Original Article

In silico analysis of polymorphisms in microRNAs that target genes affecting aerobic glycolysis

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Background: Cancer cells preferentially metabolize glucose through aerobic glycolysis, an observation known as the Warburg effect. Recently, studies have deciphered the role of oncogenes and tumor suppressor genes in regulating the Warburg effect. Furthermore, mutations in glycolytic enzymes identified in various cancers highlight the importance of the Warburg effect at the molecular and cellular level. MicroRNAs (miRNAs) are non-coding RNAs that posttranscriptionally regulate gene expression and are dysregulated in the pathogenesis of various types of human cancers. Single nucleotide polymorphisms (SNPs) in miRNA genes may affect miRNA biogenesis, processing, function, and stability and provide additional complexity in the pathogenesis of cancer. Moreover, mutations in miRNA target sequences in target mRNAs can affect expression.

Methods: *In silico* analysis and cataloguing polymorphisms in miRNA genes that target genes directly or indirectly controlling aerobic glycolysis was carried out using different publically available databases.

Results: miRNA SNP2.0 database revealed several SNPs in miR-126 and miR-25 in the upstream and downstream pre-miRNA flanking regions respectively should be inserted after flanking regions and miR-504 and miR-451 had the fewest. These miRNAs target genes that control aerobic glycolysis indirectly. SNPs in premiRNA genes were found in miR-96, miR-155, miR-25 and miR34a by miRNASNP. Dragon database of polymorphic regulation of miRNA genes (dPORE-miRNA) database revealed several SNPs that modify transcription factor binding sites (TFBS) or creating new TFBS in promoter regions of selected miRNA genes as analyzed by dPORE-miRNA.

Conclusions: Our results raise the possibility that integration of SNP analysis in miRNA genes with studies of metabolic adaptations in cancer cells could provide greater understanding of oncogenic mechanisms.

Keywords: MicroRNAs (miRNA); single nucleotide polymorphism (SNP); cancer

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Introduction

Cancer cells preferentially utilize glucose to generate ATP via glycolysis despite the availability of oxygen, and this phenomenon is called the Warburg effect (1). This metabolic reprogramming favors the proliferative and invasive phenotypes of many cancers (2). Increased aerobic glycolysis in cancer cells provides key carbon precursors for the synthesis of nucleic acids, phospholipids, cholesterol, and porphyrins in addition to energy (3). Moreover, glycolytic intermediates such as glucose-6-phosphate, phosphoenolpyruvate, and dihydroxyacetone act as the

precursors for nucleotide, amino acid, and lipid biosynthesis, respectively (4-6). A plethora of mechanisms have been proposed by various investigators to explain the Warburg effect in cancer cells including oncogene activation, loss of tumor suppressors, mitochondrial dysfunction, epigenetic changes, posttranslational modifications, and microRNA (miRNA) regulation. Interestingly, several genes involved in aerobic glycolysis are regulated by miRNAs (7,8).

miRNAs are gene regulators that enhance translational repression by binding, with imperfect base pairing, to complementary sequences in the 3'-untranslated regions (3'-UTRs) of their target mRNAs (9,10). Aberrant expression of miRNAs has been associated with breast cancer, prostate cancer, colorectal cancer, and several other human proliferative diseases (11-13). Interestingly, many cancer genes, including RAS family members, have target sequences that bind and are regulated by miRNAs (13). Several studies have suggested that examination of miRNA profiles may facilitate identifying tumor origin and predicting cancer relapse; hence, miRNAs can serve as reliable and superior prognostic indicators (14,15).

Single nucleotide polymorphisms (SNPs) have been studied widely to identify those that play major roles in influencing cancer, treatment prognosis, and survival (16-18). SNPs in miRNA binding sites add another layer of complexity to understanding cancer (19-21). In this regard, SNPs located in miRNA genes were studied and associated with cancer susceptibility (22). Recently, a polymorphism in the 3'-UTR of *IFNRA1* was shown to influence hepatocellular carcinoma risk, likely through miRNA (miR)-1231-mediated regulation (23). Moreover, miRNA-disrupting polymorphisms in the 3'-UTR of *BRCA1* were investigated by Pelletier *et al.* (24) to identify new genetic markers in breast cancer, and Landi *et al.* (13) reported an association between 3'-UTR polymorphisms and colorectal cancer risk. An increased risk for non-small cell lung cancer (NSCLC) was associated with an SNP in the *MIRLET*7 binding site in v-Ki-ras 2 Kirsten rat sarcoma viral oncogene homolog (KRAS) (21). Furthermore, cataloguing polymorphisms in miRNAs is crucial, and Iwai and Naraba (25) conducted a large comprehensive study analyzing 173 different miRNAs in 96 individuals. Polymorphisms were identified in various regions of ten different miRNAs (25).

