Alterations in esophageal microRNAs expression in primary esophageal achalasia by next-generation sequencing

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Abstract

Background The molecular knowledge of primary esophageal achalasia is essential for the early diagnosis and treatment of this neurodegenerative motility disorder. So it is substantial to find the main microRNAs (miRNAs), which are related to mechanisms of achalasia. Methods This study aimed to determine some patterns of deregulated miRNAs in achalasia. This case-control study was performed on 52 achalasia patients and 50 non-achalasia controls. The miRNA expression profiling was conducted on esophageal tissue samples from achalasia and non-achalasia patients, via next-generation sequencing (NGS). Differential expression of miRNAs was analyzed by edgeR software. The selected dysregulated miRNAs were additionally confirmed using RT-qPCR. Potential target genes of the down-regulated and up-regulated miRNAs were also predicted to understand the putative role of the miRNAs in achalasia. Results We identified 15 miRNAs that were significantly altered in the achalasia tissues compared to controls. Among these miRNAs, three; miR-133a-5p, miR-143-3p and miR-6507-5p were up-regulated. Also we found six miRNAs; miR-215-5p, miR-216a-5p, miR-216b-5p, miR-217, miR-7641 and miR-194-5p were down-regulated significantly. The predicted targets for the dysregulated miRNAs showed significant disease-associated pathways like neuron cell apoptosis, neuromuscular balance, neuron growth factor signaling and immune response regulation. Gene expression analysis confirmed significant downregulation of hsa-miR-217 (p-value =0.004) in LES of achalasia patients with significant enrichment in myelination process ontology. This study provides the first integrated miRNA expression profile using NGS in achalasia. Our findings introduced 15 candidate microRNAs as achalasia associated non-coding RNAs genes showing confirmed downregulation of the hsa-miR-217 in Achalasia disease. Conclusions Our results may serve as a basis for more future functional studies to discover the role of candidate miRNAs in the etiology of achalasia and their application in diagnosis and probably treatment.

Background

Achalasia is a chronic motor disorder featured by impaired lower esophageal sphincter (LES) laxity and peristalsis disturbance [1]. Achalasia Symptoms include progressive swallowing disorder, regurgitation, esophageal chest pain, aspiration, and eventually malnutrition [2]. Based on the population study, achalasia prevalence is more than 10/100 000, with a steady increase trend from 2.51/100 000 in 1996 to 10.82/100 000 in 2007. The survival of achalasia patients is significantly less than age-matched healthy people [2]. Due to nonspecific symptoms of the disease and the absence of non-invasive diagnostic tests, most patients underwent late diagnosis and ineffective treatment [3].

The pathophysiology of achalasia is a selective loss of inhibitory neurons in the myenteric network, which can interfere with the coordination of esophageal peristalsis and LES relaxation during a swallow [4]. Decreasing nitric oxide synthase and vasoactive intestinal polypeptide as inhibitory neurotransmitters in the myenteric plexus disrupt esophageal neuromuscular function in achalasia [5]. Although the exact mechanism of the disease is not fully understood, some studies have shown evidence of viral, autoimmune, and neurodegenerative association [6].

microRNAs (miRNA) are a group of small noncoding RNAs that act as gene expression regulators in disease pathways [7]. The miRNA system is involved in various physiological and pathophysiological processes with potential prognostic biomarker applications [8]. Several studies have shown alterations in the expression of miRNAs in various disorders, including cancers [9], immune-mediated inflammatory diseases [10], and nervous disturbance [11].

It has been well known that neurological communication, cholinergic signaling, and inflammation are related to achalasia pathogenesis. On the other hand, miRNAs expression influence all of these processes [12]. It suggests that studying the miRNAs expression could help us better understand the achalasia pathophysiology. Although the effects of miRNAs on several diseases pathogenesis have received considerable attention, its impact on achalasia is still unclear. So we performed NGS with an analytical approach to identify reliable candidate miRNA associated with the disease.

Methods

Participants and sampling

This matched case-control study was performed on 102 patients referred to the digestive disease clinic of Shariati Hospital in Tehran, Iran; between August 2015 and April 2016. All the primary esophageal achalasia patients (cases, N = 52 diagnosed with barium radiography, ≥ 18 years old) who referred to the clinic for the regular follow up consecutively were recruited in this study. Controls (N = 50) were selected randomly from the individuals visiting the same clinic without dysphagia or lesions in the esophagus. All cases and controls were matched by age (± 5 years) and sex. Participants underwent endoscopy and biopsy samples were removed from the LES of the esophagus by an expert clinician. The samples were stored at -80˚C for the experiments. Patients with other associated motility or non-motility disorders, malignancy, coagulopathy, were excluded.

RNA isolation and deep sequencing

Total RNA was extracted from all samples (52 cases and 50 controls) using Trizol reagent according to instruction (Invitrogen, Sweden). Based on the response to pneumatic dilatation treatment cases were divided into two groups good response and poor response. To increase the experiment power, two samples of each group were pooled and sent for miRNA sequencing. For the control group also two samples were selected and mixed equally for miRNA profiling.

