Original Article
Running Title: miR-221, miR-93, miR-21, and miR -24 Potential as Biomarkers in LSCC
Received: May 27, 2024; Accepted: November 23, 2024

Evaluation of Potential miR-221, miR-93, miR-21, and miR-24 as Molecular Markers for Laryngeal Squamous Cell Carcinoma

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Abstract

Background: Laryngeal cancer, the most prevalent head and neck malignancy, represents 2.4% of all tumors and is the 11th most common tumor worldwide. Laryngeal squamous cell carcinoma (LSCC) includes 85-90% of all laryngeal tumors and despite significant progression in screening and diagnostic approaches, occurrence and mortality has not reduced in recent decade. According to recent evidence, microRNA-221(miR-221), miR-93, miR-21, and miR-24 have possible roles in the carcinogenesis process of LSCC. In this study, we aimed to investigate any considerable changes in the micro-RNAs (miR) expression, and whether they have the potential to be a prognostic or diagnostic biomarker for LSCC.

Method: This case-control study examined the expression level of target micro-RNAs in 30 LSCC and their matched marginal healthy samples. The expression level of genes and their relationship with the patient's clinicopathological characteristics were analyzed using GraphPad Prism v.6.00, with paired t-tests and receiver operating characteristics (ROC) curve tests. A *P* value less than 0.05 was considered statistically significant.

Results: miR-24 is considerably down regulated in cancer tissues (Fold change = 0.89, *P* = 0.0213) while miR-21 (fold change: 1.16, *P* = 0.0063), miR-93 (Fold change: 1.123, *P* = 0.0448) and miR-221 (Fold change: 1.313, *P*< 0.0001) were upregulated in LSCC tumor tissue. Among these

microRNAs, only mir-221 has related to clinicopathological features and has an acceptable ROC area (0.7860), suggesting its potential as a diagnostic marker.
Conclusion: The results of this study revealed that miR-221 have moderate diagnostic accuracy and could be used as potential biomarkers in LSCC. Additional combination and confirmation with other biomarkers are suggested.
Keywords: Carcinoma, Squamous cell, MicroRNAs, Real-time polymerase chain reaction, Biomarkers

Introduction

Head and neck tumor include a wide range of cancers that originate in the oral cavity, larynx, pharynx, nasal cavity, salivary glands, and paranasal sinuses.[1] Laryngeal cancer (LC) is the most typical diagnosis of head and neck tumor, with an occurrence of 4.2% and is considered the 11th most frequent and deadly cancer. One type of LC is laryngeal squamous cell carcinoma (LSCC), which encompass nearly 85-90% of all LC.[2, 3] Diagnosis of more than 50% of cases is made in advanced stages, which decreased survival rate under 40%, while the survival rate in patients with early detection is more than 60%.[4, 5] In terms of tumor area, LC is divided into glottis, subglottis, and supraglottis and squamous cell carcinoma (SCC) is the frequent histological types. Excessive alcohol drinking and Tobacco are the important risk factors for LC progression.[6] Surgery, radiation therapy and chemotherapy are the main approaches to the treatment of LC separately or in combination with together.[7] As a result of progress in surgical techniques, radiation, and chemotherapy patients' 5-year survival rate has improved, but mortality rate is still high. Thus, early diagnosis of LC by clinicians is important.[8] Also, in spite of developed therapeutic approaches, the clinical outcome of progressive LC has not considerably improved in the last 20 years.[9] In fact, the lack of effective diagnostic or prognostic biomarkers reduces the therapeutic effect. Moreover, radiotherapy or chemotherapy resistance and high rates of regional or local recurrent after surgery are other reasons for high mortality.[10] MicroRNAs are a small non-coding and regulatory RNAs group with nearly 22 nucleotides which have a regulatory role in the post-translational phase via controlling gene expression through binding to mRNA and then inhibition or degradation of mRNA. It is obviously clear that miRNA act as oncogenes or tumor suppressor and play a significant role in different human tumors. In tumorigenesis, miRNAs can induce tumor development by low expressing tumor suppressor genes or preventing apoptosis.[11] They can also be used as markers to diagnosis, prognosis and clinical outcome of illness. Previous studies have shown that miRNAs are one of the main factors in the progression of LC using through controlling oncogenes or tumor suppressors miRNAs that are involved in apoptosis, migration, invasion, and growth.[2] MiR-221 is known to be high expressed in several malignancy, like hepatocellular carcinoma and breast cancer, inducing malignancy progression and migration.[12] MiR-93 has dual roles, acting as an oncogene in some tumors by suppressing tumor suppressors and inducing cell invasion, metastasis, and growth.[13] MiR-21 is a oncomiR, often high expressed in several tumors, like LSCC, which targets tumor suppressor genes and promote tumor progression.[14] On the other hand, miR-24 is usually low expressed in malignancies like LSCC, where it suppress cell growth and induce apoptosis.[15] Therefore, it is urgent to discover appropriate miRNAs which can be used in the effective diagnosis, prognosis and treatment of LC. In this study, we try to detect

miRNAs, which are significantly involved in the LC development, as well as find a new diagnostic and prognostic miRNA for LC early detection.

