



Gene Expression Profiling of MiRNA-190, MiRNA-588 and MiRNA-802 in Laryngeal Cancer: Diagnostic and Prognostic Values



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Abstract

Laryngeal cancer (LC) is a common poor prognostic head and neck cancer. MiRNAs are promising biomarkers for diagnosis of LC. This study aims to evaluate miRNA-190, miRNA-588 and miRNA-802 expression levels and their significance as molecular biomarkers for LC. Furthermore, a prediction model for LC late stage was created to discover patients at high risk of late stage development. Quantitative real-time polymerase chain reaction (RTqPCR) was used to investigate the expression levels of these genes in sixty cancerous tissue specimens and adjacent thirty healthy specimens. The demographic and clinical data, along with the TNM staging of LC patients were also investigated. Significant down-regulation of MiRNA-588 and miRNA-802 in LC ($P < 0.001$) showed a tumor suppressor role with the possibility of their usage as biomarkers differentiating between cancerous and healthy tissues. ROC curve analysis confirmed the diagnostic ability of miRNA-588 and miRNA-802 to discriminate between cancerous and healthy tissues. In the late stage, decreased expression level of miRNA-802 was only detected when compared with early stage ($P = 0.004$) indicating that miRNA-802 might play a protective role against progression to late stages. The multivariate analysis revealed that only stridor ($P < 0.001$) and Cervical lymphadenopathy ($P = 0.009$) might serve as independent predictors of LC late stage.

Keywords: Laryngeal cancer; MiRNAs expression; qRT-PCR; Biomarkers; LC late stage

1. Introduction

Laryngeal cancer (LC) is the most common head and neck cancer and it represents about 1–5 % of all cancers. LC occurs more frequently in males than females, with an approximate ratio of 5:1 [1]. A prior study demonstrated that LC incidence and number of deaths grew steadily from 1997 to 2017 worldwide [2]. The 5-year overall survival (OS) for LC patients reached about 60.7% from 2006 to 2012, and it has no significant improvement over the past several decades [3]. Tumor recurrence and distant metastasis, which are closely associated with the advanced tumor stages of the larynx, are the main causes of mortality from this malignancy [4].

The Tumor Node Metastasis (TNM) classification of LC is largely implicated in estimating the prognostic outcomes for LC patients and identifying the optimal therapeutic regimen for each patient [5]. Around 30 to 40 percent of patients with head and neck squamous cell carcinomas present with early stages (stage I or II) [6]. There are no consensus

predictors for advanced laryngeal cancer. This calls for in-depth investigations to find new possible biomarkers that could be used for early prediction of more aggressive outcomes. There is a growing interest in molecular biomarkers that may have diagnostic or prognostic relevance.

MicroRNAs (miRNAs) are small non-coding RNAs with various regulatory functions in almost all processes in cancer cells. The first onco-miRNA in B-cell chronic lymphocytic leukemia cells was reported in 2002. As a result, miRNAs have gained prominent importance in cancer research [7]. Many studies have linked miRNA expression to diagnostic and prognostic aspects, bolstering their potential as biomarkers [8, 9]. To date, numerous studies demonstrated dysregulated expression of miRNAs in laryngeal tissue and blood of LC patients [10].

MiRNA-190 is located on chromosome 15q22.2 [11]. MiRNA-190 has been demonstrated to function as a tumor suppressor as well as an oncogene in a variety of cancers. [12]. MiRNA-190 was shown to

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be overexpressed in pancreatic cancer, meningioma, bladder cancer and gastric cancer [13], whereas miRNA-190 was found to be down-regulated in rectal cancer, prostate cancer, hepatocellular carcinoma (HCC), breast cancer [11] and cervical cancer [14]. These findings suggested that miRNA-190 may target several genes involved in tumorigenesis and tumor progression.

MiRNA-588 is located on chromosome 6q22.32 [15]. In lung cancer, gastric cancer, breast cancer, and glioma, a tumor-suppressive role of miRNA-588 has been noticed [16]. In contrast, its oncogenic role was reported in prostate cancer, supporting its role in tumor cell proliferation [17]. MiRNA-802 gene exists on chromosome 21q22.12 [18]. As a tumor suppressor gene, miRNA-802 can prevent cancer formation and progression. MiRNA-802 expression is reduced in numerous malignancies, involving laryngeal and tongue squamous cell carcinomas [19]. However, it was found to be overexpressed in osteosarcoma, HCC, and bladder cancer [20]. This observation shows that miRNA-802 can also function as an oncogene.

