved in explaining variance in individual risk for developmental defects due to folate deficiency. However, it is difficult to elucidate cause and consequence.

3. Cancer

Folate status is linked to modulation of risk for a number of cancers [43, 44]. Aberrant DNA methylation is a feature of cancers, as are aberrant miRNA signatures [45, 46]. Folate status has been found to modulate miRNA expression in a number of model systems, and this modulation may be related to cancer risk. To date, the investigation of the relationship between miRNA and folate has primarily occurred in animal and culture model systems.

Rats fed a folate, and methyl donor-deficient diet develop hepatocellular carcinoma (HCC) after 54 weeks [47]. Livers from these animals showed increased expression of let-7a, miR-21, -23, -130, -190, and -17-92 and decreased expression of miR-122 relative to those on the control diet. Returned to the control diet at 36 weeks, returned miR-122 levels to normal and prevented the development of HCC [47]. Further analysis of this model identified downregulation of miR-34a, -16a, -181a, -200b and -127, each of which are tumour suppressor miRNA. Notably, suppression of miR-34a and -127 occurred early and continued throughout carcinogenesis [48, 49]. This was paralleled by increased liver expression of E2F3 and BCL6, proteins known to be regulated by miRNA34a and miRNA127, respectively [50].

In human lymphoblast cells (TK-6 cells) culture in folate-deficient media leads to modulation of multiple miRNA. Return to folate-sufficient conditions results in the restoration of miRNA profiles to control levels [51]. miR-222 and -22 significantly upregulated under folate-deficient conditions and were also differentially modulated in the lowest vs. highest percentile of folate intake [51]. However, this study was conducted in a small subset of patients from a larger case-control study of head and neck squamous cell carcinoma, which may be a confounding factor [51].

miR-22 has also been found to be a regulator of folate metabolism in human breast cancer cell lines (MCF-7 and T47D cells) via repression of MTHFD2 levels and subsequent reduction of S-adenosylmethionine expression. MTHFD2 is crucial for the regeneration of mitochondrial tetrahydrofolate that is needed for purine synthesis, the S-adenosylmethionine cycle, and mitochondrial glycine synthesis. Overexpression of miR-22 in cells resulted in repression of MTHFD2 mRNA and protein levels. Luciferase reporter assays demonstrated a miR-22 binding site in the 3'-UTR of *MTHFD2*. [52]. In normal (HL-7702 cells) and cancerous (QGY-7703 cells) human hepatocytes miR-22-3p/miR-149-5p has been shown to directly targeted the 3'UTR sequence of *MTHFR*. Folic acid deficiency led to an upregulation of miR-22-3p/miR-149-5p expression in both cell lines. However, the transcription of *MTHFR* and protein levels were decreased in QGY-7703 cells, but elevated mRNA expression occurred in HL-7702 cells. [53]. Additionally, miR-22, -24 and -34a have been shown to be involved in the folate pathway, predicting outcomes of pemetrexed-based chemotherapy in non-small cell lung cancer [54]. It has also been shown that miR-22 and miR-29b directly target rat *Mthfr* and *Mat1* genes, respectively. Altered expression of miRNA could, therefore, alter ICM in cancer cells, altering outcomes including susceptibility to antifolate chemotherapy.

Thymidylate synthase (TYMS) plays a crucial role in folate metabolism as well as DNA synthesis and repair and is an important target for cancer chemotherapy. High expression of TYMS is associated with resistance to 5-fluorouracil treatment. A miR-433 binding site exists in the 3'-UTR of TYMS, and overexpression of miR-433 decreases expression of TYMS mRNA and protein in HeLa cells. Furthermore, miR-433 increased inhibition of cell proliferation in HeLa cells treated with 5-fluorouracil [55]. The TYMS polymorphisms rs16430 6bp deletion/insertion, rs2790 A>G and rs1059394 C>T are each associated with miRNA binding sites and are also associated with altered risk for gastric [56] and breast cancers [57]. Larger population studies are warranted to verify these findings.

