Received: 17 July 2019 | Revised: 7 October 2019 | Accepted: 28 October 2019

DOI: 10.1111/nmo.13764

ORIGINAL ARTICLE

Neurogastroenterology & Motility NGM WILEY

microRNA-mRNA network model in patients with achalasia

Orazio Palmieri[1] | Tommaso Mazza[2] | Gabrio Bassotti[3] | Antonio Merla[1] | Salvatore Tolone[4] | Tommaso Biagini[2] | Antonello Cuttitta[5] | Fabrizio Bossa[1] | Giuseppina Martino[1] | Tiziana Latiano[1] | Giuseppe Corritore[1] | Domenica Gioffreda[1] | Orazio Palumbo[6] | Massimo Carella[6] | Anna Panza[1] | Angelo Andriulli[1] | Anna Latiano[1]

[1]Gastroenterology Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy

[2]Bioinformatics Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy

[3]Department of Medicine, Gastroenterology and Hepatology Section, Santa Maria della Misericordia Hospital, Perugia, Italy

[4]Department of Surgery, University of Campania 'Luigi Vanvitelli', Napoli, Italy

[5]Unit of Thoracic Surgery, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy

[6]Medical Genetics Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy

Correspondence
Anna Latiano, Gastroenterology Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, Viale Cappuccini 1, 71013 San Giovanni Rotondo, Foggia, Italy.
Email: a.latiano@operapadrepio.it

Funding information
This work was supported by the Italian Ministry of Health.

Abstract

Background: Achalasia is a rare idiopathic disease with a complex etio-pathogenesis still unknown. This study aimed to identify microRNA (miRNA)-mRNA regulatory networks underlying achalasia.

Methods: The investigation was performed in tissue specimens from 11 patients and five controls using the microarray technology followed by an integrated bioinformatics analysis.

Key Results: One hundred and six miRNAs were significantly up-regulated and 64 were down-regulated in achalasia patients. The expression of the most 10 differential expressed miRNAs (miR-122-5p, miR-133a-3p, miR-504-5p, miR-187-3p, miR-133b, miR-200c-3p, miR-375, miR-200b-5p, miR-200b-3p, and miR203a) was confirmed by droplet digital PCR in an independent cohort. The interactions between the significant miRNAs and their targets uncovered 14 miRNA-mRNA interacting pairs with experimentally predicted genes (ie, *FN1*, *ROCK2*, *DPYSL2*), and 35 pairs with not experimentally target genes (ie, *SULF1*, *MRVI1*, *PRKG1*); all genes were involved in *immune cell trafficking*, *skeletal and muscular system development*, *nervous system development* macro-processes.

Conclusion & Inferences: The mRNA–miRNA regulatory networks described in this study provide new insights in the genetic background of the disease, suggesting further investigations in novel pathogenic mechanisms.

KEYWORDS

achalasia, expression profile, microRNA, mRNA

1 | INTRODUCTION

Idiopathic achalasia is an esophageal disorder of unknown etiology, characterized by a degeneration of inhibitory neurons releasing nitric oxide (NO) in the myenteric plexus leading to the failure of relaxation of the lower esophageal sphincter (LES) and absent peristalsis in the lower esophageal body. The underlying etiology is generally thought to be secondary to an autoimmune response triggered by a viral infection in genetically susceptible subjects.[1] Although cure is not possible, symptom palliation can be achieved in many patients with current treatment options. Traditionally, the treatments include

Orazio Palmieri and Tommaso Mazza are contributed equally to this manuscript.

pharmacological therapy (eg, calcium channel blockers, long-acting nitrates), endoscopic interventions (endoscopic botulinum toxin injection to pneumatic dilation of the LES), and surgical interventions (surgical or peroral endoscopic myotomy, and esophagectomy).

Recently, we reported a characterized expression profile of the whole genome in patients with achalasia, pointing out genes, and pathways participating in *neuronal/muscular* processes as well as in *neuronal/immunity organismal responses*.[2] Gene expression is regulated in part by short non-coding RNAs (18-24 nucleotide), termed microRNAs (miRNAs), that cause the degradation or translational repression by binding to the 3′-untranslated regions (3′-UTR) of their target messenger RNA (mRNAs). Each miRNA has a complementary binding site on numerous mRNAs, and each gene can be regulated by many miRNAs. Emerging evidence has demonstrated that miRNAs are associated with tumorigenic processes, including cell proliferation, apoptosis, angiogenesis, and invasion via their interaction with oncogenes and anti-oncogenes.[3] The field of miRNA research is expanding rapidly with the number of miRNA-related citations increasing almost exponentially: miRNAs have been implicated in neurological, cardiovascular and autoimmune diseases, and in cancers.[4] Furthermore, in recent years, the role of miRNAs as potential biomarkers for the early detection as well as for therapeutic targets of treatments has been investigated and developed. Several reports point to miRNAs to be involved in Barrett's carcinogenesis[5,6] and in the development of the esophageal squamous cell carcinoma.[7] Recently, Shoji and colleagues performed a miRNA expression profile on esophageal mucosal biopsies from 8 patients with achalasia and 4 healthy volunteers and showed the miRNA-130a highly expressed in patients.[8]

In this study, we used miRNA microarrays to identify miRNAs with altered expression in esophageal mucosa of achalasic patients. Moreover, we intended to correlate the mRNAs previously identified to be differentially expressed (DE) in achalasic patients and controls[2] with the miRNA profiles apparent from the present investigation.

Key Points

- Genetic factors may play an important role in the development and progression of achalasia. There is little information regarding miRNA expression in achalasia.
- Differential expressed miRNAs were identified in muscle specimens of patients with achalasia compared with non-achalasic subjects. The integrative analysis of miRNA-mRNA expression data uncovered pathways possibly regulated by the identified RNAs.
- Our results show that miRNAs could potentially provide new clues to develop targeted therapies in achalasia.

2 | MATERIALS AND METHODS

2.1 | Human tissues

A total of 11 LES muscle specimens from patients with newly diagnosed achalasia were collected at the time of surgical myotomy at the IRCCS Hospital "Casa Sollievo della Sofferenza," San Giovanni Rotondo; esophageal tissue specimens were also obtained from patients with gastric cancer at the time of proximal gastrectomy and served as controls. Both patients and controls were the same of those investigated in our previous study.[2] All participants gave written informed consent, and the study was approved by the Ethics Committee of the Hospital (139/CE-28/10/2014). All methods performed in the study were in accordance with the ethical standards of the institutional and/or national research and with the Guidelines for Good Clinical Practices and the Declaration of Helsinki.