miRNA-target interactions might also be influenced by the mutations affecting the miRNA as well (26). Mutations in pri- or pre-miRNA may influence stability or processing. Mutations in the promoter of pri-mRNA or cis or trans may influence the transcription rate of mature miRNAs (26), and mutations in the seed region of the miRNAs affect target recognition (27). Finally, copy number variation might affect copies of the miRNA (26). miRNA variations in human cancer cell lines were previously demonstrated (28). Hence, many studies suggest that SNPs in miRNAs themselves provide another additional layer of complexity in carcinogenesis, and systems biology analyses of miRNA polymorphisms may be useful in the near future (22). Recently, targeting glucose metabolism in cancer cells undergoing aerobic glycolysis was suggested as a promising therapeutic strategy. Therefore, cataloguing polymorphisms in miRNAs that target genes controlling aerobic glycolysis is crucial to understanding metabolic adaptation in cancer cells. The purpose of the present study is to computationally predict the polymorphisms in miRNAs that control genes involved in aerobic glycolysis and presumably affect metabolic survival of cancer cells. To this end, we computationally analyzed polymorphisms in miRNAs that control aerobic glycolysis. The resulting catalogue may be helpful for forming hypotheses and performing experiments to develop anti-cancer therapeutics targeting aerobic glycolysis.

Materials and methods

Selection of miRNAs that control aerobic glycolysis

miRNAs predicted to target genes directly involved in aerobic glycolysis were chosen from a recent review article and are known to be deregulated in cancer cells that undergo metabolic reprogramming for survival (7) (*Table 1*). We chose miR-143, miR-223, miR-320a, and miR-375 targeting hexokinase 2 (*HKII*), solute carrier family 2 (facilitated glucose transporter), member 4 (*GLUT4*), phosphofructokinase, muscle (*PFKM*), and pyruvate dehydrogenase kinase, isozyme 1 (*PDK1*), respectively. Additionally, we chose miR-133b, miR-137, miR-326, and miR-340 targeting pyruvate kinase, muscle (*PKM2*) and included miR-29a, which targets monocarboxylate transporter (*MCT*), phosphoglycerate kinase 1 (*PGK1*), and *Enolase1*, and miR-29b that targets *MCT*. Oncogenes and tumor suppressor genes also modulate glucose metabolism indirectly, so we also included miRNAs that control these genes: miR-96, miR-18a, miR-217 (targeting *KRAS*); miR-21 (targeting p53); and miR-100, miR-126, miR-155, miR-200b/c miR-210, miR-424, miR-451 and miR-34a targeting mechanistic target of rapamycin (serine/threonine kinase)

Table 1 List of Studied miRNAs targeting genes that directly or indirectly affect aerobic glycolysis

Gene classification	miRNA	Gene/pathway target
Directly involved in aerobic glycolysis	miR-29a	Monocarboxylate transporter, phosphoglycerate kinase 1, enolase 1
	miR-133b	Pyruvate kinase 2
	miR-137	Pyruvate kinase 2
	miR-143	Hexokinase 2
	miR-223	GLUT 4
	miR-29b	Monocarboxylate transporter
	miR-320a	Muscle type phospho fructokinase
	miR-326	Pyruvate kinase 2
	miR-340	Pyruvate kinase 2
	miR-375	Pyruvate dehydrogenase kinase
Indirectly affecting aerobic glycolysis	miR-96	Kirsten rat sarcoma viral oncogene homolog (KRAS)
	miR-18a	KRAS
	miR-21	Phosphatase and Tensin homolog (PTEN), P53
	miR-25	SIRT1
	miR-30d	SIRT1
	miR-100	Mammalian target of rapamycin (mTOR)
	miR-126	Phosphoinositide 3 kinase (PI3K)
	miR-155	Hypoxia inducible factor (HIF-1α)
	miR-200b	Prolyl hydroxylase 2 (PHD2)
	miR-200c	PHD2
	miR-210	Iron-sulfur cluster assembly proteins (ISCU1/2)
	miR-217	KRAS
	miR-424	Cullin 2
	miR-451	LKB1
	miR-504	SIRT1
	miR-34a	SIRT1

