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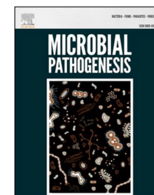

Computational prediction of microRNAs in *Histoplasma capsulatum*

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ABSTRACT

MicroRNAs (miRNAs) are small and non-coding RNAs that regulate gene expression through post-transcriptional regulation. Although, the standard miRNA repository, MiRBase, lists more than 200 organisms having miRNA mediated regulation mechanism and thousands of miRNAs, there is not enough information about miRNAs of fungal species. Considering that there are various fungal pathogens causing disease phenotypes, it is important to search for miRNAs of those organisms. The leading cause of endemic mycosis in the USA is a fungal disease known as histoplasmosis, which is resulted by infection with a fungal intracellular parasite, *Histoplasma capsulatum* (*H. capsulatum*). In this work, genomes of *H. capsulatum* strains NAm1 and G217B were explored for potential miRNA like sequences and structures. Through a complex workflow involving miRNA detection and target prediction, several miRNA candidates of *H. capsulatum* and their possible targets in human were identified. The results presented here indicate that *H. capsulatum* might be one of the fungal pathogens having a miRNA based post-transcriptional gene regulation mechanism and it might have a miRNA mediated host – parasite interaction with human.

1. Introduction

Histoplasma capsulatum (*H. capsulatum*) is one of the most common human fungal pathogens on the global scale. Even though for majority of the cases, infections are not resulting in serious conditions, 10% of *H. capsulatum* infected patients suffer from life-threatening complications [1]. The risk is more severe for individuals with compromised immunity like patients with HIV and/or cancer. While histoplasmosis (the disease caused by *H. capsulatum*) appears to be the main reason of fungal pneumonia in the United States of America [2] it is a bigger problem in several developing countries especially in Latin America region [3]. In spite of the obvious consequences of its infection, not much information is available about the pathogenesis mechanisms of histoplasmosis.

H. capsulatum is a dimorphic ascomycete that grows in its hyphal form in soil and transforms into the pathogenic yeast phase when inhaled [4]. In the lung, *Histoplasma* cells infect host macrophages and replicate. Then, yeast would be carried throughout the body, most commonly infecting organs with high concentrations of the reticuloendothelial cells such as liver, bone marrow and spleen [5].

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression at the post-transcriptional level. Thousands of miRNAs have been identified in hundreds of species. The standard miRNA repository for pre-miRNA and mature miRNA sequences, miRBase (Release 22.1) lists miRNAs from 271 organisms [6]. Although miRNAs have been a popular research subject and identified for their various roles in animals and plants, not much information have been revealed for the presence and functions of miRNAs in fungi. Only a small portion of miRNA-like small RNAs have been identified in *Neurospora crassa*, *Sclerotinia sclerotiorum*, *Metarhizium anisopliae*, *Cryptococcus neoformans* and *Penicillium marneffei* [7–11].

In various fungi species including *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, *Candida albicans*, *Saccharomyces cerevisiae* and *Malassezia simpodialis,* it has been shown that extracellular vesicles (EV) mediate the transport of RNA molecules [12–14]. Since *H. capsulatum* has an argonaute protein known as QDE2 protein, it is possible that this fungal species is also capable of producing miRNAs [3]. Moreover, recent findings indicate that *H. capsulatum* is also capable of transporting both mRNAs and non-coding RNAs through EVs [3](Graphical Abstract).

The main purpose of this work is to explore genomes of *Histoplasma* strains NAm1 and G217B for candidate miRNAs that might be able to target human genes and cause a disease phenotype. Moreover, the miRNA-based regulation of *Histoplasma* by its own miRNAs is investigated. In order to explore these issues, the genomes of two *Histoplasma* strains were transcribed, folded and potential miRNA hairpins were extracted. Since there are no known miRNA sequences of *Histoplasma*, a model built based on human miRNAs is applied to all hairpins. From

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nearly 2.5 million hairpins in the genomes of *Histoplasma* strains, around 294 (0.011%) for NAm1 and 635 (0.024%) for G217B were found acceptable in respect to the human models of izMiR approach [15].

According to the overall findings, *Histoplasma* might produce human like pre-miRNAs and it may possibly transport these hairpins or mature miRNA sequences into its host cells to alter the gene expression.