The samples were sent to BGI (Beijing Genomics Institute) Company in China for miRNA sequencing. Also, Bioanalyzer 2100 (Agilent, Santa Clara, CA) was employed to measure RNA integrity number (RIN) for each sample and samples with RIN greater than 7 were considered for sequencing. RNA purification, library construction, and sequencing were conducted by BGI Company. Each library was single-end sequenced on an Illumina HiSeq 4000 platform.

Analysis of small RNA sequencing data(NGS data)

FASTQC (version 0.11.5; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to perform primary quality control of the miRNA-Seq data. Afterward, low-quality reads and adapter sequences of raw data were trimmed by Trimmomatic software (version 0.35) [13] (parameters of trailing 20, maxinfo 18:0.90 and minimum length 18). Reads with the length shorter than 18 bases were discarded after quality trimming, and the remaining reads were mapped against the Rfam database (http://rfam.xfam.org/) to eliminate unwanted noncoding RNAs (rRNAs, tRNAs, snRNAs, and snoRNA). Subsequently, the remaining reads were analyzed using miRDeep2 (version 0.0.8)[14] to quantify known miRNAs and predict novel miRNAs.For efficient read mapping, the clean reads in each sample were collapsed into a set of unique sequences with read numbers counted. Then, the unique sequences were aligned to the Ensembl GRCH37 human genome (http://grch37.ensembl.org) and miRNAs sequences (miRBase version 21). Reads aligned to known human miRNAs from miRBase (version 21) [15] were quantified using the default settings of miRDeep2 in which only one mismatch was allowed within the read. On the other hand, putative novel miRNAs were predicted using the default settings of miRDeep2. miRDeep2 predictions were filtered, with a miRDeep2 score > 1, length ≥ 50 nucleotides and predicted probability of being a miRNA > 60%. The difference in miRNAs expression (fold change) was analyzed by edgeR package (v1.4.5) of R software. Fold change with adjusted p-value or false discovery rate (FDR) less than 0.05 was considered significant.

miRNAs target prediction and gene enrichment analyses

Potential target genes of the differentially expressed miRNAs were predicted using three target prediction programs including PITA[16], TargetSpy [17] and RNAhybrid [18]. If a gene was predicted by at least two used programs, it was considered as a putative target. Since every software has different sensitivity and specificity, by this method, we tried to reduce the false positive. Every software uses various algorithms to predict miRNA targets. The default parameters of the software were applied. The 3'UTR sequences were recovered by BioMart(http://www.ensembl.org/Multi/martview) and then used for prediction. Finally, Gene Ontology (GO) and Kyoto Encyclopedia Genes and Genomes (KEGG, http://www.genome.ad.jp/kegg) were applied to analyze the potential function and pathway of target genes.

Real time-quantitative PCR (RT-qPCR) analysis

For more confirmation, the expression level of the dysregulated miRNAs was measured in the esophagus tissue of achalasia patients and control individuals by ABI 7300 real-time PCR machine (Applied Biosystems). cDNA synthesis of miRNAs was performed by the Reverse Transcription System Kit (Zist Royesh, Iran) with a miR-specific stem-loop primer [19]. the SNORD47 was measured as an internal normalization control using the 2^{-dct} method. For RT-qPCR statistical analysis differences between the two groups were tested by Student's t-test and the Mann-Whitney U test (based on normality of data distribution) in SPSS 16.0 statistical software. We accepted the difference with a probability value of less than 0.05 as statistically significant.

Results

Achalasia patient esophagus tissue express miRNA profile different from control

The summary of the clinical information of the patients is shown in Table 1. As we can see, the cases and controls did not differ significantly in age (p-value = 0.48) and sex (p-value = 0.43). For transcriptome analysis and based on the clinicians' recommendation, the miRNA sequencing results were compared between three groups: Treat 1 including sample 1 and 2 (good and excellent response to dilatation treatment in one group as good response), Treat 2 consisting of sample 3 and 4 (fair and poor response to dilatation treatment as poor response) and samples 5 and 6 were merged into Treat 3 (controls). (Table 2)

Table 1 Clinical data for 44 Achalasia patients and 45 controls

Characteristic	Patients †	Controls †	p-value
Mean Age (SD‡), year	43.5 (1.6)	45.8 (1.6)	0.48
Male/Female No. (% male)	31/21 (59.6)	26/24 (52)	0.43
Vantrappen Classification§ Excellent Good Fair Poor	n (%) 15 (28.8) 15 (28.8) 12 (23.1) 10 (19.2)		
Achalasia subtype¶ Type 1 Type 2 Type 3	n (%) 9 (17.3) 42 (80.8) 1 (1.9)		
Mean Duration (months) of symptoms (SD)	32.34 (2.06)		
Baseline symptoms Dysphagia Chest pain Regurgitation	n (%) 43 (82.7) 7 (13.5) 2 (3.8)		
† Unless otherwise indicated data are expressed as number (percentage) of patients. Percentages have been rounded and might not total 100.			
‡ SD: Standard Deviation			
§ Vantrappen Classification: Excellent, indicates no symptoms; Good, symptoms occurring less than once a week; Fair, symptoms occurring more than once weekly; and Poor, persistent symptoms [20]			
¶Achalasia subtype: Type 1 (classic) with minimal contractility in the esophageal body, type 2 with intermittent periods of panesophageal pressurization, and type 3(spastic) with premature or spastic distal esophageal contractions[21]			