Methods and Materials

Study population

This case-control study included 30 LC patients and adjacent tumor-free normal marginal tissues have been collected through laryngectomy between 2018 February to 2019 September in the hospital. The inclusion criteria were histologically confirmed LSCC without prior radiotherapy, chemotherapy or surgery. The exclusion criteria were metastatic or recurrent cancer. All samples were directly moved to the solution with ribonuclease inhibitor (QIA Gene Cat NO: 76104) and stored at -80 ° C for total RNA extraction. The general characteristics of the contributors are shown in table 1.

RNA isolation, cDNA synthesis and real-time polymerase chain reaction (PCR)

Total RNA was extracted using TRIzol reagent (Roche Cat NO: 11667165001) as stated by manufacturer's guidelines. Later, the Nanodrop tool was used to determine the quantity and quality of RNA (Thermo Fisher Scientific, USA). Samples with an A260/A280 ratio between 1.8 and 2.1 were used for next analysis. RNA was stored at -80°C until cDNA synthesis. For complementary DNA synthesis, stem loop method and 2x RT-PCR Pre-Mix (Taq) (Universal cDNA synthesis kit (BioFACTTM, Seoul, South Korea) was used as stated by the manufacturer's guidelines. Quantitative PCR (Biosystem Applications, Step one, USA) was performed using SYBR Green Master mixture (Takara, Korea). The reaction conditions were done as an initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 25 seconds. Moreover, U6 was determined as an internal control to normalize miRNAs expression level. 2 -ΔCT method was used to determine the relative quantification of miRNA expression.

Statistical analysis

Statistical analysis was done using Graph Pad Prism v.6.00 program to determine the microRNAs gene expression panel in the cancer tissues and marginal normal tissues with impaired t-test. Receiver operating characteristics (ROC) curve analysis assessed the diagnostic potential each miRNA as a biomarker.

Ethical approval

This study was approved by the Ethics Committee, Tabriz University of Medical Sciences, and the universal principles of the Helsinki Declaration were applied to the study (ethical code: IR.TBZMED.REC.1398.999). All participants read and signed a written informed consent before enrollment. All information were maintained in a secure, password-protected database, only accessed by authorized personnel.

Results

We designed specific primers and quantitative poly chain reaction (qPCR) to evaluate miRNAs expression, the potential roles of which in the carcinogenesis pathways have been demonstrated in earlier research (Table 2).

miR-21, mirR-93, and miR-221 are up-regulated and miR-24 was down-regulated in LC

We analyzed the relative expression of miR-93, miR-221and miR-21 in 30 LSCC tissues compared with marginal normal tissues using U6 as internal control. Results indicated overexpression in miR-21 (Fold change: 1.16 ± 0.02, $P = 0.0063$, Figure 1), mirR-93 (Fold change: 1.123 ± 0.02, $P = 0.0448$; Figure 1) and miR-221 (Fold change: 1.313 ± 0.02, $P < 0.0001$; Figure 1). We also found that miR-

24 expression level was low in cancer tissues compared with cancer-free peripheral tissues (Fold change: 0.89 ± 0.02, P = 0.0213, Figure1).

Association of miR-221 expression level in clinicopathological characteristics of patients with LSCC

The over expression of miR-221 were significantly correlated with the clinicopathological characteristics like distant metastasis, Lymph node metastasis and the stage of the cancer (P < 0.0001) (Table3).

miR-221 as a diagnosis marker in LSCC

We used ROC curve to analyze miR-21, mirR-93, miR-221 and miR-24 specificity and sensitivity as a new biomarker in LSCC. The results determined a ROC region biomarker index 0.6855, 0.6385, 0.7860 and 0.6439 in LSCC patients, respectively (Figure 2). Among these miRNAs, miR-221 has more specificity and sensitivity to discriminate cancer tissues from healthy marginal (Area: 0.7860) (Figure 2).

Discussion

In present study, we found considerable dysregulation expression of four miRNAin LSCC tissues compared with normal marginal tissues. We detected that mir-221, miR-93, and miR-21 were highly expressed and miR-24 was low expressed in LSCC tissues compare with adjacent tumor tissues. Also, we found a relationship between miR-221 expression levels and clinicopathological characteristics of LSCC patients. Also, ROC analysis indicated that miR-221 could be used as a diagnostic marker in LSCC.