2.2 | RNA isolation for microarray analysis

The detailed methodologies have been presented elsewhere.[2] Briefly, after isolation the tissues were quickly frozen in liquid nitrogen and stored at −80°C. Total RNA for microarrays was extracted using TRIzol (Invitrogen) and purified with the RNeasy Mini Kit (QIAGEN), in accordance with the manufacturer's protocol.

2.3 | mRNA microarray

cDNA, synthesis, labeling, and hybridization were carried out according to manufacturer's instructions (Affymetrix). Gene expression profiling was performed using GeneChip Human Gene 2.0 ST Arrays as described above.[2]

2.4 | Validation of associated target genes using real-time PCR

A total of 14 genes (*CAV1*, *CHRM2*, *DMPK*, *IL18*, *MRVI1*, *MYH11*, *MYOCD*, *NCAM1*, *PRKG1*, *PRNP*, *SLC2A4*, *SRF*, *SULF1*, and *TLR4*) were selected for validation on the basis of the association levels and analyzed in 16 tissues of achalasia patients and 10 controls by a real-time polymerase chain reaction. The reactions were performed by using TaqMan Gene Expression assay—ABI7900HT (Applied Biosystems), according the manufacturer's instructions. Samples were analyzed with ABI 2.4 software, and the relative gene expression was normalized to the reference GAPDH. For the statistical analysis was used the *t* test. All the analyzed genes were statistically associated with the disease ($P \leq .03$) (Figure S1).

2.5 | miRNA microarray

mirRNA profiling was performed at the laboratory of research of the Medical Genetics Unit in San Giovanni Rotondo, Italy. 500 ng of total of RNA was labeled using the FlashTag™ Biotin HSR RNA Labeling

Kit (Affymetrix) according to the manufacturer's recommendations. First, poly(A) tailing was carried out at 37°C for 15 minutes in a volume of 15 mL reaction mix containing 1X Reaction Buffer, 1.5 mL MgCl2 [25 mM], 1 mL ATP mix diluted 1:500, and 1 mL PAP enzyme. Second, FlashTag Ligation was performed at room temperature for 30 minutes by adding 4 mL of 5X FlashTag Ligation Mix Biotin and 2 mL of T4 DNA Ligase into the 15 mL of reaction mix. To stop the reaction, 2.5 mL of Stop Solution was added. Samples were hybridized overnight to the Affymetrix GeneChip miRNA 4.0 array and then washed and stained using standard Affymetrix protocols.

2.6 | Absolute quantification by droplet digital PCR

The droplet digital PCR (ddPCR) assays were performed using the QX200 ddPCR system (Bio-Rad Laboratories). RNA was reverse transcribed to cDNA using TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied BioSystems; cat. No. 4427975), according to the manufacturer's instructions. An aliquot of the synthesized cDNA (5 μL) was added to 10 μL of digital PCR™ supermix (Bio-Rad Laboratories), 1 μL of TaqMan primer/probe mix (Applied BioSystems), and 4 μL of RNase-free water. Each ddPCR assay mixture (20 μL) with 70 μL of QX100 Droplet Generation oil was loaded into a plastic cartridge (Bio-Rad Laboratories). The cartridge was subsequently placed inside the QX200 droplet generator (Bio-Rad Laboratories). The droplets were then transferred to a 96-well plate (Eppendorf), and the PCR amplification was carried out on the C1000 Touch Thermal Cycler (Bio-Rad Laboratories). Thermal cycling conditions were as follows: 95°C for 10 minutes, then 40 cycles of 94°C for 30 seconds and 60°C for 1 minutes, followed by two final steps at 98°C for 10 minutes, and a 4°C indefinite hold. The PCR products were quantified using QX200 droplet reader (Bio-Rad Laboratories) and automatically analyzed by its associated QuantaSoft software. The fraction of PCR-positive droplets was quantified assuming a Poisson distribution. A negative control containing water instead of cDNA was included to ensure no contamination in all reagents.

2.7 | Bioinformatics analysis of m- and miRNA microarrays data

Differential Expression Analysis was performed by the ANOVA test implemented in Partek® Genomics Suite® software v6.6 (2016 Partek Inc). MiRNAs were considered differentially expressed (DE) between contrasts if their differences in expression were significantly (*P*-value <.05) equal or above 2-fold (absolute values). The potential effect sizes were simulated from 1.25 to 3.0 by step 0.25. With the significance threshold (α) set to 0.01 and the power (1−β) to 0.8, a sample size of 16 was guaranteed to capture 100% of genes that changed by 2-fold as statistically significant (Figure S2). Sample distribution among groups was assessed using principal component analysis (Figure S3). The false discovery rate (FDR) was controlled using the Benjamini-Hochberg step-up procedure.

Experimental miRNA/mRNA interactions were retrieved from the "Validated Target Module" of miRWalk 2.0 database (release February 2016).[9] Functional and pathway enrichment analyses were performed on the differentially expressed miRNAs with Ingenuity Pathway Analysis (QIAGEN Inc). Enriched biological functions were matched against those published in Table S1-S9 of Palmieri et al[2] Spearman correlations between miRNA and mRNA expression values were computed through R software (version 3.4.2). The study design and the overall strategy are illustrated in Figure S4.

3 | RESULTS

3.1 | miRNA expression

A GeneChip miRNA 4.0 microarray platform (www.affymetrix.com), designed to interrogate all mature miRNA sequences in miRBase Release 20 (http://microrna.sanger.ac.uk), was used to compare miRNA expression profiles between achalasic and control tissues. The study revealed 170 DE human miRNAs between the two groups (Figure 1A): the comparison resulted in 106 up-regulated and 64 down-regulated miRNAs in achalasic patients. Among all DE miRNAs, we extracted the top 10 up-regulated and the 10 down-regulated miRNAs with the lowest t fold-change value of 8.96 and −17.31, respectively (Figure 1B, Table 1).

3.2 | Validation of potential miRNAs using ddPCR

In order to validate the previous results, we conducted absolute quantification by ddPCR of expression levels of miRNAs in an independent validation set of 34 tissues from new achalasic patients and 10 tissues from patients with gastric cancer (controls). Based on their relative expression patterns and potential relevance to tissue function or disease, five of the 10 selected miRNAs were from the up-regulated miRNAs (miR-122-5p, miR-133a-3p, miR-504-5p, miR-187-3p, and miR-133b) and five from the down-regulated ones (miR-200c-3p, miR-375, miR-200b-5p, miR-200b-3p, and miR203a): data from this validation cohort were in accordance from those with the exploratory cohort of patients (Figure 2).

3.3 | Candidate genes potentially regulated by the DE miRNAs

To search for validated gene targets for the miRNAs, we combined DE miRNAs extracted from the present work and DE mRNAs highlighted in our previous work[2] by targeting information (Figure S4B): a total of 3660 interacting miRNA-mRNA pairs were obtained, of which 1747 exhibited the same expression direction, and 1913 the opposite one (Table S1).