Table 2 Next-generation sequencing read counts and Mapping Result for individual samples

Post processing grouping	Clinical outcome after dilatation		Sample ID	Total Reads	Mapped Reads	Mapped (%)
Treat 1	Good response to treatment	Excellent	Sample_1	30378288	15879232	0.523
		Good	Sample_2	26809904	13712314	0.511
Treat 2	Poor response to treatment	Fair	Sample_3	29249887	14814455	0.506
		Poor	Sample_4	30445887	16872768	0.554
Treat 3	Without treatment / Control	Control 1	Sample_5	24835372	10948105	0.441
		Control 2	Sample_6	29473046	13101058	0.445

Our findings showed that 254 miRNAs were detected as differentially expressed and only the expression level of 15 miRNAs was significantly altered (11 down-regulated and 4 up-regulated) in the achalasia tissues altogether (Treat 1 or Treat 2) in compared to controls (Table 3). The miRNA expression profiling analysis indicated that most of the dysregulated miRNAs were down-regulated in the achalasia tissues. Among these miRNAs, three; miR-133a-5p (Adjusted p-value for Treat1 < 0.001 & Adjusted p-value for Treat2: 0.005), miR-143-3p (Adjusted p-value for Treat1: 0.001 & Adjusted p-value for Treat2: 0.011) and miR-6507-5p (Adjusted p-value forTreat1: 0.001 & Adjusted p-value for Treat2: 0.016) were up-regulated. Also we found six miRNAs; miR-215-5p, miR-216a-5p, miR-216b-5p, miR-217 and miR-7641 with Adjusted p-value < 0.001 and miR-194-5p (Adjusted p-value for Treat1: 0.01 & Adjusted p-value for Treat 2: 0.005) were down-regulated significantly in both Treat 1 and 2 (Fig. 1). our results showed no significant differential expression miRNAs was observed between two groups of achalasia (Treat 1 and 2) regarding the therapy outcome, however the good responding group (Treat 1) showed significant down-regulation of four miRNAs: hsa-miR-135a-5p, hsa-miR-4488, hsa-miR-122-5p, hsa-miR-4449 and significant up-regulation of hsa-miR-3609 compare to non-achalasia groups. On the other hand, the significant down-regulation of the hsa-miR-383-5p (Adjusted p-value = 0.001) was seen in poor responding achalasia group (Treat 2) in comparison to non-achalasia groups (Table 3). These findings highlight the potential prognostic value of the targeted miRNAs for therapy outcome prediction.

Table 3 Fifteen significant up-regulated and down-regulated miRNAs in achalasia tissues (Treat 1 & Treat 2) versus control tissue

MicroRNA	Treat 1				Treat 2			
	FC†	log2FC†	p-value	Adjusted p-value	FC†	log2 FC†	P value	A p-value*
hsa-miR-217	↓0.020	-5.644	3.98E-10	2.46E-07	↓ 0.31	-1.69	9.68E-09	1.5E-06
hsa-miR-216a-5p	↓ 0.062	-4.011	1.05E-09	2.65E-07	↓ 0.047	-4.411	1.02E-10	3.14E-08
hsa-miR-7641	↓ 0.155	-2.689	1.28E-09	2.65E-07	↓ 0.160	-2.644	2.28E-09	4.71E-07
hsa-miR-216b-5p	↓ 0.08	-3.644	2.06E-09	3.18E-07	↓ 0.04	-4.644	7.52E-13	4.65E-10
hsa-miR-215-5p	↓ 0.240	-2.059	8.98E-07	0.000111	↓ 0.193	-2.373	2.39E-08	2.95E-06
hsa-miR-135a-5p	↓ 0.173	-2.531	2.14E-06	0.00022	-	-	-	-
hsa-miR-194-5p	↓ 0.368	-1.442	0.000174	0.010725	↓ 0.353	-1.502	7.62E-05	0.005888
hsa-miR-4488	↓ 0.323	-1.630	0.000571	0.029432	-	-	-	-
hsa-miR-122-5p	↓ 0.231	-2.114	0.000723	0.03438	-	-	-	-
hsa-miR-4449	↓ 0.302	-1.727	0.000835	0.036864	-	-	-	-
hsa-miR-133a-5p	↑ 35	5.129	2.89E-06	0.000255	↑ 19	4.248	7.62E-05	0.005888
hsa-miR-143-3p	↑ 6.702	2.744	1.74E-05	0.001345	↑ 5.173	2.371	0.000166	0.011374
hsa-miR-6507-5p	↑ 44	5.459	2.36E-05	0.00162	↑24	4.585	0.000261	0.016122
hsa-miR-3609	↑ 4.6	2.202	0.00038	0.021343	-	-	-	-
hsa-miR-383-5p	-	-	-	-	↓ 0.133	-2.910	1.28E-05	0.001317

