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MicroRNA expression profiles in the esophagus of children with caustic stenosis: A pathway towards esophageal cancer?

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ABSTRACT

Background: Eighty percent of caustic ingestions occur in children and esophageal neoplasms may develop as a late complication of such injury. The identification of biomarkers is a promising strategy to improve early diagnosis of esophageal cancer or caustic lesions that are at an increased risk of progression.
Study design/aims: This study aimed at identifying global microRNA (miRNA) expression changes in esophageal mucosa from children with caustic stenosis. The study included 27 biopsy samples from 15 patients. Samples were divided into two groups, according to the time elapsed after injury ($N = 15$ in Group A, with less than five years of follow-up and $N = 12$ in Group B, with more than five years of follow-up). miRNA expression profiles were determined in each lesion, compared with normal esophageal tissues from control group. We used the TaqMan Human MicroRNA Arrays (Thermo Fisher) platform. Furthermore, bioinformatic algorithms were used to identify miRNA target genes and biological pathways including miRNAs and their target genes potentially associated with esophageal disease.
Results: Thirteen miRNAs were significantly deregulated (9 over- and 4 underexpressed) in patients from Group A. In patients from Group B, two miRNAs were over- and two were underexpressed. Of note, miR-374 and miR-574 were deregulated in Group B patients and have been linked to esophageal tumorigenesis. We identified signal transduction and transcription factor networks with genes strongly related to development and progression of esophageal cancer.
Conclusion: miRNAs identified here contribute to a better understanding of pathways associated with malignant transformation from caustic stenosis to neoplastic lesions. This study may serve as a basis for validation of miRNAs, including miR-374 and miR-574, as potential biomarkers of early cancer detection.

Caustic esophageal injury remains a serious public health problem in the pediatric population, especially in developing countries. Approximately 80% of the documented caustic injuries occur in children [1–3]. Caustic soda is responsible for 1/3 of all esophageal strictures related to caustic ingestion [4].

Esophageal neoplasms may develop as a late complication of caustic injury [1–10]. Approximately 1% to 4% of cases of esophageal squamous cell carcinoma (ESCC) are thought to be associated with previous caustic ingestion [11].

Therefore, the identification of tissue-specific biomarkers is ideal for early diagnosis of ESCC associated with caustic injuries [12]. microRNAs (miRNAs) are potent gene expression regulators and key players in cancer-related mechanisms [13]. Multiple studies have shown that miRNA expression levels in tumor tissues are up- or downregulated to various degrees [14–18]. The major biological function of miRNAs is post-transcriptional mRNA regulation, which can significantly impact disease pathways [13,19–21].

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miRNAs are stable molecules, resistant to RNAse degradation, and easily detected in small tissue samples, making them suitable for profiling studies. By searching for miRNA alterations, it is possible to identify target genes as well as pathways modulated by miRNAs and potentially associated with disease [22]. Therefore, identification of deregulated miRNA expression and target genes is a potential step towards better characterization of cancer biology and to better understand the process of carcinogenesis. Global miRNA expression analysis on esophageal tissues from individuals with a history of caustic ingestion in childhood may lead to the identification of miRNAs as biomarkers for early detection of esophageal cancer.

To the best of our knowledge, there are no previous reports on molecular, genetic or epigenetic changes on caustic lesions leading to esophageal cancer. A recent (December 30th, 2019) PubMed search was performed using the keywords: [esophageal cancer AND caustic injury], and resulted in 99 publications, of which none included any type of genetic or epigenetic molecular analysis.

Therefore, the main goal of this study was to identify miRNA expression changes in esophageal mucosa tissues of children following caustic stenosis, and to identify miRNA target genes and biological pathways relevant to tumorigenesis.

1. Materials and methods

1.1. Ethics statement

This study was performed in accordance with the ethical standards of the Declaration of Helsinki and according to national and international guidelines. Our study has been approved by the Research Ethics Boards of the Faculty of Medicine (FMB), UNESP, Botucatu, SP (REB #1089452/2015).