To the best of our knowledge, there is no information concerning the role and the expression levels of miRNA-190 and miRNA-588 in LC. Furthermore, miRNA-802 is poorly investigated in this type of malignancy. It is important to study the expressions and biological functions of those miRNAs to understand the development and progression of LC. Consequently, our study was designed to examine the gene expression levels of the above-mentioned miRNAs in LC patients and to investigate their role as predictors of LC late stages.

2. Experimental

2.1 Patients

This case-control study was conducted at the Medical Biochemistry and Molecular Biology Department, the Faculty of Medicine, Mansoura University and Mansoura University Hospitals between February 2021 and March 2022. The present study was approved by the Institutional Research Board (IRB) of Faculty of Medicine, Mansoura University (Approval number: MDP.20.12.52). All patients provided written informed consent.

This study involved sixty LC patients with a confirmed diagnosis of primary LC performed by clinical examination and laryngeal biopsy during direct laryngoscope (DL) at the otorhinolaryngology department, Mansoura University Hospitals. Patients with perioperative death, patients who received preoperative radiotherapy or chemotherapy, and patients with a history of any chronic disease were excluded from the study.

LC patients were subdivided into 21 cases of LC early stages (stages I / II) and 39 cases of late stages (stages III / IV). The TNM staging was performed based on the recommendations of the American Joint Committee on Cancer (AJCC) as well as the Union for International Cancer Control (UICC) [21]. (9 were Stage I, 12 were Stage II, 20 were Stage III, 17 were Stage IVA and 2 were stage IVB cancers).

Preoperative variables such as age, sex, smoking, and the presence or absence of all of the following data: hoarseness of voice (HOV), dysphagia, stridor, breathing troubles, cough, and cervical (cx) lymphadenopathy were obtained from medical records retrospectively. A neck ultrasound was performed to assess the condition of the larynx and cervical LN.

2.2 Specimens collection and preservation

Following laryngectomy, fresh paired tissues for the LC and control groups were promptly excised. For the LC group, tissues were removed from the center of the tumor mass with a morphologically presumed viable tumor tissue free of bleeding, necrosis, and fibrosis. For the control group, thirty samples were collected from non-cancerous laryngeal tissues of the same LC patients after laryngectomy. The final histological diagnosis of cancerous and non-cancerous tissues was confirmed by an expert pathologist. All specimens were immersed immediately in an adequate volume of RNA Later "RNA Stabilization Reagent (Qiagen, Germany)" (about 10 μ L reagent for each 1 mg tissue), followed by an overnight incubation at 4°C and then samples were preserved at -80°C until RNA was extracted.

2.3 RNA extraction

Total microRNAs were extracted using a microRNA Extraction Kit (Qiagen, Germany) based on both phenol/guanidine-based cell lysis and spin column-based purification of total RNA based on the manufacturer's recommendations [22]. By measuring absorbance at 260 and 280 nm, the concentration and purity of extracted RNA were evaluated using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, USA). We only used samples with A260/A280 ratios ranging from 1.80 to 2.00. Following isolation, the RNA was stored at -80°C till the time of reverse transcription.

2.4 Complementary DNA (cDNA) synthesis

In a 20 μ l reaction volume, cDNA was synthesized from 1 μ g of total RNA using SensiFAST™ cDNA Synthesis Kit (Bioline, UK) according to manufacturer's guidelines [23]. The thermal cycler (Applied Biosystem, USA) was used to set up the reaction in the following order: primer annealing for

10 minutes at 25°C, reverse transcription for 15 minutes at 42°C, and inactivation for 5 minutes at 85°C. The samples of cDNA were stored at -20°C.

2.5 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The relative expression levels of miRNA-190, miRNA-588, and miRNA-802 in cancerous and healthy laryngeal tissues were ascertained via qRT-PCR using a real-time PCR apparatus (Applied Biosystem 7500, USA). A 20 µl total reaction volume was made as 10 µl SYBR Green Lo-ROX Master Mix (2×)(Enzymomics, Korea), 6 µl nuclease-free water, 1 µl forward primer, 1 µl reverse primer, and 2 µl of cDNA.