†FC, Fold change; *A p-value§, Adjusted p-value

Functional annotation of the candidate miRNAs

The biological process of GO and KEGG pathways of the fifteen candidate miRNAs were analyzed and the list of the most significantly enriched terms are summarized in Table 4. Interestingly, GO analysis showed that the differentially expressed genes were involved in the neuron apoptotic process (Adjusted p-value = 0.004); neuron death (Adjusted p-value = 0.006) and immune response regulation (Adjusted p-value = 0.008) were targeted by hsa-miR-143-3p. We also found that genes involved in the cellular response to oxidative stress (Adjusted p-value = 0.011), cell aging (Adjusted p-value = 0.011), axon regeneration and development (Adjusted p-value = 0.031) and myelination (Adjusted p-value = 0.031) were significantly enriched by hsa-miR-217. Moreover, KEGG analysis showed that genes involved in Glioma (Adjusted p-value = 0.0001) and Sphingolipid signaling pathway (Adjusted p-value = 0.0006) were the most highly represented pathways enriched by hsa-miR-143-3p. Also genes involved in cancers including non-small cell lung cancer(Adjusted p-value = 0.001 for hsa-miR-143-3p, Adjusted p-value = 0.004 for hsa-miR-217), prostate (Adjusted p-value = 0.004 for hsa-miR-217), colorectal (Adjusted p-value = 0.001 for hsa-miR-143-3p), bladder (Adjusted p-value = 0.001 for hsa-miR-143-3p) and endometrial cancers (Adjusted p-value = 0.004 for hsa-miR-217), were significantly enriched by the predicted target genes (Table 4).

Table 4

The most significant enriched terms (potential function and pathway of target genes) based on biological process GO† enrichment (white rows) and KEGG‡ p
associated with Achalasia

miRNA	enriched Term	Target genes
hsa-miR-217	Non-small cell lung cancer- Homo sapiens- hsa05223	E2F3;KRAS;FOX3;FHIT
	Endometrial cancer- Homo sapiens- hsa05213	TCF7L2;PTEN;KRAS;FOXO3
	Negative regulation of cell aging (GO:0090344)	PTEN;SIRT1;MARCH5
	Cellular response to oxidative stress (GO:0034599)	NR4A2;TP53INP1;FOXO3;SIRT1;HIF1A;EZH2
	Prostate cancer- Homo sapiens-hsa05215	TCF7L2;PTEN;E2F3;KRAS
	Regulation of myelination (GO:0031641)	TCF7L2;PTEN;TNFRSF21
hsa-miR-216b-5p	Melanoma- Homo sapiens- hsa05218	CDK6;CDK4;MAPK1;KRAS;FGF10
	Pathways in cancer-Homo sapiens-hsa05200	CDK6;FZD5;TPM3;CDK4;COL4A4;FZD9;TCEB2;MAPK1;KRAS;FGF10
	Signaling pathways regulating pluripotency of stem cells-Homo sapiens_hsa04550	SMAD1;FZD5;FZD9;MAPK1;LHX5;KRAS
	Non-small cell lung cancer-Homo sapiens-hsa05223	CDK6;CDK4;MAPK1;KRAS
hsa-miR-215-5p	Cell cycle-Homo sapiens-hsa04110	RB1;CDKN2D;CDKN2A;BUB1B;CDC7;TTK;CDC14A;ANAPC10;CDC20;ORC4;ORC1;CCNE1;RAD21;M
hsa-miR-143-3p	Glioma-Homo sapiens- hsa05214	PDGFRA;MDM2;AKT1;MAPK1;BRAF;CALM3;KRAS;HRAS;IGF1R
	Sphingolipid signaling pathway- Homo sapiens- hsa04071	CERS4;SGPL1;SPTLC2;PPP2R5E;BCL2;AKT1;MAPK1;KRAS;TNF;HRAS
	MicroRNAs in cancer- Homo sapiens-has 05206	TRIM71;PDGFRA;DNMT3A;PTGS2;MAPK7;ERBB3;FSCN1;MDM2;BCL2;MAPK1;KRAS;HRAS;CD44
	Non-small cell lung cancer-Homo sapiens-hsa05223	AKT1;MAPK1;BRAF;KRAS;FHIT;HRAS
	Colorectal cancer-Homo sapiens-hsa05210	SMAD3;BCL2;AKT1;MAPK1;BRAF;KRAS
	Bladder cancer-Homo sapiens-hsa05219	MDM2;MAPK1;BRAF;KRAS;HRAS
	Regulation of neuron death (GO:1901214)	ERBB3;UBE2V2;BCL2;AKT1;XIAP;KRAS;BRAF;HRAS;TNF;BB
	Regulation of neuron apoptotic process (GO:0043523)	ERBB3;UBE2V2;BCL2;XIAP;KRAS;BRAF;HRAS;TNF;BBC3
	Negative regulation of neuron death (GO:1901215)	ERBB3;UBE2V2;BCL2;AKT1;XIAP;KRAS;BRAF;HRAS
	Immune response regulating cell surface receptor signaling pathway GO:0002768)	PDGFRA;NCKAP1;PLEKHA1;YWHAB;LIMK1;ERBB
hsa-miR-6507-5p	cytokinesis_(GO:0000910)	RACGAP1;PRC1;NEK7;MYH9;CEP55;RHOB
hsa-miR-135a-5p	Jak STAT signaling pathway-Homo sapiens-hsa04630	PIAS4;MYC;MPL;BCL2;STAT6;JAK2
	Signaling pathways regulating pluripotency of stem cells-Homo sapiens-hsa04550	BMPR2;APC;MYC;JAK2;SMAD5;SKIL
	Colorectal cancer-Homo sapiens-hsa05210	APC;MYC;BCL2;BIRC5
	MicroRNAs in cancer-Homo sapiens-hsa05206	MARCKS;BMPR2;APC;ROCK1;MYC;BCL2;IRS2
	TGF beta signaling pathway-Homo sapiens-hsa04350	BMPR2;ROCK1;MYC;SMAD5
	Cellular response to BMP stimulus (GO:0071773)	HEYL;GATA6;SMAD5