Laryngeal tumor is the most prevalence head and neck tumor that represents 2.4% of all cancers worldwide with high mortality rate. Squamous cell histology is the most frequency type of LC, and nearly 40% of patients have diagnosed in late stage of the disease.[16] According to the evidence, in the early stages, the rate of survivals of patients with LC is acceptable (80-90%), while the rate of survival of patients in the late stages is less than 50%.[17] Thus, the effective method to the LC treatment is early diagnosis.[18] MicroRNAs are a class of small non-coding RNA molecules contributed to the gene expression regulation. Dysregulation of these biological processes is the hallmark of many diseases like cancers, and imbalance of miRNA biogenesis may lead to tumorigenesis. The identification of several miRNAs, which acts as oncogenes or tumor suppressors, highlights the potential ability of miRNAs in the prognosis, diagnostic and therapeutic applications.[11] It is urgent to find potential biomarkers to prognosis and diagnosis to improve the therapy effect in LC. The microRNAs selected for our study were dysregulated in different types of cancer, and are involved with pathogenesis of cancer.

Li Xu et al. have revealed that the expression level of miR-24 in cell lines or LSCC tissues was considerably lesser than in a normal keratinocyte cell line or matched normal tissues. They confirmed that miR-24 re-expression suppress proliferation and promote LSCC cells apoptosis. Additionally, miR-24 overexpression boosts LSCC sensitivity to radiotherapy. They also indicated that X-linked inhibitor of apoptosis protein (XIAP) could be a miR-24 target, and anomalous XIAP and miR-24 expression may be involved with intensive LSCC growth and development.[19] Xie et al. informed that miR-24 control (suppress) XIAP expression to promote the apoptosis in tumor cells.[20] In another study, Guo et al. found that miR-24 ectopic expression considerably prevents cell invasion and growth via straight targeting S100A8 in LC.[21] Mir-221 was another microRNA evaluated in this study. Our findings show that miR-221 is considerably high expressed in LSCC tissues and associate with clinicopathological features such as lymph node metastasis and

distant metastasis. These results are consistent with Prior research that identified miR-221 as an oncogene in several tumors, like hepatocellular carcinoma and breast cancer. Sun et al. proved that suppression of miR-221 expression restricts cell growth, development and induce apoptosis in Hep-2 cells. Moreover, they showed that miR-221 expression prevention decreased tumor volume and weight in xenograft mouse models. Also, they discovered a significant association between Apaf-1 gene overexpression and low expression of the miR-221 in vivo and in vitro. These results indicated that Apaf-1 gene is a novel target of miR-221.[22] In another study, Park et al. revealed that the suppression of miR-221 enhanced the survival rate in tumor xenograft mice (hepatocellular carcinoma) as compared with the control group.[23] In another study, Hussein et al. revealed considerable miR-221 overexpression that led to Apaf-1 gene low expression in LSCC tissues in contrast to normal marginal laryngeal tissues. Moreover, there was a considerable association among Apaf-1 gene low expression, miR-221 up-regulation, and clinical stage metastasis.[24] Kan et al. found that co-suppression of the miR-21 and miR-221 decreased cell growth and promotes apoptosis in Hep-2 cells.[25] Xie et al. has shown that prevention of miR-221 expression in Hep-2 cells induces apoptosis and reduce cell growth.[26] But Shi et al. reported different result from previous studies. They confirmed that after transfecting of miR-221 into Hep-2 cells, development and growth of Hep-2 cells decreased and apoptosis induced, and it acts through the PI3K/AKT signaling pathway.[27]

Also, we revealed that miR-21 expression increased in tumor tissues in comparison with the marginal tissues. Wang et al. showed that miR-21 exosomal (oncomir) expression and long non-coding RNA (lncRNA) HOTAIR (oncolnc) in serum was significantly upregulated in LSCC patients as compared with vocal cord polyps. Expression profile of HOTAIR and miR-21 is useful to distinguish the benign tumor from malignant tumor in laryngeal disease with highly sensitive and specific.[28] Liu et al. showed that miR-21 high expressed in LC tissues and suppressed the expression of BTG2 (tumor suppressor) in LSCC tissues. Furthermore, low expression of miR-21 restricted Hep-2 cells progression. Therefore, they suggested that miR-21 may be used as an acceptable marker for the diagnosis of LSCC.[29] Also, Hu et al. indicated that miR-21 was overexpressed and miR-375 was low expressed in LSCC tissues, as compared with the normal tissues. They also reported that patients with high miR-21 expression or low miR-375 expression in cancer tissues shows weaker prognosis than patients with higher miR-375 expression or lower miR-21 expression. Ideally, the expression ratio of miR-21 / miR-375 was highly specific and sensitive for anticipating LSCC and potentially can be used in LSCC clinical settings.[30]