2. Material and methods

Data processing, pre-miRNA prediction and mature miRNA detection workflows were created by using the Konstanz Information Miner (KNIME) platform [16].

2.1. Data acquisition

The genome files of *Histoplasma* strains were obtained from NCBI:

- NAm1 (NAm1-GCF_000149585.1_ASM14958v1_genomic)
- G217B (G217B-GCA_000170615.1_ASM17061v1_genomic)

Mature miRNA sequences of human (2654 sequences) were downloaded from miRBase Release 22.1.

Total of 35,729 *Histoplasma* mRNA sequences were acquired from NCBI.

Histoplasma capsulatum NAm1 CDS data (9313 sequences) were downloaded from EnsemblFungi.

G217B expression datasets from 87 experiments were obtained from NCBI SRA database (SRX5175079 - SRX5175165) [17].

Required workflows and files for izMiR [15] miRNA prediction software were downloaded [18] and used in KNIME.

2.2. Pre-miRNA prediction

Genome sequences of NAm1 and G217B were transcribed (T- > U) and divided into 500 nt long fragments with 250 overlaps [15]; resulting in 132,268 fragments for NAm1 and 165,272 fragments for G217B. Then these fragments were folded into their secondary structures by using RNAfold [19] with default settings and hairpin structures were extracted (for NAm1 1,089,886 hairpins; for G217B 1,505,323 hairpins in total).

After removing the duplicate sequences and filtering the hairpins based on the sequence length (min: 40 max: 500), 237,762 hairpin sequences for NAm1 and 328,985 for G217B were obtained for further analysis.

2.3. Mature miRNA prediction and targeting

Hairpins passing defined thresholds for pre-miRNA prediction scores of izMiR were split into smaller sequences of maximum 23 nt length with 6 nt overlaps in ranging lengths. Then, these fragments were filtered based on minimum length of 15 and their location on the hairpins (sequences involving loop nucleotides were removed). Target search of these remaining 940 NAm1 and 2405 G217B candidate mature miRNAs were performed against *Histoplasma* and human genes by using psRNATarget tool with default settings [20]. Also, the potential of known human mature miRNAs' from miRBase for targeting *Histoplasma* genes were tested.

2.4. Gene Ontology

The targets of *Histoplasma* generated miRNAs in human were further analyzed for their Gene Ontology (GO). To achieve this, PANTHER Classification System (http://www.pantherdb.org) was used [21].

3. Results

3.1. MiRNA prediction

For the extracted hairpins, features required to run izMiR software were calculated and prediction analysis was performed (Fig. 1). Then, hairpins were filtered based on their AverageDT score (minimum 0.99).

In order to show the influence of filtration based on the prediction score values, the total number of hairpins from the strains with equal or higher miRNA scores at varying cutoff values was plotted (Fig. 2). When a stricter scoring threshold is applied, the number of hairpins passing the defined cutoff score and labeled as a potential miRNA decreases dramatically. Setting the cutoff value as 0.99 resulted in 294 NAm1 and 635 G217B unique miRNA hairpin candidates (Figs. 1 and 2). Sequences are provided in Supplementary File.

From these hairpins, 940 NAm1 and 2405 G217B mature miRNA candidates were extracted and their potential targets for human and *Histoplasma* genes were investigated.

Machine learning based miRNA prediction approach of izMiR calculates a score for all of the candidate hairpins and label them accordingly. If the score is lower or equal to 0.5 than those hairpins with that

