

Identification of tumor suppressor gene LHPP-based 5-microRNA signature that predicts the early and mid-stage esophageal squamous cell carcinoma: a two-stage case-control study in the Chinese Han population

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Research Article

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Abstract

Global Cancer Statistics 2020 estimated that there were 544,076 deaths from esophageal cancer. It has been confirmed that phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) is an important tumor suppressor gene in many solid tumor tissues, but its expression in esophageal squamous cell carcinoma (ESCC) has not been reported. Furthermore, this study explored LHPP-based microRNA (miRNA) signatures for predicting early and mid-stage ESCC. We used next-generation sequencing to analyze and screen out the miRNAs differentially expressed in ESCC tissues. Besides, we explored the expression profile of miRNAs involved in LHPP regulation through miRDB, TargetScan, and miRanda online prediction systems. A model for ESCC prediction was established based on a testing cohort of 75 ESCC patients and 75 healthy controls. Finally, the predictive model was evaluated using a validation cohort of 45 ESCC patients and 45 healthy controls. Compared with adjacent tissues, LHPP protein levels in early and mid-stage ESCC tumor tissues were significantly decreased ($P < 0.01$). Next-generation sequencing and three biological online prediction systems indicated that miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, and miR-195-5p regulate LHPP expression. The levels of the above five miRNAs and squamous cell carcinoma antigen (SCC) in plasma were significantly increased in the early and mid-stage ESCC patients ($P < 0.05$). Then, an ESCC prediction model combining these five miRNAs and SCC was established. Finally, in an external validation cohort, the model exhibited a high discriminative value (sensitivity/specificity: 84.4%/93.3%). The combined noninvasive model we constructed has potential implications for the noninvasive diagnosis of early and mid-stage ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is a malignant tumor disease of the digestive system [1]. The fatality rate of esophageal cancer is ranked sixth among various malignant tumors. The early symptoms of esophageal cancer are not obvious, so its early screening and diagnosis mainly rely on traumatic methods, including the detection of esophageal exfoliated cells and gastrointestinal endoscopy [2, 3]. These screening technologies will cause trauma to subjects during the inspection process, making it difficult to achieve large-scale population screening. Hence, exploring diagnostic biomarkers is of great significance for early and mid-stage ESCC patients.

Endogenous microRNAs (miRNAs) are crucial players in many biological processes [4]. MiRNA mainly degrades the target gene mRNA or inhibits its translation through complete or incomplete pairing with the 3' untranslated region (3' UTR) of the target gene [5, 6]. Recent studies have suggested that miRNAs, as oncogenes or tumor suppressor genes, may play important roles in the gene regulation of the occurrence, development, invasion, metastasis, and angiogenesis of various tumors [7, 8], and are related to the occurrence and development of ESCC.

In 2018, Hindupur et al. identified a novel tumor suppressor gene phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) [9]. This suggests a new direction for non-invasive screening of early and mid-stage ESCC. In this study, first, we detected the expression levels of LHPP in

the tumor tissues of ESCC patients. Next, we used next-generation sequencing to analyze and screen out the miRNAs differentially expressed in ESCC tissues, and combined it with bioinformatics software to predict the expression profile of miRNAs involved in LHPP regulation. Then, we analyzed and determined the correlation between LHPP and miRNA in the plasma of ESCC patients. Finally, we established an early and mid-stage ESCC diagnostic regression model in a testing cohort, and performed an external verification in a verification cohort. In conclusion, we found that the combined detection of miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, miR-195-5p, and squamous cell carcinoma antigen (SCC) in plasma has a diagnostic effect on early and mid-stage ESCC.

Material And Methods

Enrollment and basic data collection of patients with locally advanced LSCC

A total of 137 early and mid-stage ESCC patients were included from January to December 2016 under the approval of the Ethics Committee of The Huaian Hospital of Huaian City (no.: k16.0112) and Taizhou People's Hospital Affiliated to Nantong University (no.: ks201601-002-1), according to the Declaration of Helsinki. The exclusion criteria for ESCC patients are (1) Tumor history. (2) The tumor stage is stage IV. (3) The patient refused to sign the informed consent form. (4) History of radiotherapy or chemotherapy. (5) The patient's clinical data is incomplete. In the end, a total of 120 ESCC patients were enrolled, including 75 patients from Huaian Hospital of Huaian City (testing cohort) and 45 patients from Taizhou People's Hospital Affiliated to Nantong University (verification cohort). During the same period, 120 healthy controls were collected.

Next-generation sequencing technology and bioinformatics analysis for miRNA expression profile exploration

The next-generation sequencing was tested by Shanghai Kangcheng Biological Engineering Co., Ltd. (Shanghai, China). The Illumina-Hiseq 4000 sequencing platform (Illumina, USA) was used for sequencing. After the sequencing is completed, the original sequencing sequence is obtained, and then bioinformatics analysis is performed. The two important indicators (Fold change and *P*-Value) in the volcano map can intuitively and reasonably screen out the miRNA genes that are differentially expressed between the two samples. The t-test was used to analyze the genes that were significantly differentially expressed between the two samples. The abscissa of the volcano plot is log₂ (fold change), and the ordinate is -log₁₀ (*P*-value). In this study, we also used miRDB, TargetScan, and miRanda software to predict the regulatory miRNA upstream of LHPP.

Cell culture and transfection

We purchased KYSE-30 (RRID: CVCL_1351) from BeNa Culture Collection (BNCC, Beijing, China) for *in vitro* research. KYSE-30 was inoculated in EMEM complete medium (BNCC338137, BNCC, Beijing, China) mixed with FBS. MiR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, miR-195-5p mimics, and si-LHPP were obtained from Guangzhou Changyu Biotechnology Co., Ltd.

Luciferase reporter assay

The 3' UTR sequence or mutant sequence of LHPP was cloned into the pMIR-REPORT vector. MiRNA mimics or mimic controls were transfected into KYSE-30 together with the reporter plasmid and pRL-SV40.

RNA pull-down assay

RNA pull-down assay was performed as described before [10]. The nucleic acids of LHPP contained in the pulled-down complexes were analyzed by qRT-PCR.

Immunohistochemical detection of LHPP

ESCC tissue was taken and cut into 4 μ m sections. Xylene was deparaffinized, washed 3 times with PBS, and microwaved for 15 min. Primary antibody (LHPP, Abcam, ab116175, 1:500) was added to the sections. Then, add a secondary antibody (1:2000). Color development with DAB, counterstaining with hematoxylin, routine dehydration, transparency, and mounting with neutral resin.