1.2. Patient samples

Patients who met inclusion criteria were those who were less than 15 years of age and with a history of hospital admission owing to an esophageal lesion induced by caustic ingestion, who have been subjected to upper digestive endoscopy followed or not by esophageal dilatation, or esophagectomy. Participants were selected over a 17-year period. Patients were excluded if they did not have endoscopic biopsies performed at any time during follow-up, or if there were no surgical specimens of the esophagus.

Formalin fixed, paraffin embedded (FFPE) esophageal samples from patients were retrospectively collected from the Department of Pathology, FMB, UNESP. Regarding the use of FFPE samples for miRNA expression analysis, Azzalini et al. [23] have shown that miRNAs are analyzable in tissues fixed with different procedures, including buffered formalin, a procedure widely used in pathology laboratories, worldwide.

Samples were divided into two groups according to the time elapsed after the injury; Group A consisted of samples from children with less than five years of follow-up after caustic injury, and Group B included samples from patients with more than five years of follow-up after injury. Samples from a total of 15 patients were obtained. These samples were paired by age, gender, and year of biopsy with macroscopically and histopathologically normal esophageal tissues (Control Group). These control tissues were obtained from children who have been subjected to routine endoscopy. Our pediatric surgery service follows the recommendations from the American Society for Gastrointestinal Endoscopy [24] for obtaining biopsy specimens from at least the duodenum, stomach, and esophagus during pediatric endoscopy.

Histological analysis of biopsies was performed before RNA extraction, to ensure the epithelial predominance of tissues collected. Macrodissected areas used for miRNA expression analysis did not contain inflammatory cells.

1.3. RNA extraction

Prior to RNA extraction, we performed needle macrodissection on formalin fixed paraffin embedded (FFPE) tissue blocks using the stereo microscope Leica EZ4 (Leica Microsystems, Wetzlar, Germany). RNA was isolated using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion/Life Technologies, Carlsbad, CA, USA), following a previously reported protocol [25]. RNA samples were quantified using NanoDrop 8000, following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA).

1.4. Quantification of miRNA expression

miRNA expression levels were quantified using the TaqMan® Human MicroRNA Array Card (TLDA) assay (card A v3.0) (Thermo Fisher, Waltham, MA, USA), as previously described [26,27] according to the manufacturer's protocol. Global data normalization was performed using Expression Suite software (Thermo Fisher, Waltham, MA, USA), and miRNA expression profiles were determined using RQ Manager v.1.2 software (Thermo Fisher, Waltham, MA, USA).

Original, raw data are available on Gene Expression Omnibus (GEO), under accession number GSE134360. Data may be accessed using the token: gdgpwaqifboxnmf.

1.5. Statistical analyses

Statistical analyses were performed to correlate deregulated miRNA expression of Groups A and B *versus* Control. Categorical variables were described using frequencies and percentages, and continuous variables were summarized using mean (range) and median values. Statistical analyses were performed using SAS software version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA). A statistically significant difference was defined as $p < 0.05$.

1.6. miRNA target prediction analysis

Significantly deregulated (Fold Change [FC] ≥ 1.5 and $p < 0.05$) miRNAs in both groups *vs.* the corresponding control esophageal tissues were subjected to target prediction analysis using the computational tool microRNA Data Integration Portal (http://ophid.utoronto.ca/mirDIP/) v.4.1.11.1, database version 4.1.0.3 [28,29]. mirDIP analysis used as criteria for target selection results with “very high” (top 1%) scores for interaction probability. miRNA–mRNA interactions were validated using Integrated Interactions Database version 2018–11 (http://iid.ophid.utoronto.ca/) [30]. In addition, pathway analysis was performed using ToppGene Suite (https://toppgene.cchmc.org/) [31] to identify statistically enriched pathways.