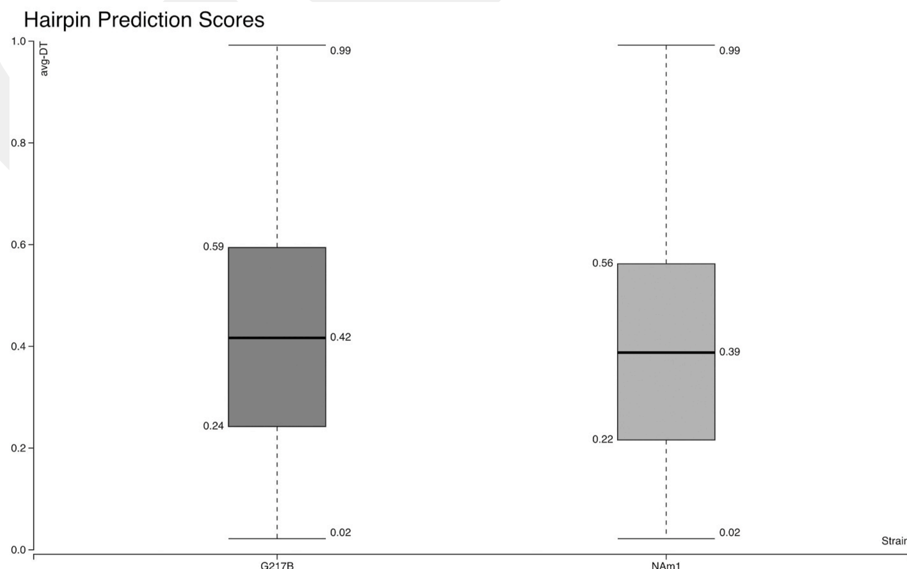

Fig. 1. Box-plots for pre-miRNA prediction scores of G217B and Nam1 hairpin sequences. Y-axis represents AverageDT scores.

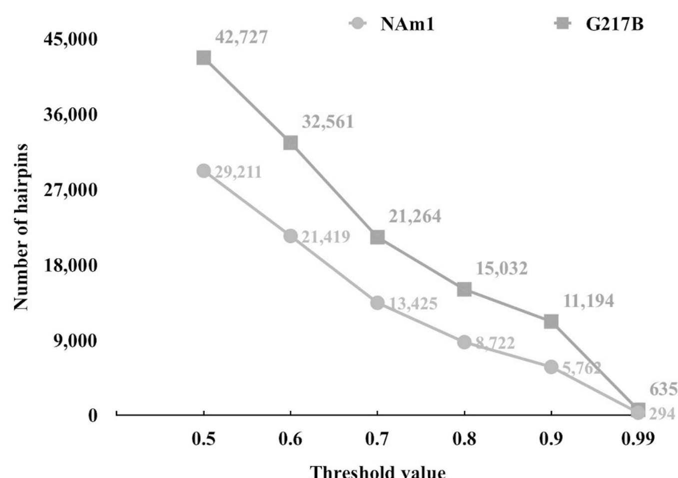

Fig. 2. Total number of hairpins at different prediction score thresholds. Y-axis indicates the number of *Histoplasma* hairpins at defined threshold values (x-axis).

score values would be assigned as non-miRNA and the sequences with higher score than 0.5 are potential miRNAs (Fig. 2). In order to find out the influence of this score on the number of total hairpins assigned as miRNAs, the miRNA threshold score versus the number of hairpins from both *Histoplasma* strains with equal or higher scores at different cutoff values were plotted in a graph (Fig. 2). As expected, when scoring threshold increases, the number of potential miRNAs decreases greatly. At a cutoff of 0.9 there are about 11,000 miRNA G217B and 6000 NAm1 candidates (Fig. 2). These numbers seem quite big compared to about 2000 known human miRNAs listed in miRBase, so the threshold value was set as 0.99 for this study.

To test whether any of the known mature miRNAs from 271 organism listed in miRBase match to any of the predicted hairpins in *Histoplasma,* a similarity search was performed based on the Levenshtein distance calculations in KNIME. There was no significant similarity between hairpin sequences so mature sequences were also compared. Among 940 NAm1 and 2405 G217B mature miRNA candidates, only a single predicted mature sequence coming from 2 NAm1 hairpins found to be significantly similar to a mouse mature miRNA (mmu-miR-466i-5p) from miRBase and a miRNA from *Echinops telfairi* (Ete-Mir-574_5p) listed in MirGeneDB [22]. Considering the low confidence of this mouse miRNA (in miRBase), it is not clear if its similarity with NAm1 miRNA candidate has a critical meaning.

Table 1
Total number of miRNAs targeting events in different scenarios. MiRNA: the organism that produces the miRNAs, # MiRNA: total number of different miRNAs involved in targeting events, Target: targeted genes' organism, Target Genes: total number of genes used from the target organism for targeting analysis # Targets: total number of different genes (mRNAs, CDS) targeted by corresponding miRNAs.