About miR-93, the role of miR-93 in tumor is controversy; this issue is because of its two-fold (oncogenic or tumor promoting) role in different cancers which is based on the type of the tumor. The miR-93 expression is increased in some human cancers and leads to the expression alteration of main tumor-correlated genes.[31] In our study, we also found that the mir-93 is overexpressed in tumor tissues in comparison with marginal tissues. Xiao et al. found that overexpression of the miR-93 prevents CCNG2 gene (tumor suppressor) expression via directly targeting CCNG2 3′UTR in the Hep-2 cells. They also indicated that miR-93 has an oncoming role which promotes the development, migration, and apoptosis in LC cells.[32] Li et al. reported that miR-93-5p overexpression promote tumor cell migration and progression in non-small cell long cancer (NSCLC) via inducing phosphatidylinositol 3- kinase/ protein kinase B (PI3K/Akt) pathway and restrain of p21,

phosphatase and tensin (PTEN), and liver kinase B 1 (LKB1) expression.[33] Li et al. indicated that miR-93 and miR-106b control cell proliferation via preventing PTEN expression from PI3K/Akt pathway in breast cancer and these miRNAs could be used as the possible therapeutic target for breast cancer treatment.[34] Also, Vila-Navarro et al. found that MiR-93 involved with poor prognosis in pancreatic tumor and stimulates cancer development through targeting microtubule dynamics. They also indicated that miR-93 or its direct targets (YES1, MAPRE1, CRMP2) are novel possible therapeutic targets for pancreatic ductal adenocarcinoma.[35] Chu et al. indicated that miR-93 overexpression in doxorubicin sensitive cells increased cellular progression and reduced breast cancer cells sensitivity to doxorubicin.[36]

Although our study indicates the potential of miR-221, miR-93, and miR-21 as diagnostic biomarkers for LSCC, it is limited by the relatively small sample size, and the single-center design. Upcoming studies should contain larger, multi-center cohorts to validate these findings. Furthermore, functional studies are essential to explain the biological mechanisms underlying miRNA dysregulation in LSCC. Also, as another limitation of this study, the serum level of these miRNAs is very important.

Finally, we found a correlation between miR-221 expression level and lymph node metastasis and metastasis. However, its moderate diagnostic potential, shown by an area under curve of 0.7860, recommends that miR-221 has sensitivity and specificity, it is better to be used with other biomarkers for precise diagnosis. Combining miR-221 with other biomarkers could improve diagnostic accuracy for LSCC. Additional studies should focus on confirming these results in larger, more diverse cohorts as well as exploring the mechanistic role of miR-221 in LSCC pathogenesis.

Conclusion

The present study show that miR-221, miR-93, and miR-21 up-regulated which have diagnostic biomarker potential, with miR-221 having the highest diagnostic precision. MiR-24 is significantly down regulation in LSCC tissues as compared with normal marginal tissues. MiR-221 could be used as a diagnosis marker in LSCC. These results indicate the importance of miR-221 in LSCC diagnosis and proposed its potential for use in clinical practice.

Acknowledgments

We would like to appreciate the Clinical Research Development Unit of Imam Reza General Hospital, Tabriz, Iran for their cooperation to conduct this study.

Funding

This study was funded by Tuberculosis and Lung Disease Research Center, Tabriz University of Medical Science, Tabriz, Iran.

Authors' Contribution

H.A: Conceptualization, Study design, Writing - Original Draft; H.Z: Study design, Writing - Original Draft, conducted literature searches and selected relevant articles; M.A: Study design, Conducted the literature search, selected relevant articles, and critically analyzed the information and reviewed the final draft; V.Z: Study design, Data gathering, Analyzed and synthesized information from selected articles; D.SH: Study design, drafting and reviewing the manuscript, Approved the final version of the manuscript; H.S.J: Study design, drafting; M.R: Study design, project administration, conceptualization and design of the work, writing review and editing; All authors have contributed to the conception or design of the work or the data acquisition and analysis, or interpretation of data for the work. Also, all authors read or reviewed and approved the final manuscript and agreed to be

accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest

None declared.