2. Results

2.1. Patient demographic and clinical data

We analyzed 27 biopsies from 15 patients with esophageal caustic lesions. These samples were divided into two groups, 15 in Group A (less than five years of follow-up since injury) and 12 in Group B (more than five years of follow-up since injury). Given that patient follow-up was >17 years, some of them have samples included in both groups. Demographic data and information on clinical features are shown in Table 1.

2.2. Deregulated miRNA expression in esophageal samples exposed to caustic ingestion

miRNA expression analysis of Group A cases showed that 13 significantly deregulated (FC ≥ 1.5 and $p < 0.05$) miRNAs (9 over- and 4 underexpressed) compared with corresponding macroscopically and

Table 1
Patient demographics and clinical features.

Patient ID	Age (months)	Sex	Follow-up (years)	Dilatation (*n*)	Esophagitis at biopsy (number of biopsies)
1	16	M	7	11	2 (4)
2	28	M	6	18	1 (2)
3	40	M	3	17	2 (2)
4	36	F	5	2	1 (2)
5	17	M	10	30	1[a] (1)
6	28	F	4	0	4 (4)
7	13	F	13	9	1 (2)[a]
8	37	F	2	0	0 (1)
9	24	M	3	0	0 (1)
10	43	F	14	3	0 (3)
11	180	F	1	4	1[a] (1)
12	20	M	3	0	0 (1)
13	25	M	1	7	1 (1)
14	172	M	1	1	1 (1)
15	31	M	2	14	1 (1)

M: male; F: female. All patients suffered from accidental caustic ingestion, except patients 11 and 14 whose ingestion was intentional. Caustic substance was sodium hydroxide (NaOH) in all patients, except patient 2, who was exposed to sulfonic and hydrofluoric acid. Group A samples (less than 5 years of follow-up) included 15 biopsies from 11 patients (patient IDs 1, 2, 3, 4, 5, 6, 7, 9, 11, 14 and 15) and Group B samples (more than five years of follow-up) included 12 biopsies from 7 patients (patient IDs 1, 6, 7, 8, 10, 12 and 13). Three patients were restudied (patient IDs 1, 6, 7), since biopsies from these 3 patients were investigated in Group A and Group B.
[a] Indicates patient subjected to esophagectomy.

histologically normal esophageal tissues. In Group B, four miRNAs were significantly deregulated (FC ≥ 1.5 and $p < 0.05$) (2 over- and 2 underexpressed) in the caustic samples compared with normal esophageal tissues. Results are shown in Table 2.

2.3. Predicted miRNA target genes and biological pathways

miRDIP analysis of the 13 miRNAs deregulated in Group A samples resulted in a total of 14,565 predicted interactions and 7521 miRNA target genes (S1 Table). In Group B samples, the four deregulated miRNAs were predicted to have 2449 interactions and 2277 target genes (S2 Table). Pathways analysis for target genes in Group A samples showed 352 pathways (FDR $p < 0.01$) with 69 most enriched pathways, based on the number of genes (at least 100 genes targeted by deregulated miRNAs) (S3 Table). In Group B samples, pathway analysis showed 121 pathways (FDR $p < 0.01$) with five most enriched pathways with over 100 genes targeted by deregulated miRNAs (S4 Table). Among the pathways identified, axon guidance, vesicle-mediated transport, membrane trafficking, EGFR signaling, and pathways in cancer were enriched with a large number of miRNA-regulated genes in lesions from both groups A and B. Interestingly, adaptive immune response as enriched in group A lesions and signaling by interleukins were enriched in group B lesions. Enriched pathways in both groups are shown in Fig. 1.