MiRNA	# MiRNA	Target	Target Genes	# Targets
NAm1	836	*Histoplasma capsulatum* (NCBI)	35,729	21,210
G217B	2176	*Histoplasma capsulatum* (NCBI)	35,729	27,525
NAm1	836	NAm1 CDS (Ensembl)	9313	8078
G217B	2176	NAm1 CDS (Ensembl)	9.313	8918
NAm1	836	Human	21,233	14,673
G217B	2176	Human	21,233	17,496
Human	2498	*Histoplasma capsulatum* (NCBI)	35,729	30,243
Human	2498	NAm1 CDS (Ensembl)	9313	9161

3.2. Targeting

While mature miRNA candidates of NAm1 and G217B were used to find their targets in *Histoplasma* mRNAs and human genes, mature miRNAs of human were applied on *Histoplasma* mRNAs (Table 1). Note that Table 1 lists all possible interactions between miRNAs and their target candidates.

While 3014 human genes were targeted only by G217B miRNAs, 715 gene targets were specific to NAm1 strain (Figs. 3 and 4, Supplementary Files).

The targeting between miRNAs and mRNAs might be a simple one to one regulation; but they can also show complex many to many relations. Table 2 shows that for some human genes like SH3TC2 there are more possible miRNA – target binding predictions than total number of miRNAs. For instance, 51 different miRNA sequence from NAm1 strain that are involved in 70 targeting events for SH3TC2. This could be due to the binding of certain miRNAs to alternative sites of this gene.

Majority of predicted targets of miRNA-like sequences of Histoplasma in NAm1 coding sequences obtained from EnsemblFungi were hypothetical proteins (Table 3). Among the targets with known functions most common ones were DNA binding and pre-mRNA processing proteins (Table 3).

3.3. GO analysis

In order to find which pathways in human might be affected by *Histoplasma* miRNAs, PANTHER Classification System was applied to targeted human genes. The targets of Nam1 and G217B strains were uploaded to the website (http://www.pantherdb.org) and investigated. Based on the results presented in Table 4, Figs. 3 and 4, and Supplementary File, a wide range of human genes with various molecular functions and pathways could be targeted by these strains of *Histoplasma*.

3.4. Expression analysis

In order to see if the predicted miRNAs are actually expressed in certain conditions, NCBI SRA Database was searched for *H. capsulatum* strains. There is a recent paper that performed RNA sequencing on G217B strain to observe temperature response [17]. The raw data produced in that study was downloaded from SRA and used as database for a basic BLAST [23] search where predicted miRNAs were used as query sequences. Note that, this method is not a proper expression level measurement but it is sufficient to show if these miRNA candidates have any potential to be expressed.

For 404 hairpin sequences (329 from G217B and 75 from NAm1), there were 100% matches with the expression data. Most of these potential pre-miRNAs are found only once or a few copies in the overall expression dataset while there were some examples showing perfect matches to more than 100 expressed sequences. Since the sequences of the expression data had the same length (51 nt), in most of the matchings (293 out of 404) predicted hairpin sequences were longer. Based on the miRBase hairpin data, among 271 species, the shortest known hairpin is 39 nt and the biggest one is 2354 nt (for human minimum: 41, maximum: 180).

G217B hairpin G217B-mir-341 had 500 copies (count) in the overall expression data (199 copies in SRR8364739, 169 copies in SRR8364740 and 132 copies in SRR8364741). When this 301 nt long hairpin was searched in NCBI BLAST server, it only showed 90.98% identity to a gene which is from *Histoplasma capsulatum* (*Ajellomyces capsulatus* nitrosative stress induced transcription 91 (NIT91) gene, partial sequence, Sequence ID: DQ350746.1, Length: 610). These hairpin and transcript sequences share only 121 nt. To further check if the smaller version of the hairpin which shows similarity to a gene might be a better miRNA candidate, sequence from the first 133 nt of the hairpin (Fig. 5) was used to form secondary structures in RNAfold WebServer [19].