The sequences of the primer sets used were as follows: miRNA-190 (Homo sapiens; PCR amplicon: 84bp; RefSeq: NR_029709.1): forward: 5'-TGAGAAGAAGTATGTAGCCACC-3', reverse: 5'-GATTCTTCTCCGGTGAAGAC-3' [24]; miRNA-588 (Homo sapiens; PCR amplicon: 79bp; RefSeq: NR_030316.1): forward: 5'-CGTCTTACCACCATGGAGAAGGC-3', reverse: 5'-AAGGCCATGCCAGTGAGCTTCCC-3' [25]; miRNA-802 (Homo sapiens; PCR amplicon: 91bp; RefSeq: NR_030414.1): forward: 5'-CGTTGTGTAGCTTATCAGACTG-3', reverse: 5'-AATGGTTGTTCTCCACTCTC -3'[26]; U6 primers (Homo sapiens; PCR amplicon: 94bp; RefSeq: NR_004394.1): forward: 5'-CTCGCTTCGGCAGCACA- 3', reverse: 5'AACGCTTCAGGAATTTGCGT -3'[24]. U6 snRNA was used as the ubiquitous control gene. The quality of the primers was evaluated by the NCBI Primer-Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

We employed the following protocol: initial denaturation step at 95°C for 10min, then 45 cycles of three steps: denaturation step at 95°C for 10 sec, annealing at 60°C for 15 sec and elongation step at 72°C for 15sec. Melting curve was used to confirm the product's specificity. After adjusting the baseline and the threshold, data was obtained and analyzed from the amplification plot.

2.6 Interpretation of results

The data was provided as relative quantification (RQ) of the target miRNA, and the relative expression of miRNAs was computed using the comparative Ct method (2-ΔΔCt method) [27] and standardized to the U6 snRNA level and relative to the calibrator (control samples).

2.7 Statistical analysis

SPSS software (version 26.0, IBM, Chicago, IL, USA) was utilized to enter and analyze the data. The Chi-square test (or Fisher's exact test) was applied to compare qualitative data. The Shapiro-Wilk H test

was used to determine the normality of quantitative data. In case of normally distributed data, the quantitative data were compared by the independent samples t-test and expressed as mean ± SD, whereas non-normally distributed data were compared by the Mann-Whitney U test and expressed as median and interquartile range (IQR). The receiver-operating characteristic (ROC) curve was applied to assess the ability of the three miRNAs to differentiate between the cancerous and non-cancerous laryngeal tissues, as well as the early and late stages of LC. Using only one predictor, univariate logistic regression was performed to predict the likelihood of progression to LC late stages. Afterward, a prediction model was made using multivariate logistic regression to find significant independent predictors of LC late stages and reporting their OR (95% CI). P values less than 0.050 were considered statistically significant for any of the tests employed.

3. Results

3.1 Patient characteristics

Routine demographic and clinical data of LC patients are shown in Table 1. The mean age was 62.52 ± 9.36 years. There were 54 (90%) males and 6 (10%) females. Among the sixty LC patients enrolled in this study, 52 were smokers and only 8 were nonsmokers. All cases (100%) were presented by HOV; Cx lymphadenopathy was the least frequent clinical presentation, present only in 38.3% of LC patients. Dysphagia, respiratory problems, coughing, and stridor were all present in 66.7%, 63.3%, and 48.3% of cases, respectively. The early stages represented 35% of the cases, while the late stages represented 65% of the cases.

3.2 Analysis of miRNA-190, miRNA-588 and miRNA-802 gene expressions in laryngeal tissue specimens

MiRNA-190, miRNA-588, and miRNA-802 expression levels were first evaluated in both cancerous and non-cancerous laryngeal tissues. There was no statistically significant difference in the levels of miRNA-190 expression between the LC and control groups (P= 0.383) (Figure 1A). The expression levels of miRNA-588 were significantly lower in the LC group (median: 0.076; IQR: 0.035–0.36) than in the control group (median: 0.4; IQR: 0.14–1.18; P< 0.001) (Figure 1B). Regarding the expression of miRNA-802, there was a statistically significant difference between the LC group (median: 0.12; IQR: 0.047–0.32) and controls (median: 0.56; IQR: 0.16–1.19; P< 0.001) (Figure 1C).