Circulating miRNA in serum and plasma may be biomarkers or aetiological agents in cancer onset and progression [58]. In a study of pancreatic cancer patients, serum miR-16 was upregulated, and miR-103 downregulated in patients relative to controls (after adjustment for age, sex, and smoking); however, after additional adjustment for folate, only miR-103 was significantly different. Multifactor dimensionality reduction analysis showed a significant interaction for miR-16, folate, and smoking. The interaction between miR-16 and folate was also verified in AsPC-1 cells [59]. Similarly, a study of colonic adenomatous polyp patients, folate status was found to correlate with miR-21 levels in serum, with modification of this relationship by MTHFR genotype [58]. It is not clear if these relationships are related to carcinogenesis or not, however, they may have implications for the use of miRNA as cancer biomarkers.

Given the link between dietary methyl group donors and regulation of DNA methylation, and the miRNA-folate associations described above, further investigation into these associations and their consequences are clearly warranted.

Recently, the attachment of miRNA to folate, resulting in molecules referred to as FolamiRs, has been investigated as a mechanism to enhance delivery of exogenous miRNA as a therapy [60]. FolamiR-34 has been shown to be rapidly taken up by breast cancer cells in vivo and in vitro, and by tumours in a model of lung cancer, leading to slower progression of disease. This innovation may allow the administration of exogenous miRNA directly to rapidly dividing cancer cells, without the use of toxic vehicles [60].

4. Neurodegenerative diseases

Epidemiological studies link folate status to altered risk for neurodegenerative diseases, including Alzheimer's disease [61-67]. This may occur via methylation pathways, or due to the inverse association between folate and homocysteine levels. Mechanisms may be direct or indirect and include promotion of inflammation [68], competition with inhibitory neurotransmitters [69], and damage to neuronal cells [67, 70-74].

Amyloid- β protein (A β) is the core protein of neuritic plaques. A β is generated by the sequential cleavage of the amyloid precursor protein (APP) via APP cleaving enzymes. Folic acid deficiency enhances A β accumulation in the brains of APP/PS1 mice. Analysis of miRNAs predicted to target these genes revealed several miRNA candidates that were differentially modulated by folic acid deficiency. In APP/PS1 mouse brains and N2a cells with folic acid-deficient treatment, miR-106a-5p, miR-200b-3p and miR-339-5p were downregulated, and their target genes APP and BACE1 were upregulated [75]. Another study found that folic acid regulated the expression of miR-126-3p and miR-339-5p, which target ADAM9 and BACE1, respectively. As such, the effect of folic acid on A β deposition may relate to modulation of APP metabolism by decreasing expression of APP cleavage enzymes, which may be regulated by miRNA [76]. However, these associations are yet to be investigated directly in human cohorts and are hindered by issues of tissue accessibility and timing of diagnosis.

5. Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) encompasses chronic liver conditions characterized by steatosis, inflammation, fibrosis, and liver injury. The liver is the primary organ for folate metabolism and storage. As such, folate status may be related to the progression of NAFLD and its associated metabolic complications. However, case-control comparisons have found no differences in folate or homocysteine levels in NAFLD patients compared to healthy controls [77].

Dietary methyl donor deficiency induces liver injury that resembles human nonalcoholic fatty liver disease (NAFLD), including an altered expression of miRNA. In mouse models of methyl-donor deficiency induced liver damage, the magnitude of miRNA expression changes are strain specific, with greater modulation found in WSB/EiJ mice, compared to A/J mice. WSB/EiJ mice also exhibited more severe liver injury. Methyl donor deficient mice displayed upregulated expression of miR-134, miR-409-3p, miR-410, and miR-495, as well as an activation of hepatic progenitor cells and fibrogenesis. The same miRNA were upregulated in undifferentiated progenitor hepatic HepaRG cells compared to in fully differentiated HepaRG cells. Additionally, miR-134, miR-409-3p, miR-410 and miR-495 were upregulated in the plasma of WSB/EiJ mice fed the methyl donor deficient diet, while expression was not altered in A/J mice [78]. Conversely, folic acid supplementation has been shown to reduce the number of inflammatory foci, and lipid vacuoles in the liver of mice fed a high-fat diet [79].