QRT-PCR assay

The total RNA was extracted with Trizol and reversed into cDNA. The cDNA and primers were taken and amplified according to the steps of the reverse transcription kit. The primer sequences are listed in Table 1.

Statistical analysis

All data were sorted in Excel and analyzed by SPSS 19.0. Data were calculated as mean \pm standard deviation and were plotted using GraphPad Prism 6.0. Comparisons between groups were performed using one-way ANOVA and *t*-test.

Results

Flow diagram of early and mid-stage ESCC diagnostic regression model establishment

The flow diagram of this study is shown in Fig. 1. According to uniform inclusion and exclusion criteria, a total of 120 patients with early and mid-stage ESCC were included. These 120 patients were distributed in two cohorts for the model establishment and external verification.

The expression of LHPP in the early and mid-stage ESCC is significantly reduced in the testing cohort and the verification cohort

Compared with paired adjacent normal tissues, LHPP protein levels in the early and mid-stage ESCC tissues were significantly decreased ($P<0.01$, Fig. 2a). Subsequently, we collected 120 ESCC patients and 120 healthy controls (75 ESCC patients and 75 healthy controls in the testing cohort; 45 ESCC patients

and 45 healthy controls in the verification cohort), and detected the LHPP mRNA levels in the plasma (Fig. 2b). Compared with healthy controls, LHPP mRNA levels in the early and mid-stage ESCC were significantly decreased in the testing cohort ($P<0.01$, Fig. 2c) and the verification cohort ($P<0.01$, Fig. 2d).

MiR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, and miR-195-5p are involved in the regulation of LHPP in the early and mid-stage ESCC tissues

We used R package DESeq2 (version 1.6.1) software, and the next-generation sequencing technology results showed that there were 791 miRNA expression differences between early and mid-stage ESCC tissues and paired adjacent normal tissues. Among them, there are 26 miRNAs with differences greater than 1.5-fold change and $P<0.05$ (Fig. 3a). A total of 17 miRNAs are highly expressed and 9 miRNAs are lowly expressed in early and mid-stage ESCC tissues (Fig. 3b). Then, we respectively applied miRanda, miRDB, and Targetsca online prediction systems, and find out the miRNA profiles predicted by each system with LHPP as the target protein (Fig. 3c). A total of 6 miRNAs were predicted, namely miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, miR-195-5p, and miR-6838-5p. Therefore, we finally chose these 6 miRNAs for further research.

Next, we applied the dual-luciferase reporter gene method and RNA pull-down assay to verify the targeting relationships between miR-15b-5p (Fig. 4a), miR-424-5p (Fig. 4b), miR-497-5p (Fig. 4c), miR-363-5p (Fig. 4d), miR-195-5p (Fig. 4e), miR-6838-5p (Fig. 4f), and LHPP. Therefore, we finally chose miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, and miR-195-5p.

MiR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, miR-195-5p, and SCC in early and mid-stage ESCC were significantly increased in the testing cohort and the verification cohort

We then detected above six miRNAs expression levels in 75 early and mid-stage ESCC patients and 75 healthy controls in the testing cohort (Fig. 5), and 45 early and mid-stage ESCC patients and 45 healthy controls in the verification cohort (Fig. 6). Compared with healthy controls, the levels of plasma miR-15b-5p (Fig. 5a, Fig. 6a), miR-424-5p (Fig. 5b, Fig. 6b), miR-497-5p (Fig. 5c, Fig. 6c), miR-363-5p (Fig. 5d, Fig. 6d), miR-195-5p (Fig. 5e, Fig. 6e), and SCC (Table 2) in early and mid-stage ESCC were significantly increased ($P<0.01$), while there was no significant difference in the expression of miR-6838-5p ($P=0.579$, Fig. 5f; $P=0.205$, Fig. 6f).

Establishment of early and mid-stage ESCC diagnostic regression model

For the diagnosis of early and mid-stage ESCC, ROC curve analysis showed that the AUC of miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, miR-195-5p, and SCC were 0.816, 0.778, 0.532, 0.637, 0.803 and 0.702, respectively (Fig. 7 and Table 3). The detailed information of diagnostic performances was listed in Table 3.

Each of the above-mentioned variables was included in the multivariate logistic regression model. The final diagnostic regression model was: Logit (P) = 3.370-0.125(miR-15b-5p)-0.096(miR-424-

5p)-0.103(miR-497-5p)-0.641(miR-363-5p)-1.227(miR-195-5p) -0.513(SCC). The identification value of this model was high with an AUC of 0.851 (Fig. 7 and Table 3), and the probability was 0.725.

External verification of the early and mid-stage ESCC diagnostic regression model

In the external validation group (Table 2), the probabilities of 42 (out of 49) subjects were higher than 0.725, and the probabilities of 38 (out of 41) subjects were lower than 0.725 (Fig. 8). The sensitivity/specificity for early and mid-stage ESCC were 85.7%/92.7%, respectively.

Discussion

At present, the commonly used tumor detection methods, such as X-ray, ultrasound, PET-CT, MRI, and other imaging methods, still have limitations in clinical application due to problems such as accuracy, resulting in delays in tumor detection. In addition, radioactive detection methods, such as X-ray, CT, and PET-CT, are also not suitable for frequent detection of the population. However, the method of puncture is time-consuming, complicated to operate, and causes great harm to the patient's body. In the past ten years, early accurate tumor screening has developed rapidly due to its advantages of early diagnosis and early treatment, non-invasiveness, accuracy, and dynamics. For patients, early accurate tumor screening can not only relieve pain, improve prognosis, and increase the cure rate, but also greatly reduce the cost of treatment and the economic burden.

Studies have found that LHPP has a significant tumor suppressor effect, and its potential tumor suppressor mechanism has been revealed in many cancers [9, 10]. The Cancer Gene Atlas data show that the median survival of bladder cancer patients with low LHPP expression decreased by nearly 2 years in patients with high LHPP expression [10]. After down-regulating the expression of LHPP, the protein histidine phosphorylation level in the cancer cell will increase significantly, which will trigger uncontrolled cancer cell proliferation [10]. However, LHPP expression in ESCC has not been reported. In the current study, we found for the first time that LHPP mRNA levels in early and mid-stage ESCC tissues were significantly lower in both the test and validation cohorts compared with adjacent normal tissues.