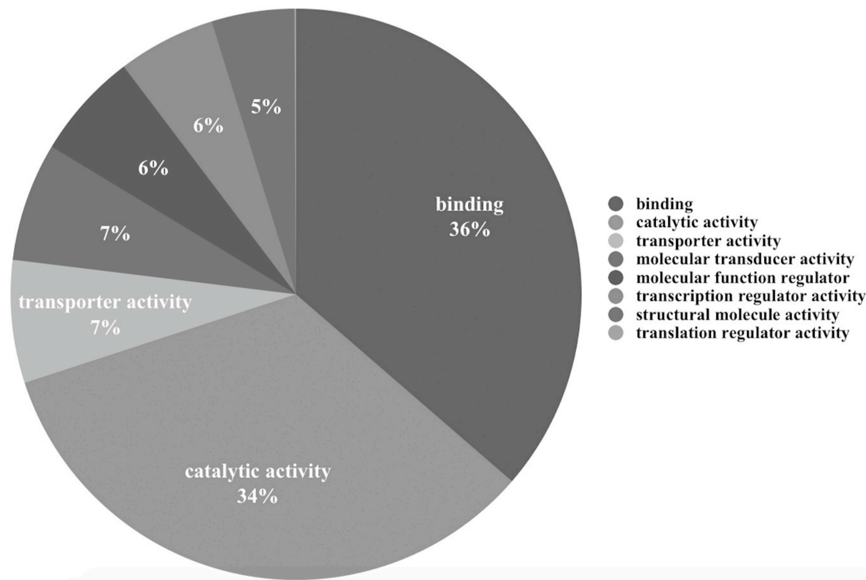

Fig. 3. Pie - chart of molecular functions of the genes targeted by only G217B miRNAs. Labels on the right part are sorted in descending order making the chart direction in a clockwise order. Translation regulatory activity shows the lowest values (1%).

Based on the structures, it is very unlikely that shorter sequence of the hairpin would be a proper miRNA candidate (Fig. 5).

4. Discussion

MiRNAs have been shown to be involved in various cellular processes. Previously, it has been shown that miRNAs might be generated from non-coding transcription units as well as protein-coding transcription units [24] so for this study miRNA positions are not considered. Since a single miRNA could target up to hundreds of mRNAs and an mRNA might be targeted by many different miRNAs, investigating miRNA mediated post-transcriptional gene regulation is a complicated task. In recent years, possible targeting events between host mRNAs and its parasites' miRNAs have been proposed as an alternative communication method, which further increased the complexity of miRNA analyses [25,26].

Even though histoplasmosis cases have been occurring worldwide, disease seems to be concentrated in endemic regions such as North American area [4]. As the fungus responsible for this infection, *Histoplasma capsulatum*, shows significant genotypic and phenotypic differences within its strains, two of them G217B and NAm1 (North America 1) were analyzed in this study. Contrary to known miRNAs of animals and plants that are involved in various cellular functions ranging from development to stress response, the possible roles of miRNAs in fungi remain to be established. However, based on the results presented in Table 4, *Histoplasma* miRNAs might target human cytokines which are important for immune response against infection of this pathogen. Although these findings need further validation through wet-lab