† GO, Gene Ontology; ‡KEGG, Kyoto Encyclopedia of Genes and Genomes; *A p-value, Adjusted p-value

miRNA	enriched Term	Target genes
	Response to BMP (GO:0071772)	HEYL;GATA6;SMAD5
	neuron_projection_regeneration_(GO:0031102)	BCL2;APOA1;JAK2
	Axon development (GO:0061564)	BCL2;APOA1;JAK2
	Axon regeneration (GO:0031103)	BCL2;APOA1;JAK2
	Positive regulation of ntrinsic apoptotic signaling pathway (GO:2001244	PIAS4;SIAH1;BCL2;SKIL
hsa-miR-3609	Pathways in cancer-Homo sapiens-hsa05200	ITGB1;EGLN3;PRKCB;F2R;FZD9;XIAP;HIF1A;IGF1R;TGFBR2;BMP2;CCND1;MDM2;MAPK1;CRK;AP
	Proteoglycans in cancer Homo sapiens hsa05205	ITGB1;CCND1;PRKCB;CAV1;FZD9;MDM2;RRAS2;MAPK1;HIF1A;THBS1;IGF1R
	Endocytosis-Homo sapiens hsa04144	SH3GLB1;HSPA8;RAB5B;RAB4A;ZFYVE9;SH3KBP1;CAV1;F2R;EPS15L1;IGF1R;TGFBR2;RAB11FIF
	Focal adhesion- Homo sapiens-hsa04510	RAP1B;ITGB1;CCND1;PRKCB;CAV1;XIAP;PAK6;MAPK1;CRK;THBS1;IGF1R
hsa-miR-194-5p	Adherens junction- Homo sapiens-hsa04520	TJP1;EP300;RAC1;IGF1R
	Proteoglycans in cancer-Homo sapiens-hsa05205	CAV1;FZD6;RAC1;HBEGF;IGF1R
	Focal adhesion- Homo sapiens-hsa04510	CAV1;TLN2;RAC1;IGF1R;ITGA9
	HIF-1 signaling pathway-Homo sapiens-hsa04066	CDKN1B;EP300;RBX1;IGF1R

† GO, Gene Ontology; ‡KEGG, Kyoto Encyclopedia of Genes and Genomes; *A p-value, Adjusted p-value

Novel predicted miRNAs in esophageal tissue

Interestingly, during the data analysis, we found novel potential miRNA transcripts in esophageal tissue which were expressed in at least two different pooled samples with mean read counts per group greater than five. All the rRNA and tRNA were excluded by Rfam software (http://www.sanger.ac.uk/Software/Rfam/) and the identified novel miRNAs possessed the criteria of secondary structure in RNA fold change. Following this approach, we identified 36 novel candidate miRNAs with mammalian homologues (Table S1), but no one was significantly changed in achalasia. GO analysis showed that, 8 novel microRNAs are significantly related to the neurotransmitter regulation process (Adjusted p-value = 0.03); axon development and regeneration (Adjusted p-value = 0.02), cellular response nerve growth factor (Adjusted p-value = 0.03) and inflammation process (Table 5).

Table 5

The most significant enriched terms (potential function and pathway of target genes) based on biological process GO† enrichment of the novel candidate miRNAs in esophageal tissue