Furthermore, we explored LHPP-based miRNA signatures for predicting early and mid-stage ESCC. We performed the next-generation sequencing technology and three online prediction software to screen out 5 miRNAs that target and regulate LHPP, including miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, and miR-195-5p. The above five miRNAs have also been found to function as oncogenes in clinical and basic research on a variety of tumors [11–14]. Dong et al. [11] demonstrated that miR-15b-5p level was upregulated in liver cancer tissues and cell lines. Dai et al. [12] found through experiments *in vivo* and *in vitro* that miR-424-5p promotes colorectal cancer cell proliferation and metastasis. Besides, colorectal cancer cells secret miR-424-5p into peripheral blood through exosomes. Bai et al. [13] found that miR-497-5p can promote the epithelial-mesenchymal transition of colorectal cancer cells *in vitro*. Through the Kaplan-Meier test, Zhang et al. [14] revealed that high expression of miR-363-5p was associated with poor overall survival of hepatocellular carcinoma patients. Previous studies seem to suggest that miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, and miR-195-5p may promote the occurrence and development of

tumors [11–14]. Based on the above hypothesis, can the differential value of these miRNAs for ESCC improve the non-invasive diagnostic ability of ESCC?

In this study, a total of 75 ESCC patients and 75 healthy controls were detected in the testing cohort. We found that the levels of miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, miR-195-5p, and SCC in the plasma of ESCC patients were significantly higher than those in the healthy controls. Our results are consistent with a series of previous studies [11–14], indicating that these markers may play a potential role in promoting ESCC. miRNAs are involved in many processes in the cell life cycle and affect the occurrence and development of tumors. They are present in the blood as nucleic acid-protein complexes that tolerate repeated freeze-thaw cycles and extreme pH environments and are therefore more stable than ctDNA [7]. For tumors with no typical clinical symptoms, non-specific examination, and low early diagnosis rate (such as ESCC), detecting the expression level of specific miRNAs in peripheral blood can screen out a certain type of cancer, thereby avoiding invasive biopsy of tissue cells. This means that tumors can be detected even in the early stages of "asymptomatic" tumors, allowing us to intercept them earlier.

Combined detection of tumor markers is a method recommended by the Updated National Academy of Clinical Biochemistry Guidelines [15]. For tumor detection, the accuracy of detection can be significantly improved by combining several tumor markers with outstanding performance. Taking ovarian cancer as an example [16], although the classic carbohydrate antigen 125 (CA125) marker has a sensitivity of 80% for the diagnosis of ovarian cancer, its positive rate in the early stage is less than 50%, and its specificity is less than 60%. Another indicator of ovarian cancer, human epididymal protein 4 (HE4), has been significantly improved in specificity, with a sensitivity of 67% when the specificity is 96%. The ROMA index calculated by combining CA125 and HE4 has a sensitivity and specificity of 90.1% and 87.7% for ovarian cancer. In general, the combined detection of tumor markers enables the application of tumor markers to be optimized, and the detection sensitivity and accuracy are significantly improved. For this consideration, in this study, we used ROC analysis to analyze multiple combinations of the above six markers. The results showed that the specificity for predicting ESCC was 91.4%, and the sensitivity was 81.6%. The above studies suggest that the combination of miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, miR-195-5p, and SCC in ESCC plasma has clinical value in the evaluation of ESCC.

In conclusion, this study found that the combined detection of plasma miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, miR-195-5p, and SCC in ESCC has an early warning effect on the risk assessment of early and mid-stage ESCC patients.

Declarations

Funding

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Xiang Zhao, , Xiaocun Zhu, Luoshai Wang, Yurao Chen, Ronghuai Chen, Zemao Zheng, Hengjin Yang, and Wan Xia. The first draft of the manuscript was written by Xiang Zhao, Juan Yao, and Kun Zhao. All authors read and approved the final manuscript.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval

Human study was approved by the Ethics Committee of The Huai'an Hospital of Huai'an City (no.: k16.0112) and Taizhou People's Hospital Affiliated to Nantong University (no.: ks201601-002-1).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

Not applicable.

Acknowledgments

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References

1. Kong P, Xu E, Bi Y, Xu X, Liu X, Song B, Zhang L, Cheng C, Yan T, Qian Y, Yang J, Ma Y, Cui H, Zhai Y, Zou B, Liu X, Cheng Y, Guo S, Cheng X, Cui Y (2020) Novel ESCC-related gene ZNF750 as potential Prognostic biomarker and inhibits Epithelial-Mesenchymal Transition through directly depressing SNAI1 promoter in ESCC. *Theranostics* 10:1798–1813. doi: 10.7150/thno.38210.
2. Hu Z, Ke C, Shen Y, Zeng X, Yang C (2021) Renal metastases from esophageal cancer and retroperitoneal lymphoma detected via chromosome duplications identified by fluorescence in situ