To raise hypotheses on the possible roles of the DE miRNAs, target prediction analysis was initially conducted on miRNAs that

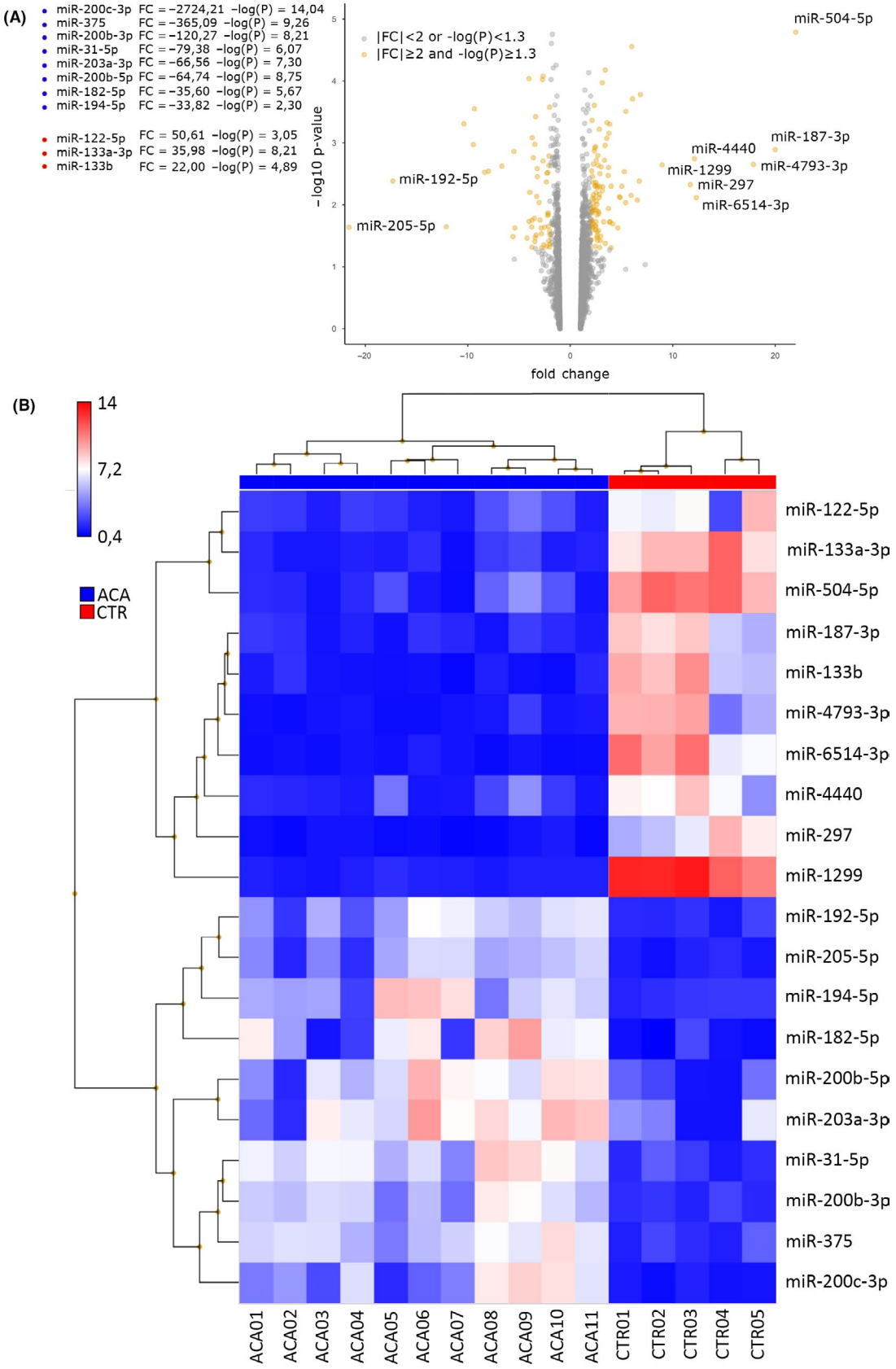

FIGURE 1 (A) Volcano plot representing the 170 differentially expressed miRNAs (orange dots) and the remaining miRNAs (gray dots). Out-of-scale miRNAs were not plotted. (B) Heatmap of the 20 most differentially expressed miRNAs

TABLE 1 Differential expression of top 20 miRNAs in patients with achalasia

miRNA	*P*-value	Fold change
hsa-miR-122-5p	.000887971	50.6074
hsa-miR-133a-3p	6.21483e-09	35.9832
hsa-miR-504-5p	1.27776e-05	22.0024
hsa-miR-187-3p	.00128763	19.997
hsa-miR-133b	1.17288e-06	18.7447
hsa-miR-4793-3p	.00222134	17.8742
hsa-miR-6514-3p	.00764932	12.2897
hsa-miR-4440	.00179836	12.1147
hsa-miR-297	.00471102	11.7204
hsa-mir-1299	.00227017	8.95824
hsa-miR-192-5p	.0041327	−17.3126
hsa-miR-205-5p	.0254648	−21.8534
hsa-miR-194-5p	.00505123	−33.8199
hsa-miR-182-5p	2.12345e-06	−35.6022
hsa-miR-200b-5p	1.77934e-09	−64.7482
hsa-miR-203a	4.99743e-08	−66.5647
hsa-miR-31-5p	8.45781e-07	−79.3838
hsa-miR-200b-3p	6.11702e-09	−120.272
hsa-miR-375	5.46529e-10	−365.096
hsa-miR-200c-3p	9.11004e-15	−2724.21

directly targeted genes known to be involved in the pathogenesis of achalasia, including *ALADIN*, *PTPN22*, *IL10*, *IL23R*, *VIPR1*, *c-kit*, *IL33*, *LTA/TNFα*, *HLA-DQA1*, *HLA-DRB1*, *TLR4*, and *IL18* which were reported as critical upstream regulators in Ref. [2] (Figure S4C): miR-197-3p and miR-346 negatively appeared to regulate the expression of *IL18* target gene, whereas miR-146a-5p, miR-146b-5p, and miR-4738-3p regulated the expression of the *TLR4* in an opposite direction (Table 2).