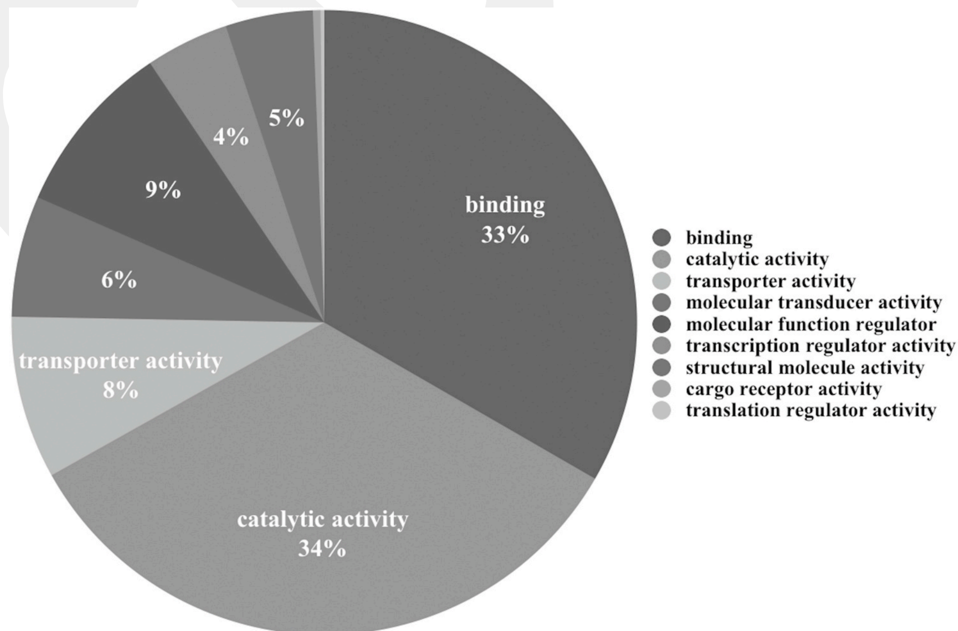

Fig. 4. Pie - chart of molecular functions of the genes targeted by only NAm1 miRNAs. Labels on the right part are sorted in descending order making the chart direction in a clockwise order. Cargo receptor activity and translation regulatory activity show the lowest values (around 1%).

Table 2
The list of targeting events between *Histoplasma* miRNAs and human genes. G217B MiRNA and NAm1 MiRNA columns indicate the number of different miRNAs from these strains targeting the specific human genes. Total counts of miRNA targeting events from respective *Histoplasma* strains shown in G217B and NAm1 Target columns. Gene Name and Synonyms of the genes were obtained from Amigo 2 [30]. The table is sorted based on G217B MiRNA and limited to top 20 genes. The full version of the table is available in the Supplementary Files.

Genes	G217B MiRNA	NAm1 MiRNA	G217B Target	NAm1 Target	Gene Name	Synonyms
SH3TC2	208	51	211	70	SH3 domain and tetratricopeptide repeat-containing protein 2	KIAA1985, PP12494
GUCY1A2	130	28	135	28	Guanylate cyclase soluble subunit alpha-2	GUC1A2, GUCSA2
LPP	123	37	125	44	Lipoma-preferred partner	
ZNF451	120	22	135	24	E3 SUMO-protein ligase ZNF451	COASTER, KIAA0576, KIAA1702
AAK1	117	47	125	50	AP2-associated protein kinase 1	KIAA1048
CSRNP3	111	39	185	67	Cysteine/serine-rich nuclear protein 3	FAM130A2, TAIP2
BRWD1	109	32	121	39	Bromodomain and WD repeat-containing protein 1	C21orf107, WDR9
DISC1	107	34	185	39	Disrupted in schizophrenia 1 protein	KIAA0457
HOOK3	107	35	116	36	Protein Hook homolog 3	
SLC7A11	107	33	196	72	Cystine/glutamate transporter	
NUFIP2	106	40	175	65	Nuclear fragile X mental retardation-interacting protein 2	KIAA1321, PIG1
QKI	106	51	365	180	Protein quaking	HKQ
ZNF264	105	26	122	30	Zinc finger protein 264	KIAA0412
TET2	104	26	170	56	Methylcytosine dioxygenase TET2	KIAA1546, Nbla00191
INO80D	102	30	105	30	INO80 complex subunit D	
SAMD12	101	36	148	54	Sterile alpha motif domain-containing protein 12	
ZNF704	100	22	104	22	Zinc finger protein 704	
FLT1	98	24	101	26	Vascular endothelial growth factor receptor 1	FLT, FRT, VEGFR1
MBNL3	98	43	240	130	Muscleblind-like protein 3	CHCR, MBLX39, MBXL
NSL1	98	23	203	47	Kinetochore-associated protein NSL1 homolog	C1orf48, DC31, DC8, MIS14

experiments, such miRNA-mediated regulations could be one of the mechanisms of fungal pathogens to evade the host immune system.

The results presented here indicate that the *Histoplasma* strains examined might have the capacity to generate functional miRNAs with a secondary structure that can be recognized and processed by miRNA biogenesis proteins such as Drosha and/or Dicer. Moreover, recent findings report that small RNA molecules were found in the extracellular vesicles (EVs) of *Histoplasma capsulatum* [3]. These miRNA-like, strain specific, short-fragments (25–40 nt) were found in EVs produced by the G217B strain [3]. Considering the fact that QDE2 protein (argonaute protein in *H. capsulatum*) was also observed to be enriched in the EVs of the G217B strain [3], it is very likely that miRNA-like small RNA - QDE2 delivery from *H. capsulatum* to the recipient cells might be a cellular communication mechanism. In the case where the recipient is a human

Table 3
The list of targeting events between *Histoplasma* miRNAs and *Histoplasma* CDS. Target description column indicates the function of targets. MiRNA column shows the number of different miRNAs from Histoplasma strains targeting the specific NAm1 CDS. Total counts of miRNA targeting events shown in Target column. The table is sorted based on Target and limited to top 25 targets.