miRNA	enriched Term	Target genes	A p-value*
2:46348793..46348872	positive_regulation_of_neurotransmitter_transport_(GO:0051590)	DTNBP1	0.024
	positive_regulation_of_neurotransmitter_secretion_(GO:0001956)		0.024
	anterograde_axon_cargo_transport_(GO:0008089)		0.024
	axon_cargo_transport_(GO:0008088)		0.03
	regulation_of_neurotransmitter_secretion_(GO:0046928)		0.03
	regulation_of_neurotransmitter_transport_(GO:0051588)		0.03
3:186787298..186787358	neuroepithelial_cell_differentiation_(GO:0060563)	MITF	0.046
6:104646203..104646269	cellular_response_to_interleukin-6_(GO:0071354)	GALT	0.039
	interleukin-6-mediated_signaling_pathway_(GO:0070102)		0.027
	response_to_interleukin-6_(GO:0070741)		0.04
7:53776229..53776317	positive_regulation_of_interleukin-8_biosynthetic_process_(GO:0045416)	PRG3	0.031
	regulation_of_interleukin-8_production_(GO:0032677)		0.031
15:60128283..60128360	neuron_projection_regeneration_(GO:0031102)	NEFL	0.02
	axon_development_(GO:0061564)		0.02
	axon_regeneration_(GO:0031103)		0.02
	anterograde_axon_cargo_transport_(GO:0008089)		0.02
	neurofilament_cytoskeleton_organization_(GO:0060052)		0.02
	axon_cargo_transport_(GO:0008088)		0.02
	response_to_axon_injury_(GO:0048678)		0.02
	positive_regulation_of_axonogenesis_(GO:0050772)		0.024
	negative_regulation_of_neuron_apoptotic_process_(GO:0043524)		0.036
	regulation_of_axonogenesis_(GO:0050770)		0.036
	negative_regulation_of_neuron_death_(GO:1901215)		0.037
	regulation_of_neuron_apoptotic_process_(GO:0043523)		0.04
	positive_regulation_of_neuron_differentiation_(GO:0045666)		0.044
	regulation_of_neuron_death_(GO:1901214)		0.044
	regulation_of_neuron_projection_development_(GO:0010975)		0.046
	positive_regulation_of_neurogenesis_(GO:0050769)		0.047
20:38425194..38425268	cellular_response_to_nerve_growth_factor_stimulus_(GO:1990090)	RAP1A	0.032
	response_to_nerve_growth_factor_(GO:1990089)	RAP1A	0.032
	positive_regulation_of_calcium_ion_transmembrane_transporter_activity_(GO:1901021)	ANK2	0.032
	negative_regulation_of_neurotransmitter_transport_(GO:0051589)	RAP1A	0.032
	nerve_growth_factor_signaling_pathway_(GO:0038180)	RAP1A	0.032
	negative_regulation_of_neurotransmitter_secretion_(GO:0046929)	RAP1A	0.032
	regulation_of_neurotransmitter_secretion_(GO:0046928)	RAP1A	0.047
6:77781479..77781527	regulation_of_intrinsic_apoptotic_signaling_pathway_by_p53_class_mediator_(GO:1902253)	RRM2B	0.004
	negative_regulation_of_signal_transduction_by_p53_class_mediator_(GO:1901797)		0.005
	regulation_of_signal_transduction_by_p53_class_mediator_(GO:1901796)		0.006
	regulation_of_intrinsic_apoptotic_signaling_pathway_(GO:2001242)		0.014

† GO, Gene Ontology; *A p-value, Adjusted p-value

miRNA	enriched Term	Target genes	A p-value*
	negative_regulation_of_apoptotic_signaling_pathway_(GO:2001234)		0.018
	regulation_of_apoptotic_signaling_pathway_(GO:2001233)		0.028
12:29562570..29562639	calcium-mediated_signaling_using_intracellular_calcium_source_(GO:0035584)	HOMER2	0.039
	regulation_of_interleukin-8_biosynthetic_process_(GO:0045414)	PRG3	0.039
	mast_cell_activation_involved_in_immune_response_(GO:0002279)	PLA2G3	0.039
	positive_regulation_of_interleukin-8_biosynthetic_process_(GO:0045416)	PRG3	0.039
	axoneme_assembly_(GO:0035082)	PLA2G3	0.042

† GO, Gene Ontology; *A p-value, Adjusted p-value

Validation of NGS results by RT-qPCR analysis

Two candidate miRNAs, which had the most expression changes in the achalasia tissues, were selected from the NGS data for RT-qPCR validation. These two miRNAs were hsa-miR-217 as one of the down-regulated and hsa-miR-143-3p as one of the up-regulated miRNAs. The analysis showed that the NGS results of hsa-miR-217 could be confirmed significantly with lower miRNA levels in achalasia tissues than in controls (p-value = 0.004) and hsa-miR-143-3p was up-regulated in achalasia tissues but it was not significant (p-value = 0.457) (Fig. 2).

Discussion

To our knowledge, this is the first study that miRNA expression in achalasia was compared to non-achalasia esophageal tissue by using the NGS approach. Our NGS data showed that 15 miRNAs had significant differential expression in the esophageal tissue of achalasia patients compared with controls. By stem-loop qPCR, we were able to validate that miR-217 was down-regulated and miR-143 was up-regulated in achalasia tissue similar to those observed in the NGS result. By using the NGS approach, we derived the complete pattern of miRNAs regulating in achalasia. In a recent study using the microarray method, researchers showed that only 2 miRNAs (miR3615p and miR130a) were increased in achalasia patients, while we did not find this increase. This difference may be because Shoji et al used microarray method for miRNA expression analysis which could appear different results from NGS sequencing. Indeed, they used middle esophageal mucosa for sampling which could potentially have different gene expression from LES [22].

Functional annotation revealed that many miRNAs that were determined in our study are involved in neuronal cell apoptosis (hsa-miR-143-3p), myelination process (hsa-miR-217) and neuronal regeneration (hsa-miR-135a-5p). In accordance with our findings, Qualman et al showed that the mechanism of esophageal dysfunction in response to neuronal destruction in Parkinson patients with dysphagia and achalasia is likely to be similar [23]. Moreover, we found that immune system regulation can be targeted by hsa-miR-143-3p which has been shown dysregulated in our patients. Although the etiology of primary esophageal achalasia remains unknown; several hypotheses suggest that factors inflammation and autoimmunity are associated with its pathogenesis [24]. The histopathology analysis of the achalasia esophagus tissues indicated the lymphocytic infiltration, myenteric inflammation, and aganglionosis during achalasia [25]. The cytotoxic autoimmune responses to the neuronal cells potentially can trigger progressive myenteric plexus neuronal apoptosis in achalasia patients [26]. Evidence suggests that miRNAs play an important role in the development of neurodegenerative diseases [12].