3.4 | Direct association of miRNAs to relevant functions through known interacting genes

We explored the involvement of DE miRNAs and their validated targets in the *smooth muscle contraction* pathway, *smooth muscle contractility* pathway, *damage to nervous tissue* pathway, *synaptic transmission of cells* pathway, *leukocyte migration* pathway, *recruitment of phagocytes* pathway, *adhesion of immune cells* pathway, *regeneration of nerves* pathway, and *synaptogenesis* pathway [2] (Figure S4D). We selected the DE genes that participate in these functions and shown to experimentally target of the DE miRNAs (Figure S4E). The results pointed out toward 8 miRNAs and 10 target mRNAs, resulting in 14 miRNAs-mRNAs interacting pairs, each representing a specific function (Table 3). Of the selected 8 miRNAs, the miR-148a-3p was found to contain a putative binding site for ROCK1 mRNA, flagging three different pathways: *adhesion of immune cells*, *smooth muscle contraction*, *and leukocyte migration*; the miR-200b-3p and miR-200c-3p were shown to influence both *ROCK2* and *FN1*, genes involved in *adhesion of immune cells* and *smooth muscle contraction* functions. Three miRNAs (miR-143-3p, miR-150-5p, and miR-182-5p) were observed to have a unique binding site for *PTGS2*, *CYT1P*, and *THBS1*. miR-130b-3p resulted to interact with *DPYSL2* and *F3* genes, and miR-155-5p with *FGF2* and *MYLK*. In addition, this analysis evidenced 6 annotated pathways (*adhesion of immune cells*, *contraction of smooth muscle*, *leukocyte migration*, *regeneration of nerves*, *synaptic transmission of cells*, *synaptogenesis*) for the down-expressed miRNAs, and 1 pathway (*contraction of smooth muscle*) for the over-expressed miRNA.

3.5 | Indirect association of mRNA-miRNA, without prior knowledge of the interaction

To finally gain insights into the functional role of the identified miRNA-mRNA pairs, we analyzed mRNAs with experimentally relevant functions identified in Palmieri et al[2], and that are not known (in the literature) to be controlled by DE miRNAs (Figure S4F). By this way, we observed 35 pairs of potential relevance which remains uncovered at present (Table 4). Four enriched pathways shared both up-regulated and down-regulated miRNAs (*smooth muscle contraction*, *smooth muscle contractility*, *synaptic transmission of cells*, *and leukocyte migration*). The *MRVI1* mRNA was found to contain binding sites for 4 miRNAs (miR-143-3p, miR-150-5p, miR-362-5p, and miR-760), as well as for *SULF1* (miR-130b-3p, miR-148a-3p, miR-200b-3p, and miR-200c-3p). Moreover, *PRKG1* and *SYDE1* appeared regulated by miR-200b-3p and miR-200c-3p. In addition, *CHRM2*, *CNN1*, *RAP1A*, *STXBP1*, and *NBEA* genes were identified as potential targets of the 9 DE miRNAs with two genes (*CHRM2* and *NBEA*) being controlled by down-regulated miRNAs, one gene (*CNN1*) by up-regulated miRNAs, and two (*RAP1A*, *STXBP1*) by both. *Adhesion of immune cells* pathway was potentially controlled by the up-regulated miR-193b-3p and miR-125b-5p, found to contain a putative binding site for *CD34* gene. On the other hand, *recruitment of phagocytes* pathway was the only one that was regulated by repressed miR-155-5p and miR-182-5p predicted to have a binding site on *F13A1* gene.

4 | DISCUSSION

Recent, attention has been focused on miRNAs, short single-stranded non-coding RNAs, which can regulate gene expression at the post-transcriptional level, and may interfere with the process of transcription influencing cellular proliferation, migration and invasion, signal transduction, autophagy, and apoptosis.[5]

Although the exploration of miRNAs was shown to be promising as potential biomarkers and screening of multiple cancers and immune disorders, only the study on the Japanese population was performed on achalasia patients so far.[8-10]

The main goal of this study was to identify miRNAs with differential expression in muscle specimens of achalasic compared with