Target Description	MiRNA	Target
predicted protein	3012	76,502
conserved hypothetical protein	3003	43,240
hypothetical protein	2894	16,000
DNA binding protein URE-B1	127	138
dynein heavy chain	129	135
pre-mRNA processing splicing factor 8	109	115
bifunctional pyrimidine biosynthesis protein pyrABCN	88	94
anucleate primary sterigmata protein B	86	93
DNA-directed RNA polymerase II 138 kDa polypeptide	83	86
activating signal cointegrator 1 complex subunit 3	79	82
xanthine dehydrogenase	75	79
3-dehydroquinate synthase	73	77
DNA-directed RNA polymerase II largest subunit	73	76
elongation factor G	69	75
60S ribosomal protein L19	70	71
antiviral helicase SKI2	64	71
cytokinesis protein sepA	70	71
ATP-dependent RNA helicase DOB1	60	70
chitin synthase 2	68	70
1,3-beta-glucan synthase component GLS1	63	66
acetyl-CoA hydrolase	63	65
ribosome biogenesis protein BMS1	49	65
DNA topoisomerase 2	61	64
endochitinase 1 precursor	58	63
nucleolar protein NOP2	60	63

Table 4
List of pathways affected by G217B and NAm1 miRNAs. G217B and NAm1 columns indicate the number of genes targeted by miRNAs of these strains and also involved in corresponding pathways. Panther pathway accessions are indicated in parentheses. The table is sorted by gene counts and limited to minimum 25 genes. Full list of the pathways and involved gene number are available in Supplementary Files.

Pathway	G217B	NAm1
Wnt signaling pathway (P00057)	108	97
Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	107	88
Integrin signaling pathway (P00034)	75	66
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	68	54
Huntington disease (P00029)	65	54
Gonadotropin-releasing hormone receptor pathway (P06664)	63	59
Angiogenesis (P00005)	57	54
CCKR signaling map (P06959)	55	43
Cadherin signaling pathway (P00012)	52	45
Alzheimer disease-presenilin pathway (P00004)	51	44
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)	44	38
TGF-beta signaling pathway (P00052)	42	34
EGF receptor signaling pathway (P00018)	41	38
Apoptosis signaling pathway (P00006)	40	34
PDGF signaling pathway (P00047)	36	39
Nicotinic acetylcholine receptor signaling pathway (P00044)	34	30
Interleukin signaling pathway (P00036)	33	31
T cell activation (P00053)	32	25
Parkinson disease (P00049)	32	22
FGF signaling pathway (P00021)	32	30
Endothelin signaling pathway (P00019)	31	27
Cytoskeletal regulation by Rho GTPase (P00016)	31	28
Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)	29	24
Muscarinic acetylcholine receptor 2 and 4 signaling pathway (P00043)	28	25
p53 pathway (P00059)	27	28
Metabotropic glutamate receptor group III pathway (P00039)	27	23
Transcription regulation by bZIP transcription factor (P00055)	26	22