Next ROC curve was explored to examine the diagnostic accuracy of the expression levels of those miRNAs to discriminate the LC group from controls. For miRNA-190 expression, the area under the ROC

curve (AUC) was 0.557 (95%CI= 0.430–0.683; P= 0.380), having no diagnostic accuracy of LC. The AUC for miRNA-588 expression was 0.735 (95%CI= 0.633–0.838; P< 0.001). The cutoff value of miRNA-588 expression (≤ 0.19) had a significant diagnostic accuracy of LC versus controls with 73.3% specificity and 68.3% sensitivity. The AUC for miRNA-802 expression was 0.733 (95%CI= 0.614–0.852; P < 0.001). The cutoff value of miRNA-802 expression (≤ 0.33) had a significant diagnostic accuracy of the LC group versus the control group with 66.7% specificity, but 78.3% sensitivity (Figure 1D and 1E). These observations confirm the ability of miRNA-588 and miRNA-802 to discriminate between cancerous and non-cancerous laryngeal tissues with moderate accuracy.

between the early and late stages (P= 0.227 and P= 0.540, respectively) (Figures 2A, 2B); whereas,

MiRNA-802 expression level was significantly lower in the late stages (median: 0.085; IQR: 0.028- 0.32) than the early stages (median: 0.2; IQR: 0.1- 0.31; P= 0.004) (Figure 2C). ROC curve analysis was applied to examine the diagnostic accuracy of the expression levels of those miRNAs to discriminate the LC early stages from late stages. MiRNA-190, miRNA-588, and miRNA-802 showed no diagnostic accuracy of the late stages versus early stages of LC (P> 0.05).

Table 1. Demographic and clinical features of LC patients

Parameters	No of patients=60
Age (years)	
Mean \pm SD	62.52 \pm 9.36
Sex	
Male	54 (90%)
Female	6 (10%)
Smoking	
Non-smoker	8 (13.3%)
Smoker	52 (86.7%)
Clinical presentation	
Hoarseness of voice	60 (100%)
Dysphagia	40 (66.7%)
Stridor	29 (48.3%)
Breathing difficulty	40 (66.7%)
Cough	38 (63.3%)
Cervical lymphadenopathy	23 (38.3%)
Stages	
Early (I / II)	21 (35%)
Late (III / IV)	39 (65%)

SD: Standard deviation. Age is represented by mean \pm SD, sex, smoking, clinical presentation, and tumor stages are represented by count and percentage.

3.3 Analysis of miRNA-190, miRNA-588, and miRNA-802 gene expression levels in the early versus late stages of LC

There were no statistically significant differences in miRNA-190 and miRNA-588 expression levels

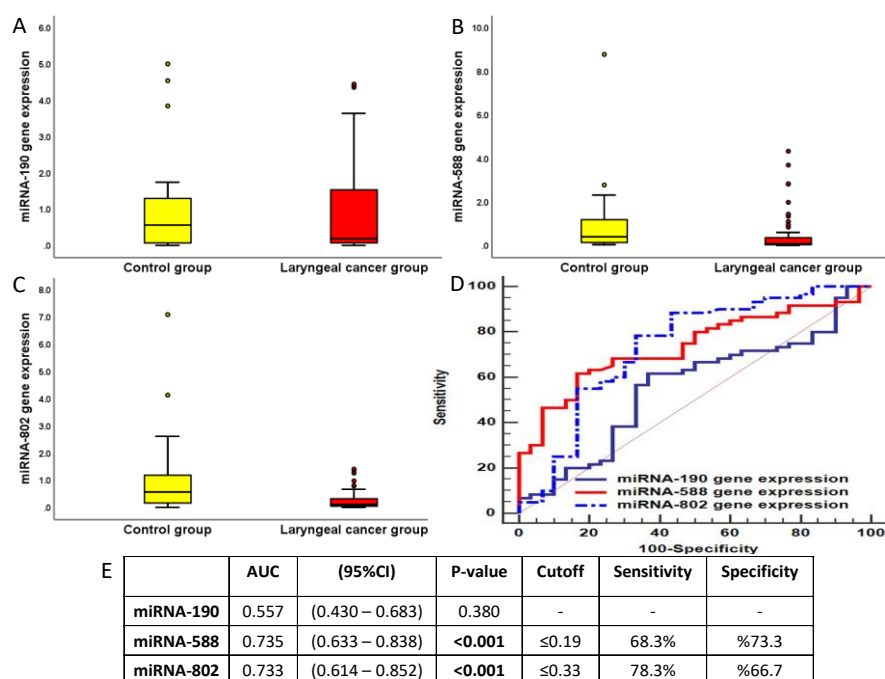


Fig. 1. Gene expression levels of miRNA-190, miRNA-588, and miRNA-802 among the control and LC groups. A) MiRNA-190 gene expression among the control and LC groups. B) MiRNA-588 gene expression among the control and LC groups. C) MiRNA-802 gene expression among the control and LC groups. D) ROC of the three miRNAs gene expression levels to discriminate between cancerous and healthy tissue. E) Validity of the three miRNAs gene expression levels to discriminate between cancerous and healthy tissues.