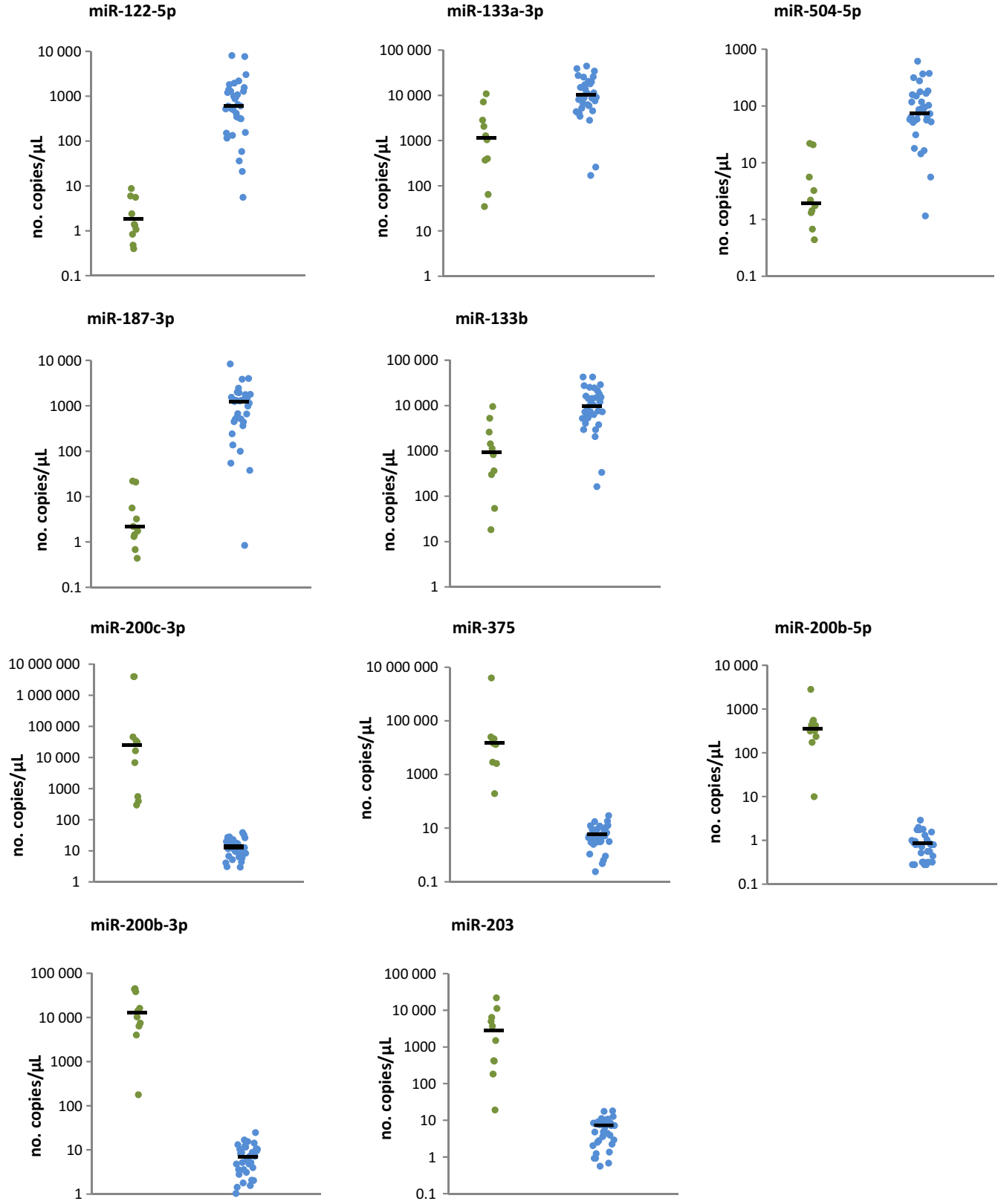

FIGURE 2 Absolute quantification for validation of miRNAs in the control group (CTR—green dots) and in patients with achalasia (ACA—blue dots). Each blot indicates the level of miRNA with increased expression (miR-122-5p, miR-133a-3p, miR-504-5p, miR-187-3p, and miR-133b) and with decreased expression (miR-200c-3p, miR-375, miR-200b-5p, miR-200b-3p, and miR203a) tested using droplet digital PCR. The median levels in both subgroups are indicated by the horizontal black bars. Values are reported in log scale (y-axis)

TABLE 2 miRNAs that directly target genes known to be involved in the pathogenesis of achalasia

miRNA	*P*-value	Fold change	Target gene	*P*-value	Fold change
hsa-miR-197-3p	.0251331	2.14151	IL18	.0139193	−4.16412
hsa-miR-346	.00219947	2.26487	IL18	.0139193	−4.16412
hsa-miR-146a-5p	.00285804	−7.98501	TLR4	.00439768	2.80933
hsa-miR-146b-5p	.00727751	−3.27947	TLR4	.00439768	2.80933
hsa-miR-4738-3p	.0237149	−2.84207	TLR4	.00439768	2.80933

non-achalasic subjects. By using microarray technology and applying stringent parameters, we detected a set of differentially expressed miRNAs that better stratify the two groups. We also employed an integrative methodology to study the correlations miRNA-mRNA expression as well as to profile uncovered pathways possibly regulated by those small regulatory RNAs. Finally, we performed ddPCR to validate the miRNA expression changes detected by microarray analysis. In our view, this original strategy of analysis may help to shed light in the genetic background of the disease, suggesting further molecular investigations in novel pathogenic mechanisms. From our knowledge, this is the first time that this approach has been used to study the relationship among levels of expression of miRNA-mRNA in achalasia patients, combining miRNAs/mRNAs microarray data from the same subjects obtained at the same time point. Moreover, despite the relatively small number of recruited samples, our sample size was able to capture 100% of genes that changed by 2-fold as statistically significant.

Microarray profiling showed 170 differentially expressed miRNAs in patients with achalasia compared with the control group. miR-133b and miR-133a-3p were the most significantly over-expressed miRNAs ($P < 1 \times 10^{-6}$), whereas miR-375 and miR-200c-3p ($P < 1 \times 10^{-10}$) were known for their greatest changes of decreased expression. The technical validation of the selected most differentially expressed miRNAs, with 5 significantly up-regulated and 5 significantly down-regulated miRNAs, by absolute quantification ddPCR analysis in independent sets, confirmed our observations.

Although none of miRNAs with altered expression find in our investigation have been previously reported in achalasia association studies, their role has been investigated in several diseases. The dysregulation of miR-133, highly expressed in differentiated muscle tissues,[11] has been shown to play a key role in several human cancers,[12-14] including gastric cancer,[15] esophageal squamous cell carcinoma,[16] and so on. In the same direction, the emerging role of the miR-375 in pancreatic islet development, glucose homeostasis, mucosal immunity, lung epithelial cell differentiation, and carcinogenesis.[17] MiR-375 was identified as a novel and promising marker specifically associated with late-stage malignant progression in Barrett's esophagus.[18] Its down-regulation has also been found in esophageal squamous cell carcinoma,[19] gastric cancer,[20] glioma,[21] and other tumors.[22,23] Similarly, the miR-200 family, which includes five members (miR-200a, miR-200b, miR-200c, miR-429, and miR-141), appeared to be down-regulated during tumor progression and acts as a key inhibitor for the epithelial-to-mesenchymal transition, tumor cell invasion, angiogenesis, fibrosis, and inflammation.[24] Dysregulation of the miR-200c was observed in a variety of cancers[25] and noteworthy, it has been reported to be associated with

TABLE 3 Direct interactions between miRNAs and differentially expressed genes identified by Palmieri et al[2]

miRNA	*P*-Value	Fold change	Target gene	*P*-value	Fold change	Spearman correlation Coefficient	*P*-value Spearman correlation coefficient	Associated function
hsa-miR-130b-3p	.00238111	−6.67243	F3	3.8372E-05	−2.85418	0.591176	.0158756	Adhesion of Immune Cells
hsa-miR-200b-3p	6.117E-09	−120.272	FN1	8.4309E-06	6.48845	−0.576471	.0194151	Adhesion of Immune Cells
hsa-miR-200c-3p	9.11E-15	−2724.21	FN1	8.4309E-06	6.48845	−0.702941	.00238695	Adhesion of Immune Cells
hsa-miR-148a-3p	.00106562	−9.46446	ROCK1	9.7059E-09	2.87048	−0.511765	.0427234	Adhesion of Immune Cells
hsa-miR-155-5p	.00028234	−9.35332	MYLK	2.1241E-09	4.77208	−0.670588	.00446721	Contraction of Smooth Muscle
hsa-miR-143-3p	2.7885E-05	5.99802	PTGS2	0.00072751	4.20947	0.744118	.000948283	Contraction of Smooth Muscle
hsa-miR-148a-3p	.00106562	−9.46446	ROCK1	9.7059E-09	2.87048	−0.511765	.0427234	Contraction of Smooth Muscle
hsa-miR-200b-3p	6.117E-09	−120.272	ROCK2	6.3494E-07	2.08408	−0.508824	.0441352	Contraction of Smooth Muscle
hsa-miR-200c-3p	9.11E-15	−2724.21	ROCK2	6.3494E-07	2.08408	−0.791176	.000261946	Contraction of Smooth Muscle
hsa-miR-150-5p	.00037641	−3.37612	CYTIP	0.00361137	−2.87295	0.558824	.0244365	Leukocyte Migration
hsa-miR-148a-3p	.00106562	−9.46446	ROCK1	9.7059E-09	2.87048	−0.511765	.0427234	Leukocyte Migration
hsa-miR-155-5p	.00028234	−9.35332	FGF2	1.584E-06	9.23272	−0.570588	.0209907	Regeneration of Nerves
hsa-miR-130b-3p	.00238111	−6.67243	DPYSL2	3.2539E-05	2.48739	−0.561765	.023537	Synaptic Transmission of Cells
hsa-miR-182-5p	2.1235E-06	−35.6022	THBS1	4.2668E-05	3.63309	−0.588235	.0165397	Synaptogenesis