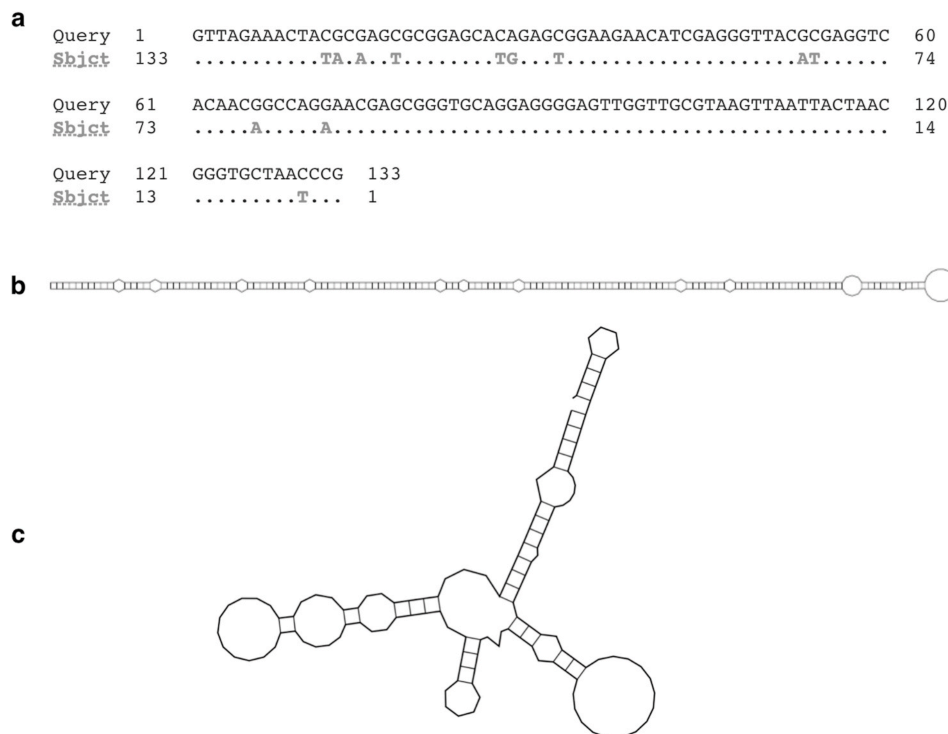

Fig. 5. Alignment and structures of G217B-mir-341. Panel A shows the pairwise alignment of the hairpin and gene (DQ350746.1). Part B shows the secondary structure of overall sequence of this predicted hairpin (500 nt, minimum free energy: −207.20 kcal/mol) and C shows the secondary structure based on the first 133 nt of the hairpin (minimum free energy: −32.10 kcal/mol).

cell, it is possible that fungal RNAs would regulate the expression of human genes, which might be an example of cross-species gene regulation mechanisms.

Based on the results of Histoplasma miRNA-like sequences' capacity to target Histoplasma genes, various pathways including transcription, translation and replication might be regulated through miRNA actions. For instance, DNA-directed RNA polymerase targeting would affect transcription while ribosomal proteins targeting could change translation rate. Interestingly, the most targeted 3 genes from NAm1 CDS data were identical for Histoplasma and human miRNAs; DNA binding protein URE-B1, dynein heavy chain and pre-mRNA processing splicing factor 8 (Table 3). However, this is most likely due to the length of these sequences. Although this work is mainly focused on interactions between host and parasite through miRNA-like sequences, the obtained results show that miRNA mediated gene regulation might be involved in a wide range of processes of *Histoplasma capsulatum* strains.

RNA interference studies in fungi has shown that there are three main functions for small non-coding RNAs: defense, gene regulation and heterochromatin formation [27]. Among these, gene regulation might be one of the main actions of Histoplasma miRNA-like sequences based on the data presented in this study. Biogenesis of miRNAs are achieved through diverse pathways in different organisms. In fungi, like *Neurospora crassa* [28] and *Schizosaccharomyces pombe* [29], generation of some miRNA-like sequences are dependent on Dicer like RNAse III domain-containing proteins while there are also reports of Dicer-independent mechanisms. In general, factors like Dicer, QDE-2, QIP (exonuclease), and MRPL3 are required for the generation of functional miRNA-like sequences in fungi [8,28], Since it has been shown that Histoplasma EVs contain various RNAi machinery elements [3], it could be possible for the pathogen to generate miRNA-like sequences without depending on the host enzymes.

5. Conclusion

The present study demonstrated the capacity of two *H. capsulatum* strains to produce functional miRNAs. The miRNA candidates are predicted to target various human genes, the expression of which could be modulated to control numerous pathways. Nevertheless, further examination of other *Histoplasma* strains for miRNA profiles and experimental validation of predictions are required in order to obtain better understanding of miRNA actions of these fungal species.

Author contributions

All authors contributed to the study conception and design, material preparation, data collection and analysis. All authors read and approved the final manuscript.

Data availability

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Mendeley Data: https://doi.org/10.17632/gsdhvbf8nr.1

Acknowledgments

None.

Declaration of competing interest

None Declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2020.104433.

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