- hybridization in urine exfoliated cells: First 2 case reports.** Medicine (Baltimore) **100**:e24010. doi: 10.1097/MD.00000000000024010.
3. Wang Y, Xie Z, Lu H (2020) **Significance of halofuginone in esophageal squamous carcinoma cell apoptosis through HIF-1 α -FOXO3a pathway.** Life Sci **257**:118104. doi: 10.1016/j.lfs.2020.118104.
 4. Shin S, Jung Y, Uhm H, Song M, Son S, Goo J, Jeong C, Song JJ, Kim VN, Hohng S (2020) **Quantification of purified endogenous miRNAs with high sensitivity and specificity.** Nat Commun **11**:6033. doi: 10.1038/s41467-020-19865-9.
 5. Luo D, Huang Z, Lv H, Wang Y, Sun W, Sun X (2020) **Up-Regulation of MicroRNA-21 Indicates Poor Prognosis and Promotes Cell Proliferation in Esophageal Squamous Cell Carcinoma via Upregulation of lncRNA SNHG1.** Cancer Manag Res **12**:1–14. doi: 10.2147/CMAR.S221731.
 6. Yang H, Su H, Hu N, Wang C, Wang L, Giffen C, Goldstein AM, Lee MP, Taylor PR (2020) **Integrated analysis of genome-wide miRNAs and targeted gene expression in esophageal squamous cell carcinoma (ESCC) and relation to prognosis.** BMC Cancer **20**:388. doi: 10.1186/s12885-020-06901-6.
 7. Xian Q, Zhao R, Fu J (2020) **MicroRNA-527 Induces Proliferation and Cell Cycle in Esophageal Squamous Cell Carcinoma Cells by Repressing PH Domain Leucine-Rich-Repeats Protein Phosphatase 2.** Dose Response **18**:1559325820928687. doi: 10.1177/1559325820928687.
 8. Zhang N, Liu JF (2020) **MicroRNA (MiR)-301a-3p regulates the proliferation of esophageal squamous cells via targeting PTEN.** Bioengineered **11**:972–983. doi: 10.1080/21655979.2020.1814658.
 9. Hindupur SK, Colombi M, Fuhs SR, Matter MS, Guri Y, Adam K, Cornu M, Piscuoglio S, Ng CKY, Betz C, Liko D, Quagliata L, Moes S, Jenoe P, Terracciano LM, Heim MH, Hunter T, Hall MN (2018) **The protein histidine phosphatase LHPP is a tumour suppressor.** Nature **555**:678–682. doi: 10.1038/nature26140.
 10. Li Y, Zhang X, Zhou X, Zhang X (2019) **LHPP suppresses bladder cancer cell proliferation and growth via inactivating AKT/p65 signaling pathway.** Biosci Rep **39**:BSR20182270. doi: 10.1042/BSR20182270.
 11. Dong Y, Zhang N, Zhao S, Chen X, Li F, Tao X (2019) **miR-221-3p and miR-15b-5p promote cell proliferation and invasion by targeting Axin2 in liver cancer.** Oncol Lett **18**:6491–6500. doi: 10.3892/ol.2019.11056.
 12. Dai W, Zhou J, Wang H, Zhang M, Yang X, Song W (2020) **miR-424-5p promotes the proliferation and metastasis of colorectal cancer by directly targeting SCN4B.** Pathol Res Pract **216**:152731. doi: 10.1016/j.prp.2019.152731.
 13. Bai J, Xu J, Zhao J, Zhang R (2020) **lncRNA SNHG1 cooperated with miR-497/miR-195-5p to modify epithelial-mesenchymal transition underlying colorectal cancer exacerbation.** J Cell Physiol **235**:1453–1468. doi: 10.1002/jcp.29065.
 14. Zhang J, Fan J, Zhou C, Qi Y (2017) **miR-363-5p as potential prognostic marker for hepatocellular carcinoma indicated by weighted co-expression network analysis of miRNAs and mRNA.** BMC Gastroenterol **17**:81. doi: 10.1186/s12876-017-0637-2.

15. Di Fiore R, Suleiman S, Ellul B, O'Toole SA, Savona-Ventura C, Felix A, Napolioni V, Conlon NT, Kahramanoglu I, Azzopardi MJ, Dalmas M, Calleja N, Brincat MR, Muscat-Baron Y, Sabol M, Dimitrieva V, Yordanov A, Vasileva-Slaveva M, von Brockdorff K, Micallef RA, Kubelac P, Achimash Cadariu P, Vlad C, Tzortzatou O, Poka R, Giordano A, Felice A, Reed N, Herrington CS, Faraggi D, Calleja-Agius J (2021) GYNOCARE Update: Modern Strategies to Improve Diagnosis and Treatment of Rare Gynecologic Tumors—Current Challenges and Future Directions. *Cancers (Basel)* 13:493. doi: 10.3390/cancers13030493.
16. Wang H, Liu P, Xu H, Dai H (2021) Early diagnosis of ovarian cancer: serum HE4, CA125 and ROMA model. *Am J Transl Res* 13:14141–14148.

Tables

Table 1 Primer sequences

Gene	Primers
LHPP	F: 5'-AGGCTGGGATTTGACATCTC-3' R: 5'-AGCAGGTATGGTCGCAGG-3'
miR-15b-5p	F: 5'-ATCCAGTGCCTGTCGTG-3' R: 5'-TGCTTAGCAGCACATCATG-3'
miR-424-5p	F: 5'-GCGCCAGCAGCAATTGATG-3' R: 5'-CAGCCACAAAAGAGCACAAT-3'
miR-497-5p	F: 5'-CCTTCAGCAGCACACTGTGG-3' R: 5'-CAGTGCAGGGTCCGAGGTAT-3'
miR-363-5p	F: 5'-GCAGCAACTAGAAACG-3' R: 5'-GCACTCATGCCATTGATCC-3'
miR-195-5p	F: 5'-GGAGTGTAGGCCAATACCAGA-3' R: 5'-TGCCACTTAGCAGCACAGAAA-3'
miR-6838-5p	F: 5'-GCACTCCTGGATGCCAATCT-3' R: 5'-CTCTACAGCTATATTGCCAGCCAC-3'
GAPDH	F: 5'-GCACCGTCAAGGCTGAGAAC-3' R: 5'-TGGTGAAGACGCCAGTGG-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTGCGT-3'

Table 2 Clinicopathological characteristics and clinical tumor markers in the testing cohort and the verification cohort

Variables	Testing cohort				Verification cohort			
	Healthy	Early and mid- stage ESCC	χ^2/Z	P	Healthy	Early and mid- stage ESCC	χ^2/Z	P
Number of subjects	75	75			45	45		
Age, median (IQR, year)	63 (52, 76)	65 (54, 77)	1.035	0.195	64 (51–74)	66 (53–78)	1.265	0.202
Male (%)	44 (58.67%)	42 (56.00%)	0.109	0.741	28 (62.22%)	31 (68.89%)	0.443	0.506
Smoking (%)	37 (49.33%)	41 (54.67%)	0.427	0.513	25 (55.56%)	29 (64.44%)	0.741	0.389
Drinking (%)	51 (68.00%)	48 (64.00%)	0.267	0.605	32 (71.11%)	35 (77.78%)	0.526	0.468
SCC (median, IQR, $\mu\text{g/L}$)	0.98 (0.65, 1.82)	3.66 (1.76, 6.47)	6.845	<0.001	1.13 (0.59, 1.77)	3.75 (1.82, 6.98)	7.638	<0.001
CEA (median, IQR, U/ml)	6.49 (5.48, 8.19)	6.73 (5.98, 8.54)	0.507	0.416	6.24 (5.31, 7.98)	6.32 (5.43, 9.05)	0.426	0.367
CA19-9 (median, IQR, U/mL)	27.64 (22.70, 34.82)	28.47 (21.19, 37.83)	0.876	0.197	25.04 (19.87, 31.18)	27.84 (20.70, 32.76)	0.343	0.214
The habit of eating preserved food (%)	59 (78.67%)	63 (84.00%)	0.703	0.402	39 (86.67%)	38 (84.44%)	0.090	0.764

Table 3 The diagnostic value of miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, miR-195-5p, and SCC for the early and mid-stage ESCC in the testing cohort (n = 75).