TABLE 4 Indirect association between mRNA and differentially expressed miRNA without prior knowledge of the interaction

miRNA	*P*-value	Fold Change	Target Gene	*P*-value	Fold Change	Spearman Correlation Coefficient	*P*-value Spearman correlation coefficient	Associated function
hsa-miR-125b-5p	.00348974	2.17246	CD34	7.95021E-05	4.6151	0.614706	.0112795	Adhesion of immune cells
hsa-miR-193b-3p	.0253079	2.46025	CD34	7.95021E-05	4.6151	0.602941	.0134241	Adhesion of immune cells
hsa-miR-125b-5p	.00348974	2.17246	CD34	7.95021E-05	4.6151	0.614706	.0112795	Leukocyte migration
hsa-miR-193b-3p	.0253079	2.46025	CD34	7.95021E-05	4.6151	0.602941	.0134241	Leukocyte migration
hsa-miR-130b-3p	.00238111	−6.67243	CHRM2	9.37235E-09	10.3733	−0.567647	.0218146	Contractility of smooth muscle
hsa-miR-92a-3p	.000265436	−2.01953	CHRM2	9.37235E-09	10.3733	−0.647059	.00674208	Contractility of smooth muscle
hsa-miR-130b-3p	.00238111	−6.67243	SULF1	3.01247E-06	4.16688	−0.632353	.00857758	Contractility of smooth muscle
hsa-miR-148a-3p	.00106562	−9.46446	SULF1	3.01247E-06	4.16688	−0.676471	.00400825	Contractility of smooth muscle
hsa-miR-200b-3p	6.12E-09	−120.272	SULF1	3.01247E-06	4.16688	−0.555882	.0253622	Contractility of smooth muscle
hsa-miR-200c-3p	9.11E-15	−2724.21	SULF1	3..01247E-06	4.16688	−0.855882	2.34492E-05	Contractility of smooth muscle
hsa-miR-143-3p	2.79E-05	5.99802	MRVI1	1.6432E-08	4.40056	0.608824	.0123152	Contractility of smooth muscle
hsa-miR-150-5p	.000376412	−3.37612	MRVI1	1.6432E-08	4.40056	−0.532353	.0337686	Contractility of smooth muscle
hsa-miR-362-5p	.0469489	−2.30139	MRVI1	1..6432E-08	4.40056	−0.567647	.0218146	Contractility of smooth muscle
hsa-miR-760	.00190272	2.53334	MRVI1	1.6432E-08	4.40056	0.588235	.0165397	Contractility of smooth muscle
hsa-miR-200b-3p	6.12E-09	−120.272	PRKG1	3.25098E-11	4.99205	−0.514706	.0413458	Contractility of smooth muscle
hsa-miR-200c-3p	9.11E-15	−2724.21	PRKG1	3.25098E-11	4.99205	−0.65	.00641571	Contractility of smooth muscle
hsa-miR-200b-3p	6.12E-09	−120.272	PRKG1	3.25098E-11	4.99205	−0.514706	.0413458	Contraction of smooth muscle
hsa-miR-200c-3p	9.11E-15	−2724.21	PRKG1	3.25098E-11	4.99205	−0.65	.00641571	Contraction of smooth muscle
hsa-miR-143-3p	2.79E-05	5.99802	MRVI1	1.6432E-08	4.40056	0.608824	.0123152	Contraction of smooth muscle
hsa-miR-150-5p	.000376412	−3.37612	MRVI1	1.6432E-08	4.40056	−0.532353	.0337686	Contraction of smooth muscle
hsa-miR-362-5p	.0469489	−2.30139	MRVI1	1.6432E-08	4.40056	−0.567647	.0218146	Contraction of smooth muscle
hsa-miR-760	.00190272	2.53334	MRVI1	1.6432E-08	4.40056	0.588235	.0165397	Contraction of smooth muscle
hsa-miR-760	.00190272	2.53334	CNN1	4.54199E-07	8.24569	0.544118	.0293363	Contraction of smooth muscle
hsa-miR-125b-5p	.00348974	2.17246	RAP1A	3.228E-07	3.30223	0.823529	8.83914E-05	Leukocyte migration
hsa-miR-486-5p	.000309977	5.4184	RAP1A	3.228E-07	3.30223	0.620588	.0103135	Leukocyte migration
hsa-miR-92a-3p	.000265436	−2.01953	RAP1A	3.228E-07	3.30223	−0.582353	.0179327	Leukocyte migration
hsa-miR-143-3p	2.79E-05	5.99802	STXBP1	4.81282E-05	2.00248	0.667647	.00471198	Synaptic transmission of cells
hsa-miR-200a-3p	.000494183	−10.3826	STXBP1	4.81282E-05	2.00248	−0.752941	.000761081	Synaptic transmission of cells
hsa-miR200b-3p	6.12E-09	−120.272	SYDE1	3.0052E-06	2.80904	−0.723529	.00153448	Synaptic transmission of cells
hsa-miR-200c-3p	9.11E-15	−2724.21	SYDE1	3.0052E-06	2.80904	−0.758824	.00065403	Synaptic transmission of cells
hsa-miR-31-5p	8.46E-07	−79.3838	SYDE1	3.0052E-06	2.80904	−0.6	.0140071	Synaptic transmission of cells
hsa-miR-150-5p	.000376412	−3.37612	NBEA	1.3941E-08	3.74975	−0.65	.00641571	Synaptic transmission of cells
hsa-miR-375	5.47E-10	−365.096	NBEA	1.3941E-08	3.74975	−0.681384	.00365467	Synaptic transmission of cells
hsa-miR-155-5p	.000282343	−9.35332	F13A1	0.00198383	2.9959	−0.573529	.020191	Recruitment of phagocytes
hsa-miR-182-5p	2.12E-06	−35.6022	F13A1	0.00198383	2.9959	−0.741176	.00101847	Recruitment of phagocytes

neuronal cell death.[26] miR-200b physiologically limits the endothelial nitric oxide synthase (eNOS) expression during hypoxia leading to a loss of NO bioavailability,[27] and miR-200c destabilizes *NOS3* transcript in response to oxidative stress.[28] Of note, inhibition of miRNA-200b resulted in increased neuronal apoptosis and microglia-mediated neurotoxic effect.[29] The data of the present study that show a decreased expression of miR-200b-5p, miR-200b-3p, and miR-200c-3p, led us to hypothesize their possible involvement in hypoxic response and in the context of chronic neuroinflammation suggesting a possible mechanism to the loss of ganglion cells in the esophagus and in the LES with local and systemic inflammation and subsequent loss of important neurotransmitters such as the vasoactive intestinal peptide and nitric oxide.