References

1. Argiris A, Karamouzis MV, Raben D, Ferris RL. Head and neck cancer. *Lancet.* 2008;371(9625):1695-709. doi: 10.1016/S0140-6736(08)60728-X. PMID: 18486742; PMCID: PMC7720415.
2. Li P, Liu H, Wang Z, He F, Wang H, Shi Z, et al. MicroRNAs in laryngeal cancer: implications for diagnosis, prognosis and therapy. *Am J Transl Res.* 2016;8(5):1935-44. PMID: 27347304; PMCID: PMC4891409.
3. Zhang SY, Lu ZM, Luo XN, Chen LS, Ge PJ, Song XH, et al. Retrospective analysis of prognostic factors in 205 patients with laryngeal squamous cell carcinoma who underwent surgical treatment. *PLoS One.* 2013;8(4):e60157. doi: 10.1371/journal.pone.0060157. PMID: 23593169; PMCID: PMC3617169.
4. Tomeh C, Holsinger FC. Laryngeal cancer. *Curr Opin Otolaryngol Head Neck Surg.* 2014;22(2):147-53. doi: 10.1097/MOO.0000000000000032. PMID: 24504224.
5. Zhang YF, Shen YJ, Huang Q, Wu CP, Zhou L, Ren HL. Predicting survival of advanced laryngeal squamous cell carcinoma: comparison of machine learning models and Cox regression models. *Sci Rep.* 2023;13(1):18498. doi: 10.1038/s41598-023-45831-8. PMID: 37898687; PMCID: PMC10613248.
6. Igissin N, Zatonskikh V, Telmanova Z, Tulebaev R, Moore M. Laryngeal cancer: Epidemiology, etiology, and prevention: A narrative review. *Iran J Public Health.* 2023;52(11):2248-59. doi: 10.18502/ijph.v52i11.14025. PMID: 38106821; PMCID: PMC10719707.
7. Gamez ME, Blakaj A, Zoller W, Bonomi M, Blakaj DM. Emerging concepts and novel strategies in radiation therapy for laryngeal cancer management. *Cancers*. 2020; 12(6):1651. doi: 10.3390/cancers12061651. PMID: 32580375; PMCID: PMC7352689.
8. Miller KD, Nogueira L, Devasia T, Mariotto AB, Yabroff KR, Jemal A, et al. Cancer treatment and survivorship statistics, 2022. *CA Cancer J Clin.* 2022;72(5):409-36. doi: 10.3322/caac.21731. PMID: 35736631.
9. Jenckel F, Knecht R. State of the art in the treatment of laryngeal cancer. *Anticancer Res*. 2013; 33(11):4701-10. PMID: 24222104.
10. Aftabi Y, Ansarin K, Shanehbandi D, Khalili M, Seyedrezazadeh E, Rahbarnia L, et al. Long non-coding RNAs as potential biomarkers in the prognosis and diagnosis of lung cancer: A review and target analysis. *IUBMB Life.* 2021;73(2):307-27. doi: 10.1002/iub.2430. PMID: 33369006.
11. Zarredar H, Ansarin K, Baradaran B, Shekari N, Eyvazi S, Safari F, et al. Critical microRNAs in lung cancer: Recent advances and potential applications. *Anticancer Agents Med Chem.* 2018;18(14):1991-2005. doi: 10.2174/1871520618666180808125459. PMID: 30088452.
12. Peng Y, Croce CM. The role of MicroRNAs in human cancer. *Signal Transduct Target Ther.*

2016;1:15004. doi: 10.1038/sigtrans.2015.4. PMID: 29263891; PMCID: PMC5661652.