(*mTOR*), phosphatidylinositol-4,5-bisphosphate 3-kinase (*PI3K*), hypoxia inducible factor 1, alpha subunit (*HIF-1α*), prolyl hydroxylase 2 (*PHD2*), iron-sulfur cluster assembly proteins (*ISCU1/2*), *Cullin 2*, liver kinase B1 (*LKB1*), and sirtuin 1 (*SIRT1*), respectively. In total, 26 miRNAs were included that regulate transporter genes, glycolytic enzymes, and oncogenes or tumor suppressor genes (*Table 1*). At least one miRNA controlling each gene is conserved according to miRNA viewer (http://people.csail.mit.edu/akiezun/microRNAviewer/) (29).

In silico prediction of SNPs occurring in miRNA genes

SNPs for the selected human miRNAs were retrieved from publically available databases: miRNASNP (http://www.bioguo.org/miRNASNP/) (30), dragon database of polymorphic regulation of miRNA genes (dPORE-miRNA) (http://cbrc.kaust.edu.sa/dpore and http://apps.sanbi.ac.za/dpore) (31), and miRNA SNiPer (http://integratomics-time.com/miRNA-SNiPer/) (27). miRNASNP provides a complete list of SNPs, including those in human pre-miRNAs and miRNA flanking sequences (30). It also provides information regarding SNPs in other species and target gain and loss by SNPs in miRNA seed regions or the 3'-UTR of target mRNAs (30). Furthermore, information about transcriptional regulation of miRNAs by SNPs was gathered from dPORE-miRNA, which focuses on SNPs that affect transcription factor binding sites (TFBS) and transcriptional regulation of miRNAs (31). miRNA SNiPer 3.0 was used to detect

Table 2 Summary of SNPs in selected miRNA genes analyzed by miRNASNP 2.0

miRNA	Chromosome	No. of SNPs in pre-miRNA flanking regions (up/down)	SNPs in pre-miRNA regions
hsa-miR-29a	Chr 7	13/14	0 records
hsa-miR-133b	Chr 6	15/16	rs112599381
hsa-miR-137	Chr 1	15/10	0 records
hsa-miR-143	Chr 5	20/23	0 records
hsa-miR-223	ChrX	20/13	rs34952329, rs186354597
hsa-miR-29b-1	Chr 7	19/17	0 records
hsa-miR-29b-2	Chr1	15/21	rs200396959
hsa-miR-320a	Chr8	18/31	rs200301891, rs201450610
hsa-miR-326	Chr11	15/27	rs72561778
hsa-miR-340	Chr5	32/14	0 records
hsa-miR-375	Chr2	12/14	0 records
hsa-miR-96	Chr7	19/20	rs41274239, rs73159662
hsa-miR-18a	Chr13	25/14	rs41275866, rs201226336
hsa-miR-21	Chr17	17/19	0 records
hsa-miR-25	Chr7	20/37	rs41274221, rs200616968
hsa-miR-30d	Chr8	27/25	rs185802410
hsa-miR-100	Chr11	24/18	0 records
hsa-miR-126	Chr9	37/26	rs199992070
hsa-miR-155	Chr21	17/10	rs140021681, rs200351615- seed region
hsa-miR-200b	Chr1	7/15	rs72563729
hsa-miR-200c	Chr12	10/27	0 records
hsa-miR-210	Chr11	11/18	0 records
hsa-miR-217	Chr2	33/16	rs41291173, rs150436368, rs140509279
hsa-miR-424	ChrX	11/10	0 records
hsa-miR-451	Chr17	9/6	0 records
hsa-miR-504	ChrX	5/11	0 records
hsa-miR-34a	Chr1	12/21	rs72631823, rs201359809, rs35301225

polymorphisms within miRNA genes (pre-miRNA, mature and seed regions) (27).