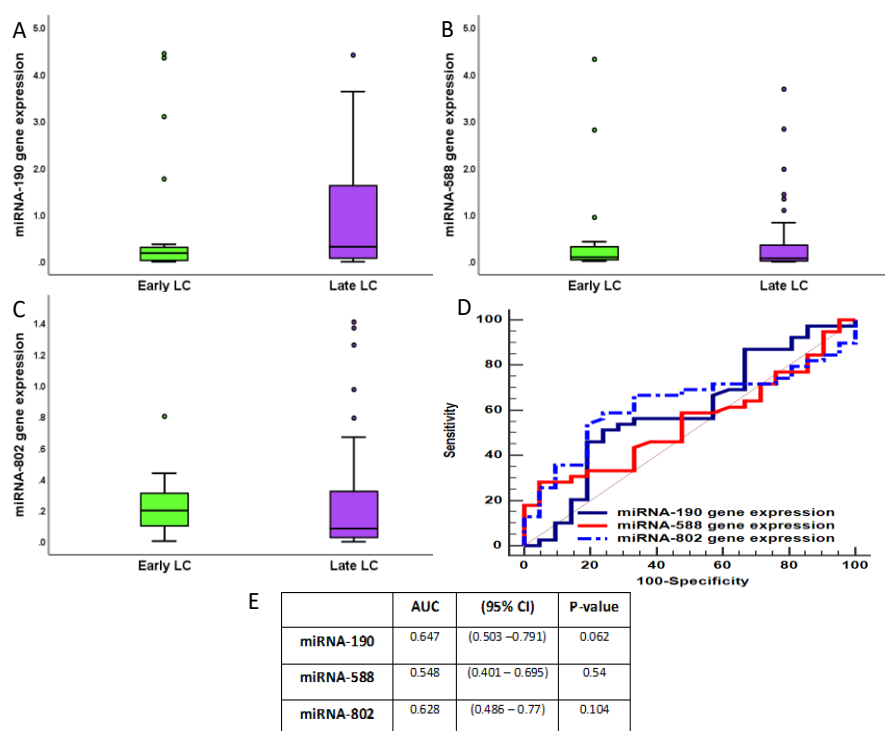


Fig. 2. Gene expression levels of miRNA-190, miRNA-588, and miRNA-802 among the early and late stages of LC. A) MiRNA-190 gene expression levels among the early and late stages. B) MiRNA-588 gene expression levels among the early and late stages. C) MiRNA-802 gene expression levels among the early and late stages. D) ROC of the three miRNAs gene expression levels to discriminate between the LC early and late stages. E) Validity of three miRNAs gene expression levels to discriminate between the LC early and late stages.

3.4 Comparison between the early and late stages of LC as regards the demographic and clinical features.

Table 2 concerning the differences in the demographic and clinical characters between LC early and late stages showed that male gender ($P=0.017$), smoking ($P=0.018$), dysphagia ($P<0.001$), stridor ($P<0.001$), breathing difficulty ($P<0.001$) and cx lymphadenopathy ($P<0.001$) were significantly associated with LC late stages. In contrast, age, HOV, and cough were not statistically significant ($P>0.05$).

Table 2. Comparison between the early and late stages of LC as

Parameters	Early stages N=21	Late stages N=39	P-value
Age (years)	64.3± 6.9	61.5± 10.4	0.254*
Sex			
Male	16 (76.2%)	38 (97.4%)	0.017**
Female	5 (23.8%)	1 (2.6%)	
Smoking			
Non-smoker	6 (28.6%)	2 (5.1%)	0.018**
Smoker	15 (71.4%)	37 (94.9%)	
Clinical presentation			
Hoarseness of voice	21 (100%)	39 (100%)	-
Dysphagia	6 (28.6%)	34 (87.2%)	<0.001**
Stridor	0 (0.0%)	29 (74.4%)	<0.001**
Breathing difficulty	7 (33.3%)	33 (84.6%)	<0.001**
Cough	11 (52.4%)	27 (69.2%)	0.196**
Cx lymphadenopathy	0 (0.0%)	23 (59.0%)	<0.001**

regards demographic and clinical features.

SD: Standard deviation. Bold numbers: Statistically significant. P-values by * Independent samples t-test (data are represented by mean ± SD). P-values by ** Chi-square test (data are represented by count and percentage).