Variables	AUC	95% CI	P	Sensitivity (%)	Specificity (%)
miR-15b-5p	0.816	0.765-0.983	<0.001	75.9	91.7
miR-424-5p	0.778	0.688-0.862	<0.001	69.1	89.3
miR-497-5p	0.532	0.468-0.593	0.331	34.4	83.7
miR-363-5p	0.637	0.574-0.762	0.002	74.1	43.9
miR-195-5p	0.803	0.713-0.879	<0.001	85.3	60.9
SCC	0.702	0.593-0.814	<0.001	54.5	83.2
Diagnostic regression model	0.851	0.815-0.910	<0.001	81.6	91.4

Figures

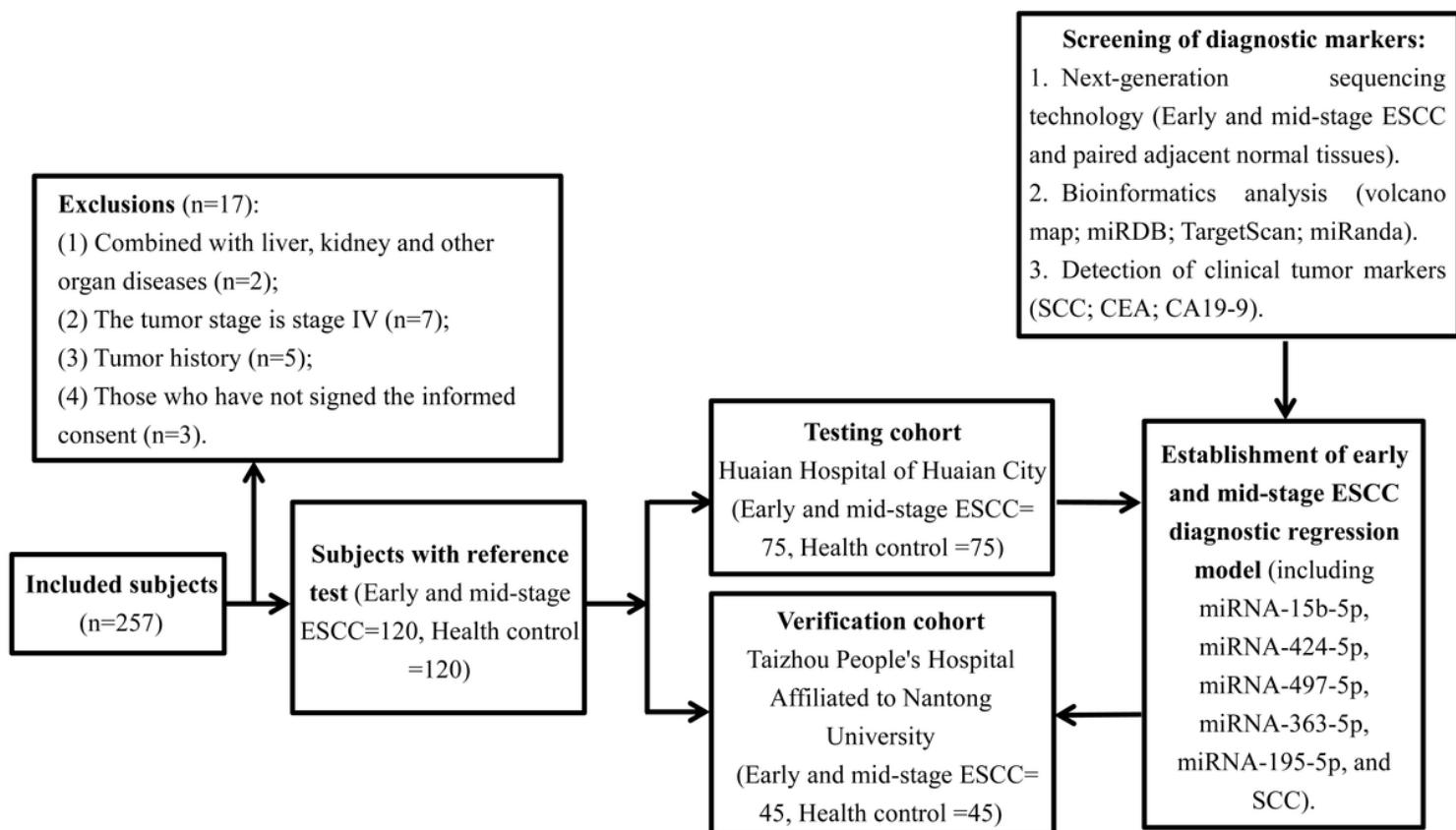


Figure 1

Flow diagram of early and mid-stage ESCC diagnostic regression model establishment.