Table 2
miRNA expression in esophageal tissue with caustic stenosis (Groups A and B) compared with normal tissue in children (Control group).

miRNA	log FC	*p* value
Group A (<5 years after caustic ingestion)		
hsa-miR-886-5p	4.05	0.016
hsa-miR-205-5p	4.03	0.024
hsa-miR-29a-3p	3.87	0.011
hsa-miR-320a	3.70	0.042
hsa-miR-145-5p	−0.54	0.01
hsa-miR-126-3p	−0.55	0.034
hsa-miR-143-3p	−0.74	0.003
hsa-miR-125a-5p	−0.82	0.006
hsa-miR-141-3p	−1.09	0.003
hsa-miR-133a-3p	−1.24	0.002
hsa-miR-103a-3p	−1.47	0.011
hsa-miR-139-5p	−1.47	0.016
hsa-miR-95-3p	−1.59	0.012
Group B (>5 years after caustic ingestion)		
hsa-miR-342-3p	3.36	0.043
hsa-miR-374b-5p	3.31	0.022
hsa-miR-574-3p	−0.51	0.019
hsa-miR-744-5p	−0.74	0.037

FC: fold change.

3. Discussion

Changes in miRNA expression are known to contribute to cancer development and progression, with different miRNA expression profiles associated with distinct biological tumor behavior [33]. The pathophysiologic mechanism of esophageal cancer related to caustic ingestion is not well understood [11]. Pathological findings have shown that the epithelium overlying an injured area is vulnerable to neoplastic transformation, especially if subjected to chemical, physical, or thermal aggression for prolonged periods. Malignant transformation is a complex process, resulting from a multistep process of dedifferentiation from native esophageal mucosa to cancer [34].

Our results showed specific changes in caustic lesions from both Groups A and B, as well as miRNA changes compatible with miRNA expression in primary tumors or cell lines established from ESCC, indicating a role of miRNA regulation and implying that common pathways may complement each other in promoting malignant transformation. Pathways of axon guidance, vesicle-mediated transport, membrane trafficking, EGFR signaling, and pathways in cancer were commonly enriched with a large number of miRNA-regulated genes in lesions with less and more than 5 years of caustic exposure. An interesting finding was the identification of adaptive immune system regulation as an enriched pathway in lesions with less than 5 years of caustic ingestion, and signaling by interleukins in lesions after 5 years of caustic ingestion. Deregulated pathways of immune response may occur in lesions after a short time of caustic exposure, and over time being observed in lesions after 5 years of exposure; the latter being more often associated with tumor development and progression [35].

Indeed, some miRNAs identified have been related to esophageal cancer. Liu et al. (2013) [36] analyzed the expression of 770 miRNAs from ESCC samples and found that 60 miRNAs were significantly deregulated (FC ≥ 2 and $p < 0.05$), with 51 miRNAs over- and nine underexpressed in cancer tissue compared with normal tissue. Our study showed that hsa-miR-126 and hsa-miR-574-3p were downregulated in Group A and B, respectively, which is consistent with the results of Liu et al. [36]. However, our study also showed that hsa-miR-143 and hsa-miR-145 were markedly downregulated in Group A, which conflicts with their findings. This inconsistency may be owing to the nonmalignant (or premalignant) state of caustic-affected esophageal cells when compared with the ESCC cells used in their study.

Decreased miR-886 expression has been previously identified in several cancer types, such as lung carcinoma [37], leukemia [38], and esophageal cancers [39], and this miRNA has been proposed to function as a tumor suppressor by inhibition of *PKR* (protein kinase RNA-activated) [38]. Lee et al. (2014) [39] demonstrated miR-886 underexpression in cancer samples compared with normal tissues. However, they found overexpression of miR-886 in samples from metaplastic or Barrett's esophagus, considered a premalignant condition. In this study, in Group A, miR-886 was overexpressed 11 fold as compared with normal samples, comparable to the previously reported results from Lee et al. [39]. This miRNA deregulation may represent an organismal response to a variety of tissue aggressions, which can occur both in patients with caustic ingestion as well as those with Barrett's esophagus and may promote oncogenesis.