3.5 Prediction of LC late stages

3.5.1 Simple (univariate) logistic regression analysis for predicting LC late stages

Univariate analysis showed that males ($P=0.018$), smokers ($P=0.017$), dysphagia ($P<0.001$), breathing difficulty ($P<0.001$), Cx lymphadenopathy ($P=0.010$), stridor ($P=0.002$) and low expression levels of miRNA-802 ($P=0.014$) were found to be significant predictors of late stages of LC (Table 3).

3.5.2 Multivariable logistic regression analysis for predicting LC late stages

Only stridor ($P<0.001$) and Cx lymphadenopathy ($P=0.009$) were statistically significant. Patients with Cx lymphadenopathy had 1.269 times higher odds to exhibit late stages of LC with 95% CI= 1.066-1.552 and patients with stridor had 1.459 times higher odds to exhibit late stages with 95% CI= 1.203-1.816. Male sex ($P=0.225$), smoking ($P=0.681$), dysphagia ($P=0.490$), and breathing difficulty ($P=0.276$) were non-significant predictors for LC late stages (Table 3).

TABLE 3. Univariate and multivariate logistic regression analysis for the prediction of LC late stages.

Parameters	Univariable		Multivariable	
	P1	OR (95% CI)	P2	OR (95% CI)
Age	0.274	0.965 (0.905-1.029)	-	-
Male	0.018	4.493 (1.294-15.594)	0.255	1.223 (0.865-1.728)
Female	ref			
Smoking	0.017	3.429 (1.248-9.423)	0.681	1.076 (0.758-1.529)
Nonsmoker	ref			
Hoarseness of voice	-	-	-	-
Dysphagia	<0.001	5.534 (2.581-11.867)	0.490	1.106 (0.831-1.472)
Stridor	0.002	3.100 (1.511-6361)	<0.001	1.478 (1.203-1.816)
Breathing difficulty	<0.001	4.302 (2.060-8.984)	0.276	1.142 (0.900-1.449)
Cough	0.200	1.554 (0.792-3.047)		
Cervical lymphadenopathy	0.010	2.312 (1.222-4377)	0.009	1.286 (1.066-1.552)
miRNA-190	0.542	1.147 (0.738-1.758)		
miRNA-588	0.816	0.934 (0.524-1.665)		
miRNA-802	0.014	0.429 (0.219-0.843)	0.237	0.910 (0.779-1.064)

Bold numbers: Statistically significant; P1 by univariate regression analysis; P2 by multivariate regression analysis.

4. Discussion

The pathogenesis of LC remains vague. There was no observed significant improvement in the 2- or 5-year OS of LC. Response to treatment is usually much better in early stages, but sometimes aggressive progression of the disease may occur. Stage IV proportion is increasing, with the risk of stage IV LC at the time of diagnosis increasing by 2.2% annually [28]. Thus, early detection of patients at high risk of LC is of great clinical importance. Consequently, investigating the molecular mechanisms and new treatment targets for LC is essential.

The current study showed that the mean age of LC patients was 62.52 ± 9.36 . Similar results have been observed by Saedi *et al*, in which the mean age of LC was 59.92 [29]. Among the sixty LC patients enrolled in this study, 54 (90%) were males and 6 (10%) were females with a 9:1 male-to-female ratio, which seems to be related to the social habit of smoking which is rare in Egyptian females. The male-to-female ratio was 2:1 in Scotland, and 9:1 in France, while in Pakistan it was 10.2:1 [30]. The differences in the male-to-female ratio may be due to genetic, environmental, and habitual factors. This study reported that fifty-two patients (86.7%) were smokers and only eight patients (13.3%) were nonsmokers. Nearer to our observation, it was found that smoking was a predisposing factor in 87.8% of the cases and it is the most crucial risk factor of LC [32].

Regarding the clinical presentation, this study illustrated that 100% of patients were presented by HOV, while dysphagia and breathing difficulty were present in 66.7% of our study group. Cough, stridor, and Cx lymphadenopathy were observed in 63.3%, 48.3%, and 38.3% of patients, respectively. Chauhan *et al* observed that the most common presenting symptom was dysphagia in 63% of patients, while 50.76% of patients were presented with HOV [33]. Khan *et al*. noticed that HOV was present in 92.86 % of cases and Cx lymphadenopathy was present in 64.3% of patients. Strider, dyspnea, and dysphagia in 37.5%, 25%, and 21.4% of his study group, respectively [30].

Previous work illustrated that miRNAs exert significant roles in the development and occurrence of LC [34]. MiRNAs have become a research focus in recent years as they hold promise for usage as biomarkers and pharmacological treatment targets for numerous cancers [10]. While certain miRNAs impair cell survival and proliferation, others enhance it. These two kinds of miRNAs, tumor suppressors, and oncogenes, may be crucial in the genesis of cancer. [35]. This study investigated three miRNAs expression levels and their relevance to LC.