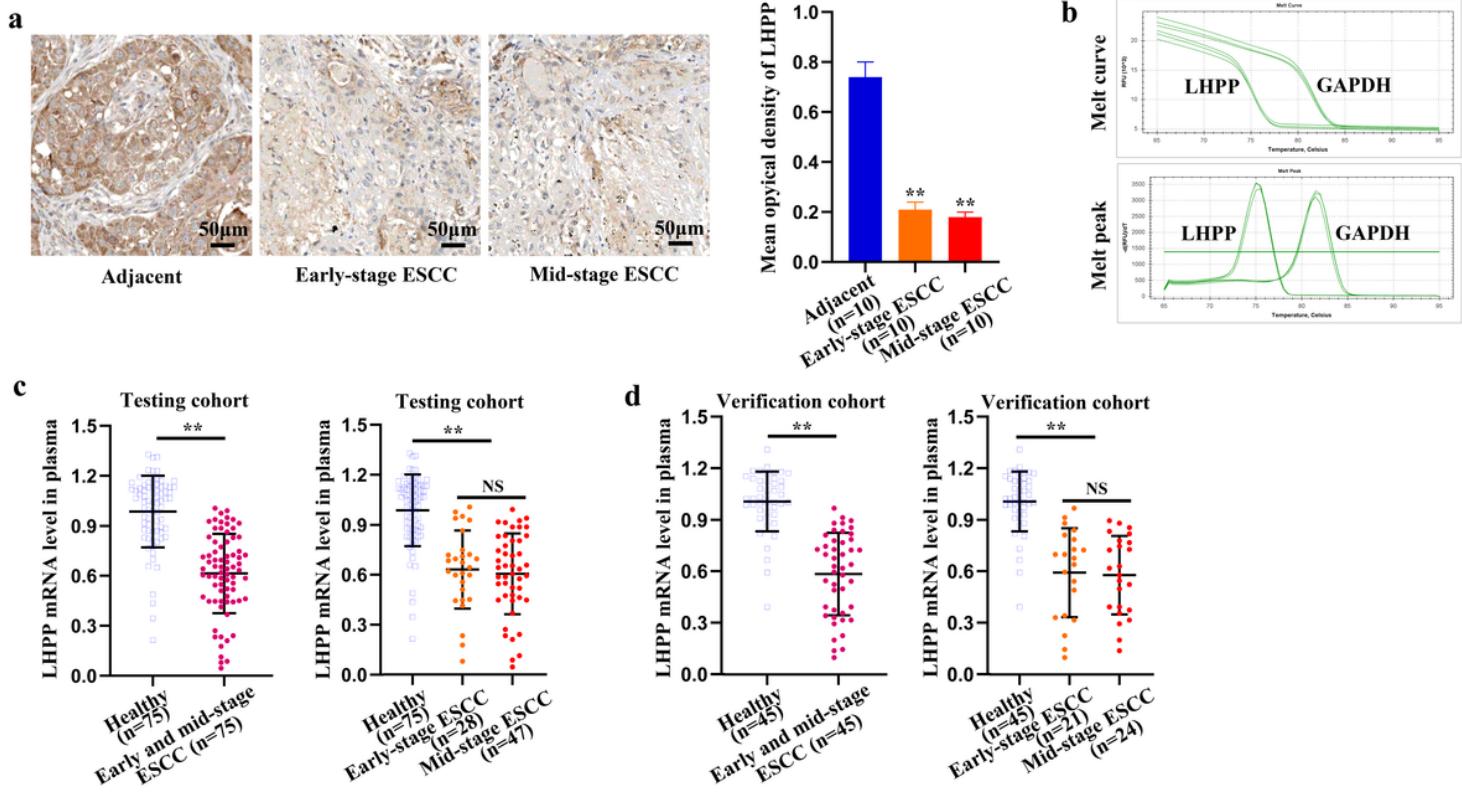


Figure 2

The protein and mRNA levels of LHPP in the early and mid-stage ESCC are significantly decreased. **(a)** Compared with paired adjacent normal tissues, LHPP protein levels in the early and mid-stage ESCC tissues were significantly decreased. Scare bar=50μm. **(b)** The qRT-PCR amplification curve of LHPP and GAPDH. **(c)** Compared with the healthy controls, the LHPP mRNA levers in the early and mid-stage ESCC were significantly decreased in the testing cohort. **(d)** Compared with the healthy controls, the LHPP mRNA levers in the early and mid-stage ESCC were significantly decreased in the verification cohort. $2^{-\Delta\Delta CT}$ method was used. ** Compared with the healthy controls, $P<0.01$.

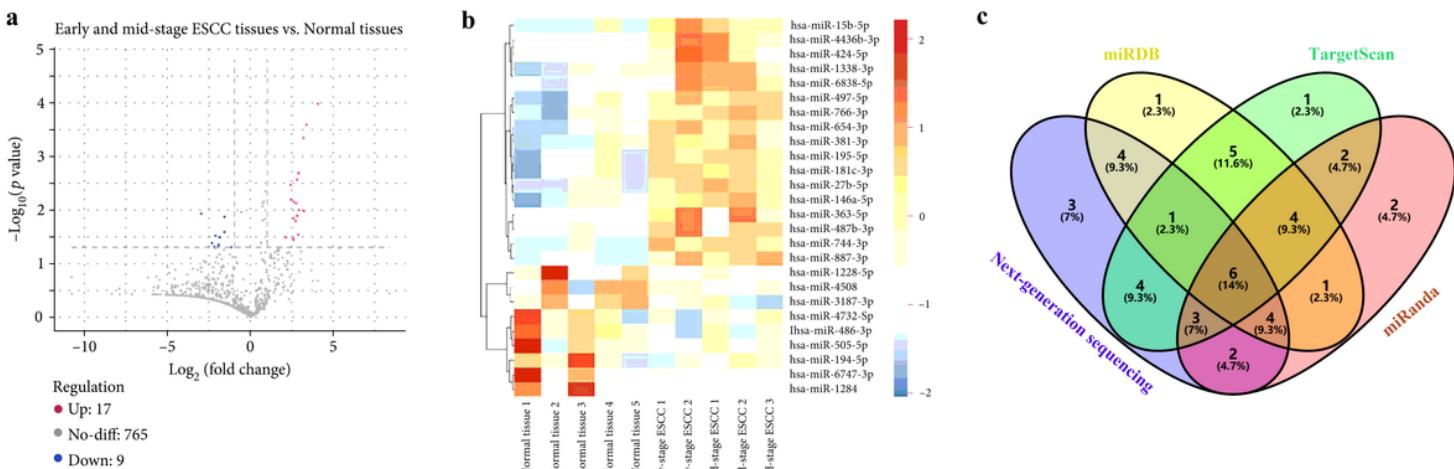


Figure 3

Next-generation sequencing technology and bioinformatics technology to analyze the targeted regulation miRNAs of LHPP. **(a-b)** Next-generation sequencing technology and volcano map of differentially expressed genes between early and mid-stage ESCC tissues and paired adjacent normal tissues. The red nodes represent up-regulation, and the blue nodes represent down-regulation. **(c)** Venn diagram was used to analyze the target miRNAs through miRanda, miRDB, and Targetscan online prediction systems.