13. Bao C, Chen J, Chen D, Lu Y, Lou W, Ding B, et al. MiR-93 suppresses tumorigenesis and enhances chemosensitivity of breast cancer via dual targeting E2F1 and CCND1. *Cell Death Dis.* 2020;11(8):618. doi: 10.1038/s41419-020-02855-6. PMID: 32796817; PMCID: PMC7428045.
14. Buscaglia LE, Li Y. Apoptosis and the target genes of microRNA-21. *Chin J Cancer*. 2011;30(6):371-80. doi: 10.5732/cjc.011.10132. PMID: 21627859; PMCID: PMC3319771.
15. Wang S, Liu N, Tang Q, Sheng H, Long S, Wu W. MicroRNA-24 in cancer: A double side medal with opposite properties. *Front Oncol*. 2020;10:553714. doi: 10.3389/fonc.2020.553714. PMID: 33123467; PMCID: PMC7566899.
16. Jones TM, De M, Foran B, Harrington K, Mortimore S. Laryngeal cancer: United Kingdom National Multidisciplinary guidelines. *J Laryngol Otol.* 2016;130(S2):S75-S82. doi: 10.1017/S0022215116000487. PMID: 27841116; PMCID: PMC4873912.
17. Wang J, Wu Y, Gao W, Li F, Bo Y, Zhu M, et al. Identification and characterization of CD133+ CD44+ cancer stem cells from human laryngeal squamous cell carcinoma cell lines. *J Cancer*. 2017;8(3):497. doi: 10.7150/jca.17444. PMID: 28261352; PMCID: PMC5332902.
18. Liu Y, Liu J, Wang L, Yang X, Liu X. MicroRNA‐195 inhibits cell proliferation, migration and invasion in laryngeal squamous cell carcinoma by targeting ROCK1. *Mol Med Rep.* 2017;16(5):7154-62. doi: 10.3892/mmr.2017.7460. PMID: 28901478.
19. Xu L, Chen Z, Xue F, Chen W, Ma R, Cheng S, et al. MicroRNA-24 inhibits growth, induces apoptosis, and reverses radioresistance in laryngeal squamous cell carcinoma by targeting X-linked inhibitor of apoptosis protein. *Cancer Cell Int.* 2015;15:61. doi: 10.1186/s12935-015-0217-x. PMID: 26106283; PMCID: PMC4477309.
20. Xie Y, Tobin LA, Camps J, Wangsa D, Yang J, Rao M, et al. MicroRNA-24 regulates XIAP to reduce the apoptosis threshold in cancer cells. *Oncogene*. 2013;32(19):2442-51. doi: 10.1038/onc.2012.258. PMID: 22733138; PMCID: PMC3460034.
21. Guo Y, Fu W, Chen H, Shang C, Zhong M. miR-24 functions as a tumor suppressor in Hep2 laryngeal carcinoma cells partly through down-regulation of the S100A8 protein. *Oncol Rep*. 2012;27(4):1097-103. doi: 10.3892/or.2011.1571. PMID: 22139384; PMCID: PMC3583566.
22. Sun X, Liu B, Zhao XD, Wang LY, Ji WY. MicroRNA-221 accelerates the proliferation of laryngeal cancer cell line Hep-2 by suppressing Apaf-1. *Oncol Rep*. 2015;33(3):1221-6. doi: 10.3892/or.2015.3714. PMID: 25586265.
23. Park JK, Kogure T, Nuovo GJ, Jiang J, He L, Kim JH, et al. miR-221 silencing blocks hepatocellular carcinoma and promotes survival. *Cancer Res*. 2011;71(24):7608-16. doi: 10.1158/0008-5472.CAN-11-1144. PMID: 22009537; PMCID: PMC3773601.
24. Hussein S, Mosaad H, Rashed HE, El-Anwar MW. Up-regulated miR-221 expression as a molecular

diagnostic marker in laryngeal squamous cell carcinoma and its correlation with Apaf-1 expression. *Cancer Biomark.* 2017;19(3):279-87. doi: 10.3233/CBM-160444. PMID: 28453462.

25. Kan X, Sun Y, Lu J, Li M, Wang Y, Li Q, et al. Co‐inhibition of miRNA‐21 and miRNA‐221 induces apoptosis by enhancing the p53‐mediated expression of pro‐apoptotic miRNAs in laryngeal squamous cell carcinoma. *Mol Med Rep.* 2016;13(5):4315-20. doi: 10.3892/mmr.2016.5048. PMID: 27035337.
26. Xie B, Ding Q, Han H, Wu D. miRCancer: a microRNA–cancer association database constructed by text mining on literature. *Bioinformatics*. 2013;29(5):638-44. doi: 10.1093/bioinformatics/btt014. PMID: 23325619.
27. Shi C, Wu YY, Wei LQ. MiR-221 affects the proliferation and apoptosis of laryngeal cancer cells through the PI3K/AKT signaling pathway. *Eur Rev Med Pharmacol Sci.* 2020;24(3):1258-63. doi: 10.26355/eurrev_202002_20180. PMID: 32096156.
28. Wang J, Zhou Y, Lu J, Sun Y, Xiao H, Liu M, et al. Combined detection of serum exosomal miR-21 and HOTAIR as diagnostic and prognostic biomarkers for laryngeal squamous cell carcinoma. *Med Oncol*. 2014;31(9):148. doi: 10.1007/s12032-014-0148-8. PMID: 25099764.
29. Liu M, Wu H, Liu T, Li Y, Wang F, Wan H, et al. Regulation of the cell cycle gene, BTG2, by miR-21 in human laryngeal carcinoma. *Cell Res*. 2009;19(7):828-37. doi: 10.1038/cr.2009.72. PMID: 19546886.
30. Hu A, Huang JJ, Xu WH, Jin XJ, Li JP, Tang YJ, et al. miR-21 and miR-375 microRNAs as candidate diagnostic biomarkers in squamous cell carcinoma of the larynx: association with patient survival. *Am J Transl Res*. 2014;6(5):604-13. PMID: 25360224; PMCID: PMC4212934.
31. Wang L, Wang Q, Li HL, Han LY. Expression of MiR200a, miR93, metastasis-related gene RECK and MMP2/MMP9 in human cervical carcinoma--relationship with prognosis. *Asian Pac J Cancer Prev*. 2013;14(3):2113-8. doi: 10.7314/apjcp.2013.14.3.2113. PMID: 23679328.
32. Xiao X, Zhou L, Cao P, Gong H, Zhang Y. MicroRNA-93 regulates cyclin G2 expression and plays an oncogenic role in laryngeal squamous cell carcinoma. *Int J Oncol*. 2015;46(1):161-74. doi: 10.3892/ijo.2014.2704. PMID: 25309979.
33. Zhou X, Wen W, Zhu J, Huang Z, Zhang L, Zhang H, et al. A six-microRNA signature in plasma was identified as a potential biomarker in diagnosis of esophageal squamous cell carcinoma. *Oncotarget*. 2017;8(21):34468. doi: 10.18632/oncotarget.16519. PMID: 28380431; PMCID: PMC5470983.
34. Li N, Miao Y, Shan Y, Liu B, Li Y, Zhao L, et al. MiR-106b and miR-93 regulate cell progression by suppression of PTEN via PI3K/Akt pathway in breast cancer. *Cell Death Dis.* 2017;8(5):e2796. doi: 10.1038/cddis.2017.119. PMID: 28518139; PMCID: PMC5520687.