Some of the miRNAs that differentially expressed in our study have been reported in association with cancers. For example, miR-217 that is down-regulated in both poor and good therapy responding achalasia groups, act as tumor suppressor factor and down-regulated in several cancers such as gastric cancer[27], pancreatic ductal adenocarcinoma[28], esophageal squamous cell carcinoma (ESCC) [29] and colorectal cancer [30]. Moreover, our findings showed less expression of hsa-miR-216 in achalasia. Reduced expression of miR-216 has also been shown in other diseases, such as non-small cell lung cancer [31], ESCC [32], nasopharyngeal carcinoma [33] and hepatocellular carcinoma [34]. Down-regulation of miR-217 and miR-216 that act as tumor suppressors, may also explain the high risk of esophageal cancer in achalasia patients. Despite the pathological differences between neurodegenerative diseases (such as achalasia) and cancers, new evidence suggests that they have similar regulatory mechanisms [35]. Therefore, it seems that further studies on common miRNAs could develop diagnostic and therapeutic plans.

Our data indicate the up-regulation of hsa-miR-143-3p in achalasia tissues. The up-regulation of miR-143 has been reproduced in $CD4^+$ T cells and highlights the importance of this miRNA in autoimmune disease [36]. It emerged that miRNAs have an essential role in the regulation of the immune system. Deregulation of certain individual miRNAs compromises immune development and response and can lead to immune disorders like autoimmunity and cancer [37]. This finding enhances the role of autoimmune in the formation of achalasia.

Based on the biological process GO and KEGG assessment in our study, it is demonstrated that PTEN and SIRT1 could be significant targets of miR-217 in achalasia (Table 4). Studies showed that PTEN tumor suppressor has a direct role in the neurodegeneration under oxidative stress conditions [38]. Also, SIRT1 levels are associated with neurodegenerative diseases, which have a progressive and severe reduction in neuronal cells [39]. These findings could be in line with the neurodegenerative role in the development of achalasia disease.

Interestingly, our findings indicated some of the other genetic factors of the candidate miRNAs which reported in other studies of achalasia. For instance, miR-122-5p that can target the HLA genes [40], hsa-miR-143-3p which can regulate IL-10 immune modulator [41] and hsa-miR-216a-5 with targeting IL-23R [42]. Accordingly, these findings highlight the role of immunity and inflammation in achalasia progression (Table S2).

Based on our study, miR-383-5p was down-regulated in achalasia patients with poor response to treatment. This miRNA might play a potential prognostic role for prediction of the response to the treatment in achalasia however more confirmation is necessary. In other studies, hsa-miR-383 is known as a tumor suppressor with a decreased level in glioma, medulloblastoma and testicular embryonal carcinoma cells [43]. Our results demonstrated that dysregulated miR-216b could target TPM, the gene which encodes beta-tropomyosin and plays an important role in regulating the calcium-dependent muscle contraction. TPM expression changes have been shown previously in the study on achalasia tissue [41]. These findings may emphasize the neuromuscular process in the pathogenesis and development of achalasia (Table 4).

Our results suggest that has-miR-135 is down-regulated only in good treatment response achalasia patients. Some studies showed that the induction of miR-135a expression in different types of cancers could suppress cell proliferation through target genes (c-MYC, STAT6, SMAD5, and BMPR2) and also miR-135a has been introduced as a potential predictor of treatment in some cancers [44]. We could show that these target genes are significant targets of has-miR-135 in achalasia. (Table 4).

We found CAV1 which involving the calcium signaling pathway could be significantly a target of hsa-miR-3609 and hsa-miR-194-5p that were differentially expressed in achalasia tissue. Our finding is in line with a study that the CAV1target gene was differentially expressed in achalasia tissue and has a possible function related to achalasia pathogenesis [41]. As we know, calcium channel blockers could support LES relaxation and/or esophageal peristalsis in achalasia patients[45].

Conclusion

In conclusion, this study shows a comprehensive analysis of miRNA expression in achalasia and may be used as a basis for future studies to explore the role of candidate miRNAs in the etiology of achalasia. We found significant down-regulation of hsa-miR-217 in LES of achalasia patients with significant enrichment in myelination process ontology. Furthermore, the NGS miRNA expression profiling might be a suitable platform to classify achalasia into responsible for dilatation treatment.

Abbreviations

LES: Lower esophageal sphincter; miRNA: microRNAs; NGS: Next generation sequencing; KEGG: Kyoto Encyclopedia of Genes and Genomes; cDNA: DNA complementary to RNA; qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction; rRNA, ribosomal RNA; tRNA, transfer RNA; ΔΔct, double delta cycle threshold; GO: Gene Ontology; PTEN: phosphatase and tensin homolog; TPM: tropomyosin; IL: interleukin

Declarations

Ethics approval and consent to participate

Declaration of Helsinki in 1995 (as revised in Edinburgh 2000) was considered throughout the designing of the study protocol and its implementation. This study was approved in the ethical committee of Golestan University of medical sciences (code= 31078693122415). Participation in this study was optional, and all participants expressed their satisfaction with written informed consent and participants' anonymity was preserved. Participants' test results were confidential and only available to the physician and the moderator of the project.