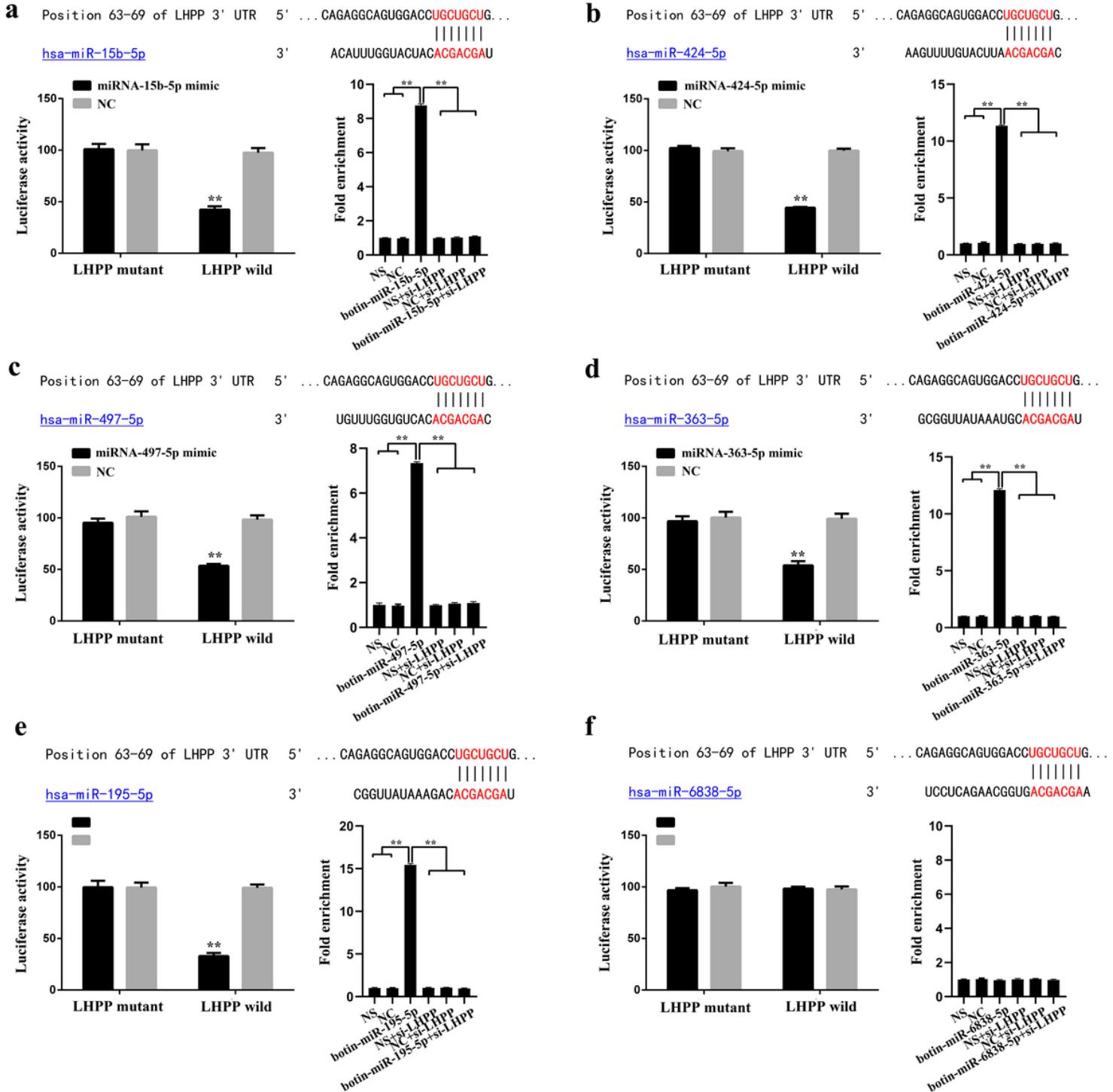


Figure 4

MiR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, miR-195-5p are involved in the regulation of LHPP expression. Luciferase reporter gene method and RNA pull-down assay were used to explore the relationship between LHPP and miR-15b-5p (**a**), miR-424-5p (**b**), miR-497-5p (**c**), miR-363-5p (**d**), miR-195-5p (**e**), and miR-6838-5p (**f**). **, $P < 0.01$.