As regards miRNA-190, there was no statistically significant difference in the expression level between

the LC and control groups ($P= 0.383$). In line with our results, miRNA-190 expression level in core biopsies of locally advanced breast cancer was non-significant [36]. MiRNA-190 dysregulation has been reported in various cancers. Up-regulation of miRNA-190 was observed in pancreatic cancer, bladder cancer, meningioma, and gastric cancer [13]. Down-regulation of miRNA-190 was found in hepatocellular carcinoma, glioma, prostate cancer, rectal cancer, and cervical carcinoma [14]. Different tumor origins might be the leading cause of discrepancies between those results. Our findings are considered preliminary theories; therefore, additional research is recommended to confirm these findings by analyzing the alterations in miRNA-190 expression in a broader population.

The significantly lower expression level of miRNA-588 in cancerous (median: 0.076) than in non-cancerous laryngeal tissues (median: 0.4) suggested a tumor suppressor effect of miRNA-588 in LC pathogenesis. In agreement with this observation, miRNA-588 was downregulated in gastric cancer (GC) [37]. Low expression of miRNA-588 was also observed in lung squamous cell carcinoma, glioma, and breast cancer [16]. Previous observations confirmed that miRNA-588 may act as a tumor suppressor gene. Inhibition of invasion, migration, and angiogenesis, and induction of apoptosis are among the mechanisms by which miRNA-588 suppresses cancer growth [38]. The down-regulation of miRNA-588 stimulates the proliferative, migratory, and invasive capabilities of osteosarcoma cells [39]. In contrast, miRNA-588 was observed to be upregulated in human prostate, and ovarian cancers [37]. Thus, miRNA-588 seems to have various functions in various pathologic conditions that might be the result of tissue- or organ-specific differences.

Various studies have shown abnormal expression of miRNA-802 in different tumors. The potential role of miRNA-802 in cancer was initially demonstrated in 2013 by examining miRNA-802 expression levels and its effects on osteosarcoma tissues and related cell lines [40]. A significant decrease in miRNA-802 expression levels in LC tissues (median: 0.12) when compared to the healthy tissues (median: 0.56) was detected. In line with our results, it was observed that the miRNA-802 expression level was decreased significantly in LC tissues than the adjacent healthy tissues. Its role as a tumor suppressor in LC is attributed to the fact that the miRNA-802 overexpression can inhibit cell viability, cause G0/G1 phase arrest in cells, and induce apoptosis [19], indicating that we can use miRNA-802 as a potential molecular biomarker and therapeutic target for LC. Down-regulation of miRNA-802 was also observed

in esophageal squamous cell carcinoma, GC, colorectal cancer (CRC), cervical cancer, breast cancer, epithelial ovarian cancer, and melanoma, confirming the tumor-suppressing effect of miRNA-802 [41]. Contrary to what was previously reported, overexpression of miRNA-802 was observed in hepatocellular carcinoma, osteosarcoma, bladder cancer, and cholesteatoma [40]. These findings reveal that miRNA-802 can also enhance tumor occurrence and development.

Early detection of LC has become an urgent need for better prognosis and overall good patient survival because it is frequently diagnosed at an advanced stage due to rapid tumor growth, the delay in seeking medical advice, misdiagnosis by the primary care physician, absence of alarming early symptoms, and the need of indirect laryngoscopy for diagnosis [9]. Discovering new diagnostic and prognostic biomarkers for LC is crucial for improving disease outcomes. Thus, we explored the ROC curve to examine the diagnostic accuracy of those miRNAs expression levels to discriminate the LC group from controls. Our results reported a moderate diagnostic accuracy of miRNA-588 (AUC: 0.735; $P < 0.001$) and miRNA-802 (AUC: 0.733; $P < 0.001$), so that miRNA-588 and miRNA-802 can be used as molecular biomarkers for discrimination between LC and control groups.

The present study also compared the demographic and clinical characters between the early and late stages of LC, in which male gender ($P = 0.017$), smoking ($P = 0.018$), dysphagia ($P < 0.001$), stridor ($P < 0.001$), breathing difficulty ($P < 0.001$) and cx lymphadenopathy ($P < 0.001$) were associated with late stages and subsequent bad prognosis of LC.