Since we had both miRNA and mRNA data available from the same muscle specimens, we were able to create miRNA-mRNA co-expression networks. Based on the gene expression data discussed in the previous paper[2] and using a bioinformatics approach, were selected miRNAs experimentally and not experimentally targets of DE genes associated with the two physiological categories of processes found significantly enriched: *neuronal/muscular* and

neuronal/immunity. Interaction networks between miRNAs and target genes revealed forty-nine miRNAs-mRNAs interacting pairs. The miRNA-200 family (miR-200a, miR-200b, miR-200c) shared several potential targets genes, of which *PRKG1*, *ROCK2*, and *SULF1* belonging to *contraction of smooth muscle* and *contractility of smooth muscle* biological processes. *SULF1* is involved in the development of a robust calcium handling system, *PRKG1* and *ROCK2* encode serine/threonine kinase proteins that regulate cytokinesis, smooth muscle contraction and relaxation, the formation of actin stress fibers and focal adhesions.[30-33]

The miR-200 family down-expression with an increased expression of *PRKG1*, by indirect association, is the interest as the functional role of interactions between these miRNAs and *PKG* binding sites have never been tested. Further studies are needed to understand the mechanism by which in pathological conditions like achalasia, miR-200 expression could be regulated by NO/cGMP/PKG signaling or whether miR-200 could modulate the contractile phenotype, proliferation, and migration of VSMCs via regulation of *PRKG1*.

Another gene involved in *contraction of smooth muscle* and *contractility of smooth muscle* pathways is *MRVI1* with several potential miRNAs binding sites (miR-143-3p, miR-150-5p, miR-362-5p, and miR-760) of which miR-150-5p and miR-362-5p were predicted to up-regulate the gene. *MRVI1* plays a role as NO/PRKG1-dependent regulator of IP3-induced calcium release. It encodes IRAG (InsP3R-associated cGMP kinase substrate) highly expressed in smooth muscle cells and platelets and considerably involved in mediating NO-dependent inhibition of calcium signaling in gastrointestinal smooth muscle contributing to NO-dependent relaxation.[34] These data suggest that investigation of alterations in genes involved in the NO-sGC-cGMP pathway and possible regulation by the identified miRNAs could be useful for the understanding of the physiological and pathophysiological regulation and modulation at diverse steps in this signaling pathway also in the esophagus of achalasia patients. They also provide treatment options for affected individuals, and these should be carefully and cautiously evaluated.[35]

Synaptic transmission of cells is another associated function contains the most predicted miRNA targets, including *DPYSL2*, *NBEA*, *STXBP1*, and *SYDE1* genes. The up-regulated genes *STXBP1*, involved in the synaptic release of neurotransmitters, and *SYDE1*, involved in cytoskeletal remodeling and cell migration and invasion, were observed as putative targets of miR-200a-3p, miR-200b-3p, and miR-200c-3p.

In summary, through microarray of miRNA-mRNA expression profiles, we discovered achalasia associated miRNAs and potentially regulated genes, many of them are reported to be associated to diverse neurodegenerative and cardiac disorders and in a variety of cancers. Our bioinformatics analysis indicates that differentially expressed miRNAs regulating genes involved in *contraction of smooth muscle* and *synaptic transmission of cells signaling* pathways might play an important role in the relaxation of smooth muscle cells and in neuronal cell death. The different expression levels of the identified miRNAs in the esophageal muscle tissue of achalasia patients might be used as a potential novel biomarkers of the disease and serve as a therapeutic target in the future. Moreover, the diverse molecular functions of the miRNAs could make them, like miR-133, a truly valuable candidate in miRNA-based gene therapy for treating such diseases.[36]

Limitations of the study might be represented by the relatively small sample size and the lack of a validation study in an independent sample, mostly due to the rarity of achalasia occurrence and the difficulty of recruiting esophageal muscle tissue during surgical myotomy. However, in our view this flaw should not limit the validity of the results considering their prediction accuracy.

On the other hand, in vitro functional validations by luciferase reporter assay of the most promising miRNAs are *in progress* in order to confirm their interactions with genes found to be potential targets in the highlight biological processes.

In conclusion, the network and pathway information presented here offer insights for the elucidation of detailed functions of mRNAs and miRNAs in the pathogenesis of the disease. However, the molecular roles that these dysregulated miRNAs play in achalasia were not completely elucidated and further studies are needed to clarify the diagnostic and prognostic potential of these miRNAs in the pathogenesis of idiopathic achalasia.

ACKNOWLEDGMENTS

The authors would like to thank all the patients and staff of the ALMA–ONLUS (Association of Patients with Achalasia) who volunteered to take part in this project. The authors are very grateful to their patients as well as the physicians and nursing staff of the Gastroenterology Unit of the "Casa Sollievo della Sofferenza" Hospital in San Giovanni Rotondo, Italy, who helped with sample collection from recruited patients.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

AL and OP conceived and designed the experiments; AL drafted and reviewed the manuscript; AM, ST, AC, FB, GM, and AA provided clinical cases and tissues; TM, TB, and GB performed data analysis; TL, GC, DG, OP, MC, and AP performed the experiments. All authors reviewed the manuscript.

ORCID

Gabrio Bassotti https://orcid.org/0000-0002-0237-1812
Anna Latiano https://orcid.org/0000-0003-3719-2061

DATA AVAILABILITY STATEMENT

The miRNA microarray data analyzed in the current study are available in ArrayExpress (www.ebi.ac.uk/arrayexpress) (E-MTAB-7333). mRNA microarray datasets are included in Palmieri et al[2] and available in ArrayExpress Annotare 2.0 database (https://www.ebi.ac.uk/fg/annotare) (E-MTAB-3962).