Among the underexpressed deregulated (FC ≥ 1.5 and $p < 0.05$) miRNAs identified in patients from Group A, miR-133a and miR-139-5p are both related to tumor suppression. miR-133a downregulation has been detected in esophageal [40], ovarian [41], bladder [42] 8),

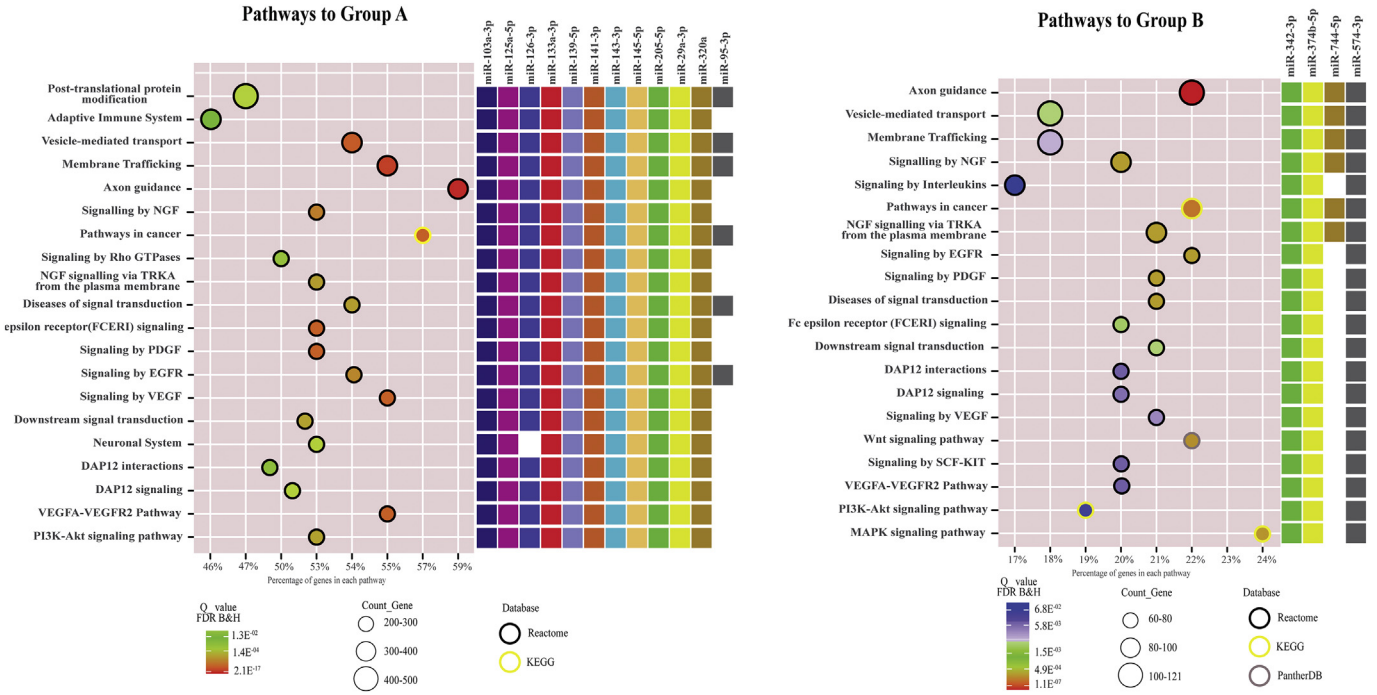

Fig. 1. Enriched pathways regulated by miRNA target genes in Group A and Group B samples. Each figure panel includes a description of the pathways (left), and which miRNAs modulate genes in each pathway (right). The percentage of genes in each pathway is shown at the bottom of each panel. Pathways are organized by statistical significance with corrected p-values (Benjamini–Hochberg procedure, to reduce the false discovery rate). Databases used are indicated. The results show that miRNAs regulate a wide range of genes with roles in mechanisms related to tumorigenesis; some pathways were commonly identified in Group A and Group B samples.

and gastric cancers [43]. The role of miR-133 in the regulation of genes that control cell migration and invasion, such as *FSCN1* (*fascin homolog 1 gene*) [44] and *SOX4* (*SRY-related HMG-box 4*) [40] has been demonstrated in ESCC cell lines. The deregulation of miR-139-5p is also a frequent event reported in ESCC and other types of cancer [45]. Liu et al. (2013) [46] showed a 14-fold decrease in the expression of miR-139-5p in ESCC samples compared with normal tissues, significantly associated with an increased risk for esophageal cancer. This suggests that the altered miR-139-5p levels in ESCC cells may trigger major downstream events in carcinogenesis, as this miRNA can suppress the cell's proliferative capability and participate in the delay of the G1/S phase transition during cell cycle progression.