In a huge study involving 4005 patients having head and neck cancer, females with laryngeal cancer had a lower recurrence rate and better prognosis compared with males [42]. Another study involved 1252 patients with LC, male gender was associated with advanced LC stages and cancer deaths [43]. Regarding smoking, a population-based study stated that smoking elevated the cancer-related death rate of head and neck cancers within 5 years of diagnosis [44]. In contrast, several studies observed no significant association between smoking and patient prognosis [45, 46]. Discrepancies between these studies may be due to small sample sizes (<200 patients) and included patients were either from single centers or clinical trials that cannot be generalized [43].

Regarding the clinical signs, Zia *et al* stated that patients complaining of breathlessness and stridor were found to have late stage tumors [31]. In agreement, another research stated that dysphagia,

breathing difficulty, stridor, and neck swelling are warning signs in the late stages of glottis cancer [47].

The differences in miRNAs expression levels between the early and late stages of LC were assessed to provide convincing molecular clues to help decrease tumor progression at the early stages and to identify novel early biomarkers of late stages. There were no statistically significant differences in miRNA-190 and miRNA-588 expression levels between the early and late stages ($P = 0.227$ and $P = 0.540$, respectively); however, the expression level of miRNA-802 was significantly decreased in the late stages (median: 0.085) than the early stages (median: 0.2; $P = 0.004$), suggesting that miRNA-802 might act as a prognostic factor in LC and its low expression levels is associated with the late stages.

The results of the current study are in agreement with previous report which revealed that the expression level of miRNA-802 is related to the outcomes of patients with esophageal cancer, in which the high expression of miRNA-802 is significantly related to the increase in (OS). Moreover, the low expression of miRNA-802 in CRC was observed to be an independent poor prognostic factor that is associated with the progression of CRC to advanced stages [41]. On contrary, shorter OS of patients with HCC was linked to the high expression levels of miRNA-802. Moreover, it was observed that patients with high blood miRNA-802 levels have much shorter median survival time compared to patients with low blood miRNA-802 levels [48]. This discrepancy may be due to different genetic and environmental factors in addition to different tumor origins.

Late stages are associated with a higher risk of local recurrence, distant metastases and increased mortality [49]. Thus, creating a prediction model for LC late stages to help identify patients at high risk of progression to late stages was the secondary outcome. Univariate analysis revealed that male gender, smoking, dysphagia, breathing difficulty, cervical lymphadenopathy; stridor, and low expression level of miRNA-802 were demonstrated to be significant predictors of LC late stages. However, only stridor and cervical lymphadenopathy were statistically significant in the multivariate analysis.

This study revealed that patients with cx lymphadenopathy had 1.269 times higher odds of exhibiting late stages of LC. In line with our results, Bradford *et al* stated that cervical lymphadenopathy is the most significant single prognostic indicator in LC [43]. Two Danish studies were also supporting the results of this study; the first one was performed by Vendelbo Johansen *et al*, on 1252 patients with laryngeal cancer, and the second one by Lyhne *et al* on 5001 patients with glottic LC [50, 51]. Our result

was also in agreement with the findings of another study performed by Nicolai *et al* on 281 LC patients in Italy, who found that Cx lymphadenopathy could be a poor prognostic factor in multivariate analysis [52]. This discrepancy may be attributed to the ethnic variations and the small sample size of patients with LC in research done by Nicolai and his colleagues. Moreover, it was illustrated that LC patients with stridor were associated with an increased risk of progression to LC late stages with 1.459 times higher odds. Zia *et al* stated that stridor is a serious symptom attributed to the extent and deep invasion of the disease. All patients complaining of stridor in his study are found to have T3 and T4 stage tumors [31].

Finally, this study provided recommendations for investigating the impact of miRNAs on the development and progression of LC on a larger scale population while taking into consideration other risk factors. This may help provide novel molecular biomarkers that could be used in accurate diagnosis of LC and early prediction of advanced stages to improve the OS of patients.

5. Conclusion

This study concluded that there were down-regulations of miRNA-588 and miRNA-802 expression levels in cancerous tissues of LC patients, proving that both of them might act as tumor suppressor genes in LC. However, miRNA-190 expression level in cancerous tissue was not significantly different from healthy tissues. Only for miRNA-802, a significant lower expression level in LC late stages was detected. Stridor and Cx lymphadenopathy were independent predictors of LC late stages.

6. Declarations

Conflicts of interest

The authors have no conflicts of interest that are directly relevant to the content of this article.

Ethics Approval

This study was approved by the Institutional Research Board (IRB) of Faculty of Medicine, Mansoura University (Approval number: MDP.20.12.52).

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