35. Vila-Navarro E, Fernandez-Castañer E, Rovira-Rigau M, Raimondi G, Vila-Casadesus M, Lozano JJ, et al. MiR-93 is related to poor prognosis in pancreatic cancer and promotes tumor progression by targeting microtubule dynamics. *Oncogenesis*. 2020;9(5):43. doi: 10.1038/s41389-020-0227-y. PMID: 32366853; PMCID: PMC7198506.

36. Chu S, Liu G, Xia P, Chen G, Shi F, Yi T, et al. miR-93 and PTEN: Key regulators of doxorubicin-resistance and EMT in breast cancer. *Oncol Rep*. 2017;38(4):2401-7. doi: 10.3892/or.2017.5859. PMID: 28765915.

Table 1. Demographic and clinicopathological characteristics of LSCC patients and healthy controls

	Number of cases
Age	
<55 years	16
≥55 years	14
Gender	
Male	19
Female	11
Tobacco exposure	
Smoker	18
Nonsmoker	12
Differentiation	
Well	19
Moderately/poorly	11
Clinical stage	
I/II	17
III/IV	13
Lymph node metastasis	
Negative	17
Positive	13
Distant metastasis	
Negative	20
Positive	10

LSCC: Laryngeal squamous cell carcinoma

Table 2. Primer sequencing used for qRT-PCR

Micro-RNA	**Stem loop**	**Forward primer**	**Reverse primer**
miR-21-5P	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGATCAACA	CGTGCTTAGCTTATCAGA	CCAGTGCAGGGTCCGAGGTA
miR-24-1-5P	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAACTGAT	CGTGCTTGCCTACTGAGC	CCAGTGCAGGGTCCGAGGTA
miR-93-5P	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACCT	CGTGCTCAAAGTGCTGTT	CCAGTGCAGGGTCCGAGGTA
miR-221-5P	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAAAATCT	CGTGCTACCTGGCATACA	CCAGTGCAGGGTCCGAGGTA
U6	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATAT	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT

qRT-PCR: Quantitative reverse transcription polymerase chain reaction; miR: Micro-RNA

Table 3. Correlation of miR-21, miR-93, miR-24, and miR-221 expression with clinicopathological features of LSCC patients

Micro-RNA	**Dysregulation type**	***P*-value**	**Related clinicopathological Features**
miR-21	Upregulated	0.0063	-
miR-93	Upregulated	0.0448	-
miR-221	Upregulated	< 0.0001	Metastasis and lymph-node metastasis
miR-24	Downregulated	0.0213	-