Overexpression of miR-374 is found in many types of cancer, such as head and neck squamous cell carcinoma [47], T-cell lymphoblastic lymphoma [48], osteosarcoma [49], and bladder urothelial carcinoma [50]. This deregulation has been associated with tumorigenesis, including the development and progression of esophageal cancer [51,52]. Wang et al. (2015) [53] found that overexpression of miR-374 significantly increased cellular proliferation in ESCC cell lines and demonstrated a possible role for this miRNA in esophageal cancer by suppressing the transcriptional activity, as well as the expression, of *Axin2* (*Axis inhibition protein 2*), a tumor suppressor. We showed that miR-374 had markedly increased expression in patient samples taken more than five years after the initial occurrence of caustic lesions, suggesting a possible role in malignant transformation.

Decreased miR-574 expression was found in the Group B samples. Deregulated miR-574 expression has been previously identified in several cancer types, including gastric, bladder, prostate, and esophageal cancers; and this miRNA may function as a tumor suppressor by controlling cell proliferation, migration, and invasion ability [15,18,54]. In prostate cancer, miR-574 was shown to be involved in the regulation of *WNT* signaling, reducing proliferation by targeting *EGFR* [55]. Okumura et al. (2016) [56] showed an association between decreased expression of miR-574 and tumor relapse and poor overall survival rate, providing further evidence for tumor suppressor effects of miR-574 in ESCC.

Some miRNAs have been found to be consistently deregulated in both types of esophageal cancer, and this histologically independent molecular aberration suggests an association with a major oncogenic function, starting early in various signaling pathways [14,18]. Altered signaling pathways may result in phenotypes of uncontrolled growth and an increased capability to invade the surrounding tissue [57]. The *WNT* signaling pathway, found in the protein–protein interaction map (PPI) for Groups A and B, is well-known in regulating self-renewal and oncogenesis in many systems, and it has been reported to play an important role in progression, metastasis, and invasion in ESCC [58,59].

Among the limitations of our work is the lack of validation of target genes in the same biopsy tissues. We were unable to perform gene expression analysis owing to sample unavailability, since biopsies were too small and not of adequate amount to perform further studies. In addition, there is a need for additional studies using larger sample sizes, in order to establish a panel of miRNAs that may be candidates for diagnostic applications, such as early detection of cancer.

Our findings build upon recent evidence in the scientific literature demonstrating the regulatory role of miRNAs expressed in esophageal cancer samples on esophageal tissue tumorigenesis. Our results highlight the influence of caustic lesions in the esophagus, beginning during the first years following injury, on the possibility of malignant transformation mediated by miRNA alterations. Based on our findings and the literature data, we hypothesize that malignant transformation of caustic esophageal lesions may occur through multiple pathways, particularly those involving *mTOR /PIK3/AKT* signaling.

4. Conclusions

miRNAs identified here may be associated with malignant transformation from caustic stenosis to esophageal cancer and may serve as future novel biomarkers of malignant progression. This study provides a theoretical basis for future research in this field with this specific pediatric population suffering from caustic exposure. Further investigation is needed to elucidate the mechanisms underlying malignant transformation associated with caustic stenosis. High-risk patients with caustic lesions may benefit from prevention and intervention strategies, including more frequent periodic screening tests to improve early detection of

cancer and treatment; such strategies will ultimately lead to better outcomes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpedsurg.2020.02.009.

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