Results

We computationally analyzed the SNPs in miRNA genes that regulate aerobic glycolysis using publically available databases. Mining the miRNA SNP2.0 database revealed several SNPs in pre-miRNA flanking regions (–1 to +1 kb) and pre-miRNA regions (*Table 2*). MiR-126 and miR-25 had the most polymorphisms in the upstream and downstream pre-miRNA flanking regions, respectively, whereas miR-504 and miR-451 had the fewest. These miRNAs target genes that control aerobic glycolysis indirectly. MiR-504 showed the fewest polymorphisms (*Table 2*) and is reported to negatively regulate p53, which highlights the need for investigation of polymorphisms in pre-miRNA flanking regions and the transcriptional regulation of miRNAs.

The results of our analysis for SNPs (nucleotide change and position) within miRNA genes (pre-miRNA, seed, and mature regions) by miRNA SNiPer are shown in *Table 3*. Both miRNASNP and miRNA SNiPer corroborate the SNPs in miRNAs with few exceptions. Additional SNPs in pre-miRNA genes were found in miR-96, miR-155, miR-

Table 3 Summary of SNPs in miRNA genes analyzed by miRNA SNiPer

miRNA	No. of SNPs	SNP information
hsa-miR-133b	1 (rs112599381)	In pre-mature 52013754, SNP (T > C)
hsa-miR-223	2 (rs34952329, rs186354597)	In pre-mature 65238767, in del (- > C); In pre-mature 65238733, SNP (G > A)
hsa-miR-326	1 (rs72561778)	In pre-mature 75046227, SNP (T > C)
hsa-miR-96	2 (rs41274239, rs73159662)	In pre-mature 129414574, SNP (A > G); In pre-mature 129414568, SNP (G > A)
hsa-miR-18a	1 (rs41275866)	In pre-mature 92003009, SNP (C > G)
hsa-miR-25	1 (rs41274221)	In pre-mature 99691200, SNP (C > T)
hsa-miR-30d	1 (rs185802410)	In pre-mature 135817129, SNP (A > G)
hsa-miR-155	1 (rs140021681)	In pre-mature 26946325, SNP (T > C)
hsa-miR-200b	1 (rs72563729)	In pre-mature 1102563, SNP (G > A)
hsa-miR-217	3 (rs41291173, rs140509279, rs150436368)	In pre-mature 56210140, SNP (G > A); In mature 56210168, SNP (T > C); In pre-mature 56210207, SNP (A > G)
hsa-miR-34a	2 (rs72631823, rs35301225)	In pre-mature 9211782, SNP (C > T); In mature 9211802, SNP (C > A,T)

25, and miR-34a by miRNASNP. We further investigated the impact of SNPs in promoter regions of miRNAs that target genes directly involved in aerobic glycolysis. RNA polymerase II is involved in the transcription of pri-miRNAs similar to protein-coding genes. The transcriptional machinery controlling miRNA biogenesis is not well understood. dPORE-miRNA provides information about the impact of SNPs in the promoter regions of human miRNA genes (intergenic as well as intragenic) and consequent transcriptional control of miRNA genes. Each miRNA gene has multiple promoters, and the promoter regions validated by the UCSC Genome browser were analyzed for SNPs. SNPs that modify TFBS or creating new TFBS in promoter regions of selected miRNA genes as analyzed by dPORE-miRNA are shown in *Table 4*.

Discussion

The genetic variation that occurs within miRNA gene sequences has profound and broad biological effects beyond those of SNPs in miRNA binding sites (27). Micro RNAs have multiple targets, and SNPs can enhance, diminish, generate, or abolish binding to target sequences (27). SNPs in miRNA-binding regions of cancer genes were intensely catalogued previously (32). However, polymorphisms in miRNA genes in relation to cancer are less well investigated. Recently, several studies attempted to elucidate the Warburg effect in relation to miRNA-linked metabolic pathways including aerobic glycolysis. Genes that directly or indirectly control aerobic glycolysis and the associated miRNAs have been reviewed elsewhere (7). Interestingly, ubiquitous loss of miR-126 and increased PI3K signaling is reported in colon cancer cell lines and primary colon tumors (33). It can be observed from *Table 2*. miR-126 had the most polymorphisms in the pre-miRNA flanking regions. Whether these polymorphisms in pre-miRNA flanking region influence the transcriptional control of miR-126 will require further investigation.