LSCC: Laryngeal squamous cell carcinoma; miR: Micro-RNA

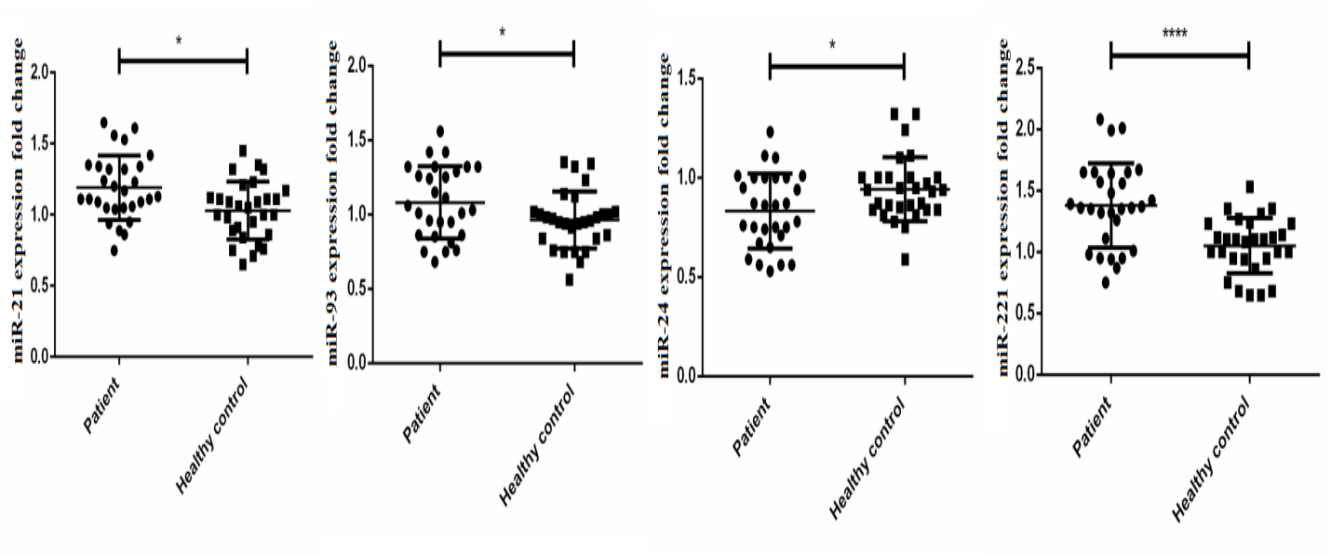

Figure 1. This figure shows the expression levels of miR-21, miR-93, miR-221, and miR-24 in LSCC tissues compared with normal marginal tissues. miR-21, miR-93, and miR-221 are significantly up-regulated, while miR-24 is down-regulated in LSCC tissues ($^{*}P < 0.05$, $^{***}P < 0.0001$).

LSCC: Laryngeal squamous cell carcinoma; miRNA: microRNA

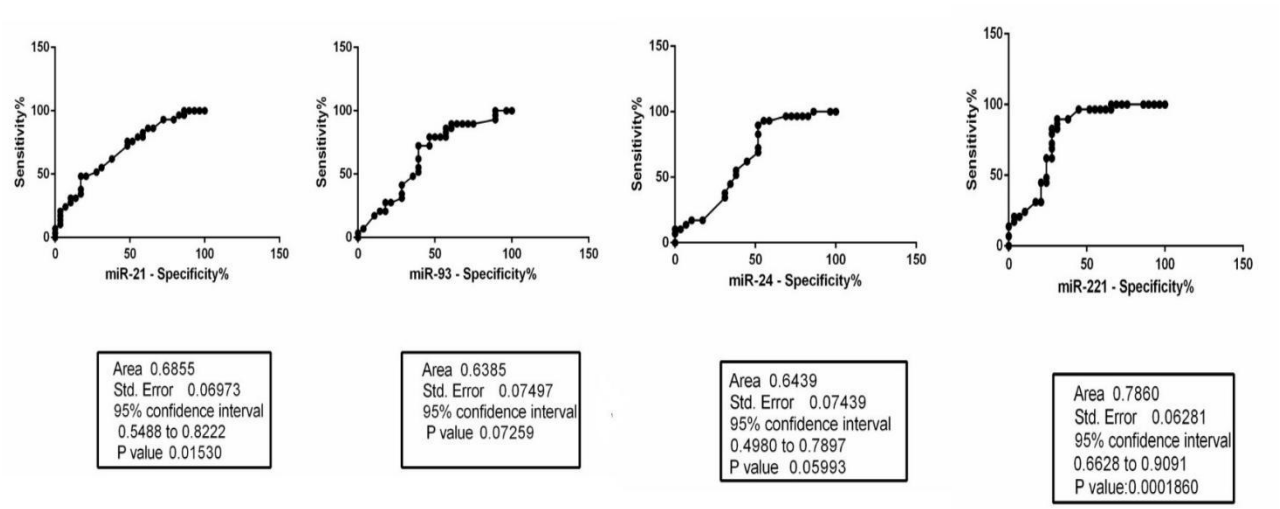

Figure 2. This figure shows the schematic illustration of ROC curve to evaluate the diagnostic potential of AUC for miRNAs.
ROC: Receiver operating characteristics; AUC: Area under the curve