REFERENCES

1. Pandolfino JE, Gawron AJ. Achalasia: a systematic review. *JAMA*. 2015;313:1841-2185.
2. Palmieri O, Mazza T, Merla A, et al. Gene expression of muscular and neuronal pathways is cooperatively dysregulated in patients with idiopathic achalasia. *Sci Rep*. 2016;6:31549.
3. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*. 2008;9:102-114.
4. Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. *Annu Rev Med*. 2009;60:167-179.
5. Kan T, Meltzer SJ. MicroRNAs in Barrett's esophagus and esophageal adenocarcinoma. *Curr Opin Pharmacol*. 2009;9:727-732.
6. Slaby O, Srovnal J, Radova L, et al. Dynamic changes in microRNA expression profiles reflect progression of Barrett's esophagus to esophageal adenocarcinoma. *Carcinogenesis*. 2015;36:521-527.
7. Mei LL, Qiu YT, Zhang B, et al. MicroRNAs in esophageal squamous cell carcinoma: potential biomarkers and therapeutic targets. *Cancer Biomark*. 2017;19:1-9.
8. Shoji H, Isomoto H, Yoshida A, et al. MicroRNA-130a is highly expressed in the esophageal mucosa of achalasia patients. *Exp Ther Med*. 2017;14:898-904.
9. Dweep H, Gretz N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat Methods*. 2015;12:697-697.
10. Ikebuchi Y, Kanda T, Ikeda H, et al. Identification of human herpes virus 1 encoded microRNAs in biopsy samples of lower esophageal sphincter muscle during peroral endoscopic myotomy for esophageal achalasia. *Dig Endosc*. 2019. https://doi.org/10.1111/den.13491
11. Rao PK, Kumar RM, Farkhondeh M, et al. Myogenic factors that regulate expression of muscle-specific microRNAs. *Proc Natl Acad Sci U S A*. 2006;103:8721-8726.
12. Akcakaya P, Ekelund S, Kolosenko I, et al. miR-185 and miR-133b deregulation is associated with overall survival and metastasis in colorectal cancer. *Int J Oncol*. 2011;39:311-318.
13. Chen XN, Wang KF, Xu ZQ, et al. MiR-133b regulates bladder cancer cell proliferation and apoptosis by targeting Bcl-w and Akt1. *Cancer Cell Int*. 2014;14:70.
14. Crawford M, Batte K, Yu L, et al. MicroRNA 133B targets pro-survival molecules MCL-1 and BCL2L2 in lung cancer. *Biochem Biophys Res Commun*. 2009;388:483-489.
15. Qiu T, Zhou X, Wang J, et al. miR-133a and miR-133b inhibit proliferation, migration, invasion and cell cycle progression via targeting transcription factor Sp1 in gastric cancer. *FEBS Lett*. 2014;588:1168-1177.
16. Kano M, Seki N, Kikkawa N, et al. miR- 145, miR-133a and miR-133b: Tumor-suppressive miRNAs target FSCN1 in esophageal squamous cell carcinoma. *Int J Cancer*. 2010;127:2804-2814.
17. Yan JW, Lin JS, He XX. The emerging role of miR-375 in cancer. *Int J Cancer*. 2014;135:1011-1018.
18. Leidner RS, Ravi L, Leahy P, et al. The microRNAs, MiR-31 and MiR-375, as candidate markers in Barrett's esophageal carcinogenesis. *Genes Chromosomes Cancer*. 2012;51:473-479.
19. Mathé EA, Nguyen GH, Bowman ED, et al. MicroRNA expression in squamous cell carcinoma and adenocarcinoma of the esophagus: associations with survival. *Clin Cancer Res*. 2009;15:6192-6200.
20. Tsukamoto Y, Nakada C, Noguchi T, et al. MicroRNA-375 is downregulated in gastric carcinomas and regulates cell survival by targeting PDK1 and 14-3-3zeta. *Cancer Res*. 2010;70:2339-2349.
21. Chang C, Shi H, Wang C, et al. Correlation of microRNA-375 downregulation with unfavorable clinical outcome of patients with glioma. *Neurosci Lett*. 2012;531:204-208.
22. He XX, Chang Y, Meng FY, et al. MicroRNA-375 targets AEG-1 in hepatocellular carcinoma and suppresses liver cancer cell growth in vitro and in vivo. *Oncogene*. 2012;31:3357-3369.
23. Zhou J, Song S, He S, et al. MicroRNA-375 targets PDK1 in pancreatic carcinoma and suppresses cell growth through the Akt signaling pathway. *Int J Mol Med*. 2014;33:950-956.
24. Liu X, Zhang J, Xie B, et al. MicroRNA-200 family profile: a promising ancillary tool for accurate cancer diagnosis. *Am J Ther*. 2016;23:e388-e397.
25. Feng X, Wang Z, Fillmore R, et al. MiR-200, a new star miRNA in human cancer. *Cancer Lett*. 2014;344:166-173.
26. Stary CM, Xu L, Sun X, et al. MicroRNA- 200c contributes to injury from transient focal cerebral ischemia by tar-geting reelin. *Stroke*. 2015;46:551-556.
27. Kalinowski L, Janaszak-Jasiecka A, Siekierzycka A, et al. Posttranscriptional and transcriptional regulation of endothelial nitric-oxide synthase during hypoxia: the role of microRNAs. *Cell Mol Biol Lett*. 2016;21:16.
28. Carlomosti F, D'Agostino M, Beji S, et al. Oxidative stress-induced mir-200c disrupts the regulatory loop among SIRT1, FOXO1, and eNOS. *Antioxid Redox Signal*. 2017;27:328-344.
29. Jadhav SP, Kamath SP, Choolani M, et al. microRNA-200b modulates microglia-mediated neuroinflammation via the cJun/MAPK pathway. *J Neurochem*. 2014;130:388-401.
30. Lincoln TM, Dey N, Sellak H. Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J Appl Physiol*. 1985;2001(91):1421-1430.
31. Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev*. 1995;75:487-517.
32. Anderson PG, Boerth NJ, Liu M, et al. Cyclic GMP-dependent protein kinase expression in coronary arterial smooth muscle in response to balloon catheter injury. *Arterioscler Thromb Vasc Biol*. 2000;20:2192-2197.
33. Boerth NJ, Dey NB, Cornwell TL, et al. Cyclic GMPdependent protein kinase regulates vascular smooth muscle cell phenotype. *J. Vasc. Res*. 1997;34:245-259.
34. Fritsch RM, Saur D, Kurjak M, et al. InsP3R-associated cGMP kinase substrate (IRAG) is essential for nitric oxide-induced inhibition of calcium signaling in human colonic smooth muscle. *J Biol Chem*. 2004;279:12551-12559.
35. Geiselhöringer A, Werner M, Sigl K, et al. IRAG is essential for relaxation of receptor-triggered smooth muscle contraction by cGMP kinase. *EMBO J*. 2004;23:4222-4231.
36. Yu H, Lu Y, Li Z, et al. microRNA-133: expression, function and therapeutic potential in muscle diseases and cancer. *Curr Drug Targets*. 2014;15:817-828.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Palmieri O, Mazza T, Bassotti G, et al. microRNA-mRNA network model in patients with achalasia. *Neurogastroenterol Motil*. 2019;00:e13764. https://doi.org/10.1111/nmo.13764