Several studies recently investigated the impact of polymorphisms in pre-miRNA flanking regions and pre-miRNA regions. Bensen *et al.* (34) identified germline variation in the 5'-region proximal to pre-miRNA gene sequences and suggested the need for further studies to validate the association with breast cancer risk among African Americans and breast cancer-specific survival. Hu *et al.* (35) studied genetic polymorphisms in pre-miRNA flanking regions in patients with NSCLC and suggested rs928508 as a prognostic biomarker of NSCLC. Xu *et al.* (36) identified the rs895819 polymorphism within pre-miR-27a as influential in NSCLC patients; this SNP may also serve as a risk factor for breast cancer in younger Chinese populations (37). A study in 240 gastric cancer patients found an association between the pre-miR-30c A/G polymorphism altering mature miR-30c expression and increased risk of

Table 4 Summary of SNPs affecting transcriptional regulation of miRNAs

miRNA	Promoter regions (UCSC genome browser)	SNPs overlapping with TFBS	SNPs causing new TFBS
hsa-miR-29a	130212046-130217109	rs17165205, rs67760466, rs7781163, rs6953968, rs57260393, rs205727, rs4728201, rs2398686, rs157908, rs6943241, rs1057289, rs5705639, rs45076964,	rs58576983, rs34908097
hsa-miR-133b	52116680-52121798	rs62408583, rs17578851, rs9349616, rs9463761, rs9463762	rs11453607, rs72542719, rs60690975, rs67745407, rs61239511, rs62408584, rs73435789, rs35825595
hsa-miR-137	98284214-98289315	rs41285698, rs2660303, rs61786696, rs61786697, rs9440399, rs72969633, rs74106014	rs58335419, rs2660304, rs72735983
hsa-miR-143	148783674-148788779	rs519814, rs519795, rs28418464, rs34311277, rs58748655, rs67363234, rs1422815, rs17710212, rs59416338, rs17723799, rs353293, rs13158382	rs34863506, rs17796757, rs17110069, rs17723793, rs4705342, rs4705343
hsa-miR-223	65150437-65155546	rs1152308, rs12394264, rs16989857, rs16989858, rs34002459, rs12853964, rs34163188, rs73213399, rs12559661, rs16989860, rs73213400, rs5965084, rs35104137	rs3848900, rs34952329
hsa-miR-320a	22158420-22163501	rs6982634, rs34392777, rs10092957, rs34101866	none
hsa-miR-326	74723784-74728878	rs72561777, rs678956, rs73492823, rs71036046, rs495193	rs60206863, rs67461553
hsa-miR-340	179374909-179374909	rs11443493, rs11746941, rs11346273, rs66616765, rs67220158, rs11443491, rs67446752, rs11346269, rs17079966, rs67369864, rs55716450, rs66962303, rs11249664, rs34823714, rs59354197, rs66823881, rs35844702, rs33940528, rs10045755, rs56691249, rs57144908	rs67789066, rs11443490, rs11346270, rs67986335, rs11443488, rs67769618, rs6601078
hsa-miR-375	219574611-219579674	rs3178492, rs61738865, rs61738865, rs73089095, rs57023019, rs61003470, rs58852567, rs72119241	rs72114580, rs61214924

gastric cancer in a Chinese population (38). SNPs in coding regions may be synonymous or non-synonymous, and utilization of several databases for predicting deleterious effects of SNPs are reported (39,40). Catalogue of SNPs (nucleotide change and position) within miRNA genes (pre-miRNA, seed and mature regions and in the promoter regions is provided in the *Tables 3,4*.

Conclusions

In summary, the data presented herein emphasize the utility of publicly available databases with information regarding SNPs in miRNA genes. Using these databases, we comprehensively catalogued the SNPs in selected miRNA genes directly or indirectly related to the Warburg effect. These SNPs can affect regulation of miRNA biogenesis and alter miRNA levels, thereby affecting genes that directly or indirectly control aerobic glycolysis. These data may inform hypotheses and experimental design for studying the Warburg effect and miRNA SNPs in cancer. Although we did not study polymorphisms in all the miRNAs currently known to affect genes controlling

aerobic glycolysis, this study provides useful information for application by the scientific community and a basis for further study.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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