Consent for publication

The analysis and aggregation of information did not contain any person’s data. So, Consent for publication “Not applicable” in this section.

Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no Competing interests.

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Author contributions

A.M., J.M, SJ.M and M.K contributed to the study conception and design. Material preparation, data collection and analysis were performed by M.G, MR.B, M.SJ, N.J, N.F and N.B. The first draft of the manuscript was written by M.G and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

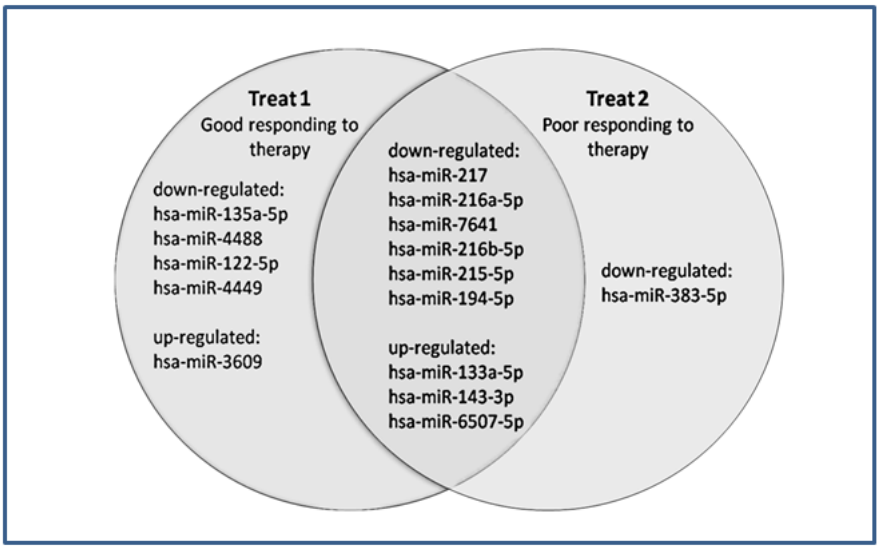

Figure 1

Candidate tissue miRNAs significantly associated with achalasia patients in comparison with controls (non-achalasia patients). Nine candidate miRNAs are common in two Treat. Treat1: achalasia samples responding to dilatation therapy. Treat 2: achalasia samples poor responding to dilation therapy.

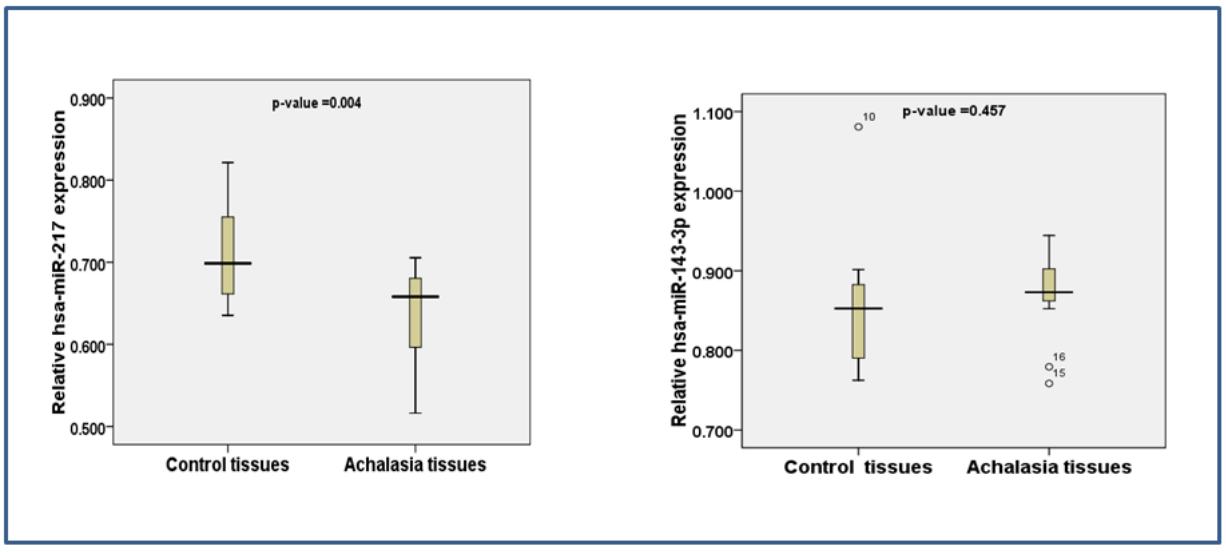

Figure 2

Relative expression of hsa-miR-217 and hsa-miR-143-3p in esophageal tissues of achalasia patients compared with controls (non-achalasia patients) by real-time PCR validation. Relative expression was calculated using 2-dct formula. Significant differences (p-value) have been shown in each graph.

Supplementary Files

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