In a study using a panel of seven genetically diverse strains of inbred male mice (A/J, C57BL/6J, C3H/HeJ, 129S/SvImJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ), the morphological changes in the livers were accompanied by differences in the levels plasma miRNAs. The levels of circulating miR-34a, miR-122, miR-181a, miR-192, and miR-200b correlated with the severity of NAFLD-specific liver pathomorphological features [80]. These observations suggest that the plasma levels of miRNAs may be used as biomarkers for noninvasive monitoring of the extent of the NAFLD-associated liver injury and susceptibility to NAFLD. Several studies have demonstrated the potential for miRNA to be used as biomarkers for NAFLD in humans [81-84]. However, additional studies are needed to demonstrate that folate and other dietary factors are not confounders in these relationships.

6. Cardiovascular disease

Low folate status may increase the risk for cardiovascular diseases via its inverse association with homocysteine. Hyperhomocysteinemia increases the risk of cardiovascular diseases, including atherosclerosis, myocardial infarction and stroke [85-89]. Potential mechanisms include promotion of low-density lipoprotein oxidation [71, 90] and inflammation [68] and damage to endothelial cells [67, 70-74].

The rs71428439 polymorphism in the region coding for pre-miR-149 is associated with an altered risk for stroke. Compared to healthy controls, a lower frequency of GG genotype, and a higher frequency of TT genotype is found in ischemic stroke patients. In the same study, The TT genotype also related to higher levels of folate [91]. Similarly, in a study of 4 polymorphisms in pre-miRNA regions (miR-146aC>G [rs2910164], miR-149T>C [rs2292832], miR-196a2T>C [rs11614913], and miR-499A>G [rs3746444]) the miR-146aG alone and the combination of miR-146aG, miR-149T, miR-196a2C and miR-499G alleles were significantly associated with the prevalence of ischemic stroke [92]. However, others have found no association [93]. Animal models are needed to investigate the mechanism of these potential associations and determine causation.

7. Conclusion

There is emerging evidence that folate status and miRNA interact in a bi-directional nature. Folate status modulates miRNA profiles, and that miRNA are involved in the regulation of ICM. By these mechanisms, miRNA may be involved in mediating the effects of folate deficiency or treatment, and may additionally be a mechanism for modulation of disease risk due to polymorphisms in the enzymes of folate metabolism. This may have consequences for developmental, neurodegenerative, cardiovascular and other diseases. In the future, modulation of folate status may be used to manipulate miRNA signatures, and exogenous miRNA therapy may be able to prevent the consequences of folate-mediated diseases. Additionally, miRNA may be used as biomarkers for disease and conditions where folate mediated pathways are at play. The majority of the evidence of associations to date is obtained from cell culture and animal models, and larger studies, including those in human cohorts, are required to better understand these mechanisms and to elucidate the direction of cause and consequence. Additional studies are also required to fully elucidate the cause and effect of some associations.

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Figure legends

Figure 1: Simplified schematic of miRNA biogenesis and mechanism of action. Pri-miRNA are transcribed from DNA in an RNA polymerase dependent manner. The enzymes Drosha and DGCR8 convert pri-miRNA to pre-miRNA, which are exported to the cytoplasm in an exportin-5 dependent manner, where it is cleaved by the enzyme Dicer leaving a miRNA duplex. Following further processing by Argonaute proteins, one strand is incorporated into the RNA-induced silencing complex (RISC), which interacts with mRNA to induce target degradation or repression of translation.

Figure 2: The potential bi-directional relationships between folate status and miRNA profiles. Folates can alter miRNA status via regulation of gene expression, which may occur via the role of folates as a source of methyl-groups for DNA methylation or other mechanisms. miRNA can, in turn, alter the expression of genes the code for DNA methyltransferases or enzymes involved in the folate-dependent one-carbon metabolism pathway.

Figure 3: Simplified Schematic of folate mediated One-carbon metabolism. Multiple enzymes are involved in the metabolism of folates, including dihydrofolate reductase enzyme (DHFR), serine hydroxymethyltransferase (SHMT), methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR) and others. miRNA may target the genes coding these enzymes and alter the substrates availability for folate dependent processes, including DNA synthesis and repair, methylation reactions and the homocysteine, glutathione and transulphonuration pathways.

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