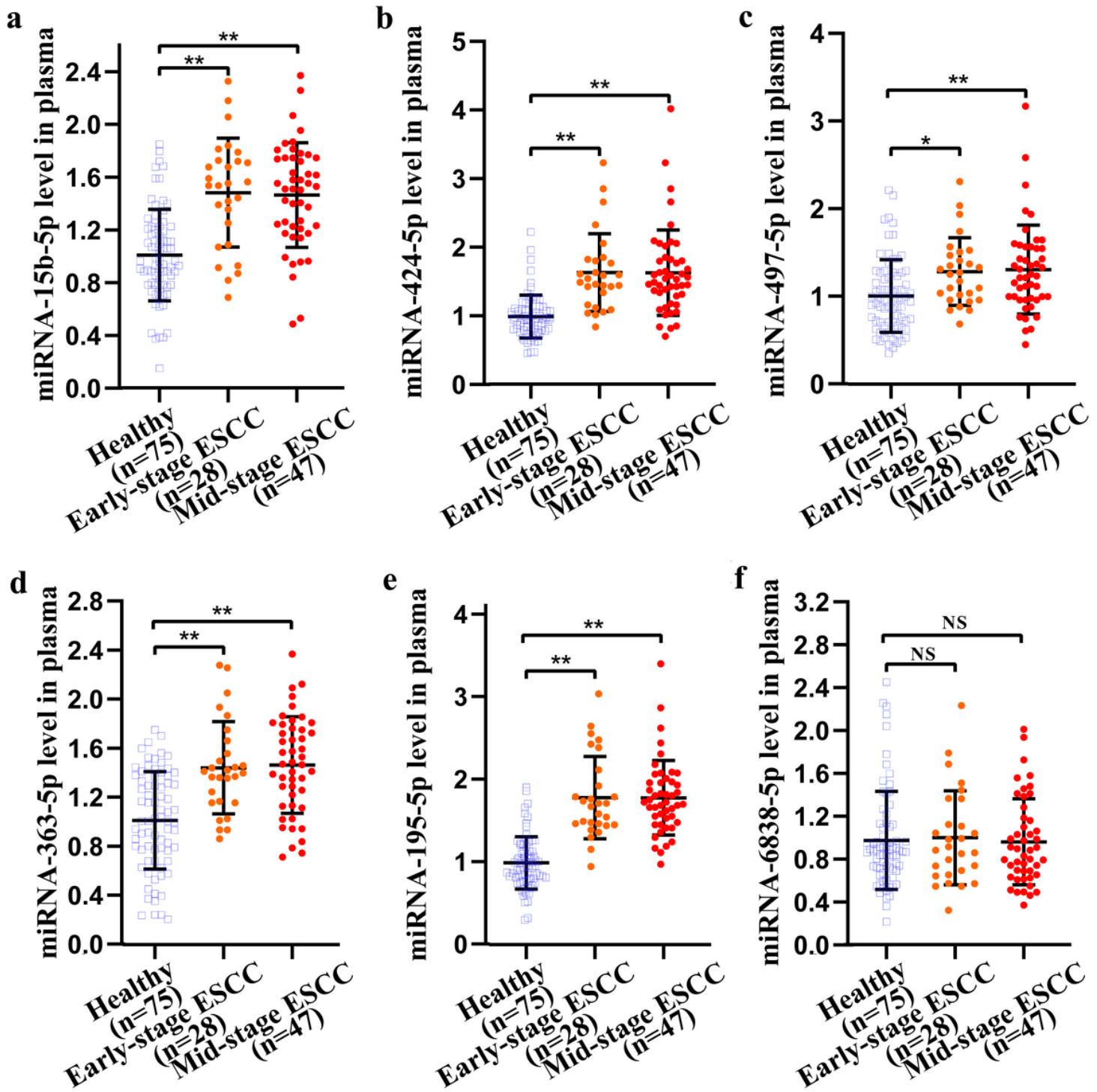


Figure 5

Plasma miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, and miR-195-5p levels of early and mid-stage ESCC patients in the testing cohort. Compared with healthy controls, the levels of plasma miR-15b-5p (**a**),

miR-424-5p (**b**), miR-497-5p (**c**), miR-363-5p (**d**), and miR-195-5p (**e**) in early and mid-stage ESCC were significantly increased ($P<0.01$), while there was no significant difference in the expression of miR-6838-5p (**f**).

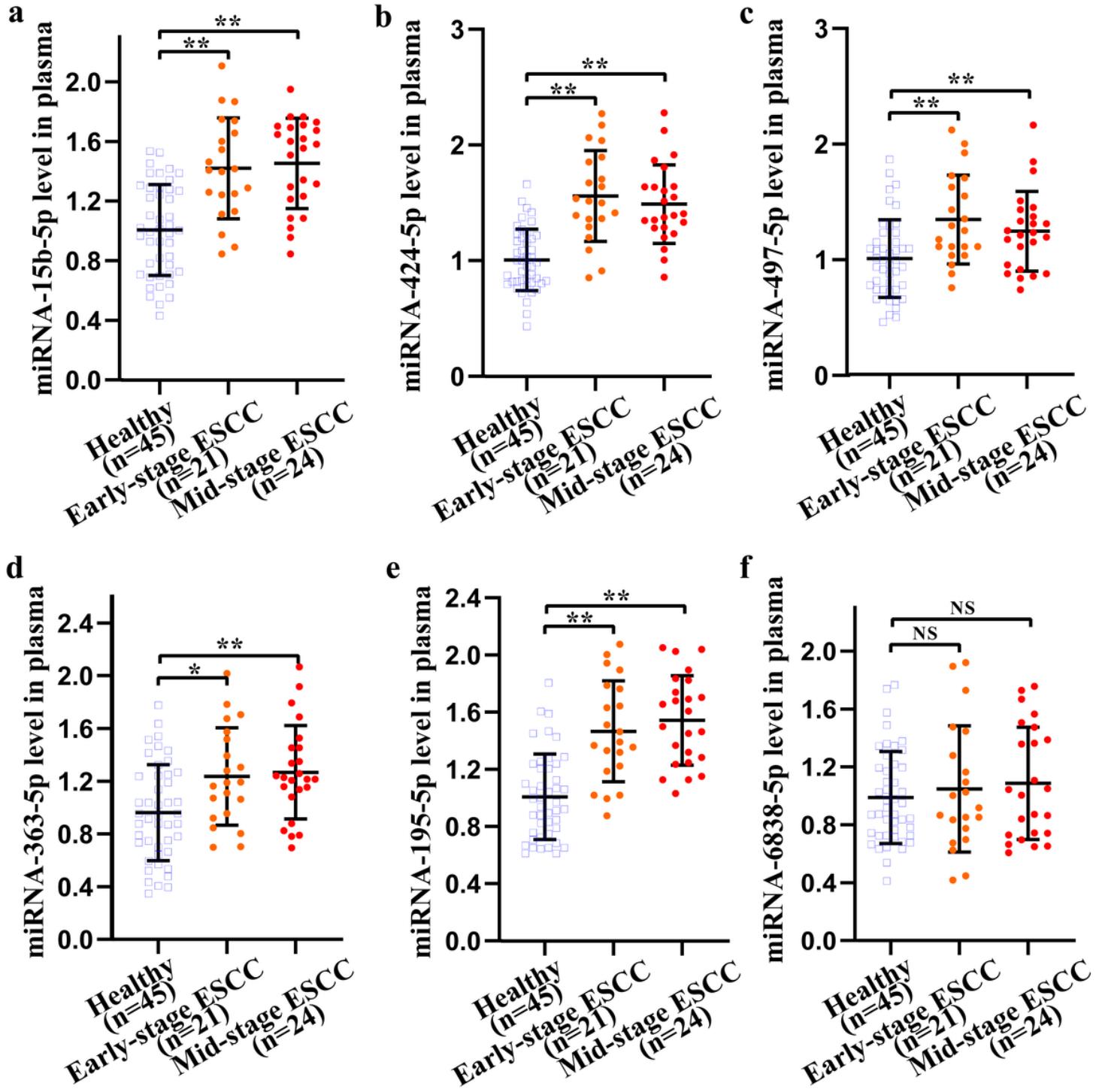


Figure 6

Plasma miR-15b-5p, miR-424-5p, miR-497-5p, miR-363-5p, and miR-195-5p levels of early and mid-stage ESCC patients in the verification cohort. Compared with healthy controls, the levels of plasma miR-15b-5p (**a**), miR-424-5p (**b**), miR-497-5p (**c**), miR-363-5p (**d**), and miR-195-5p (**e**) in early and mid-stage ESCC were

significantly increased ($P<0.01$), while there was no significant difference in the expression of miR-6838-5p (f).

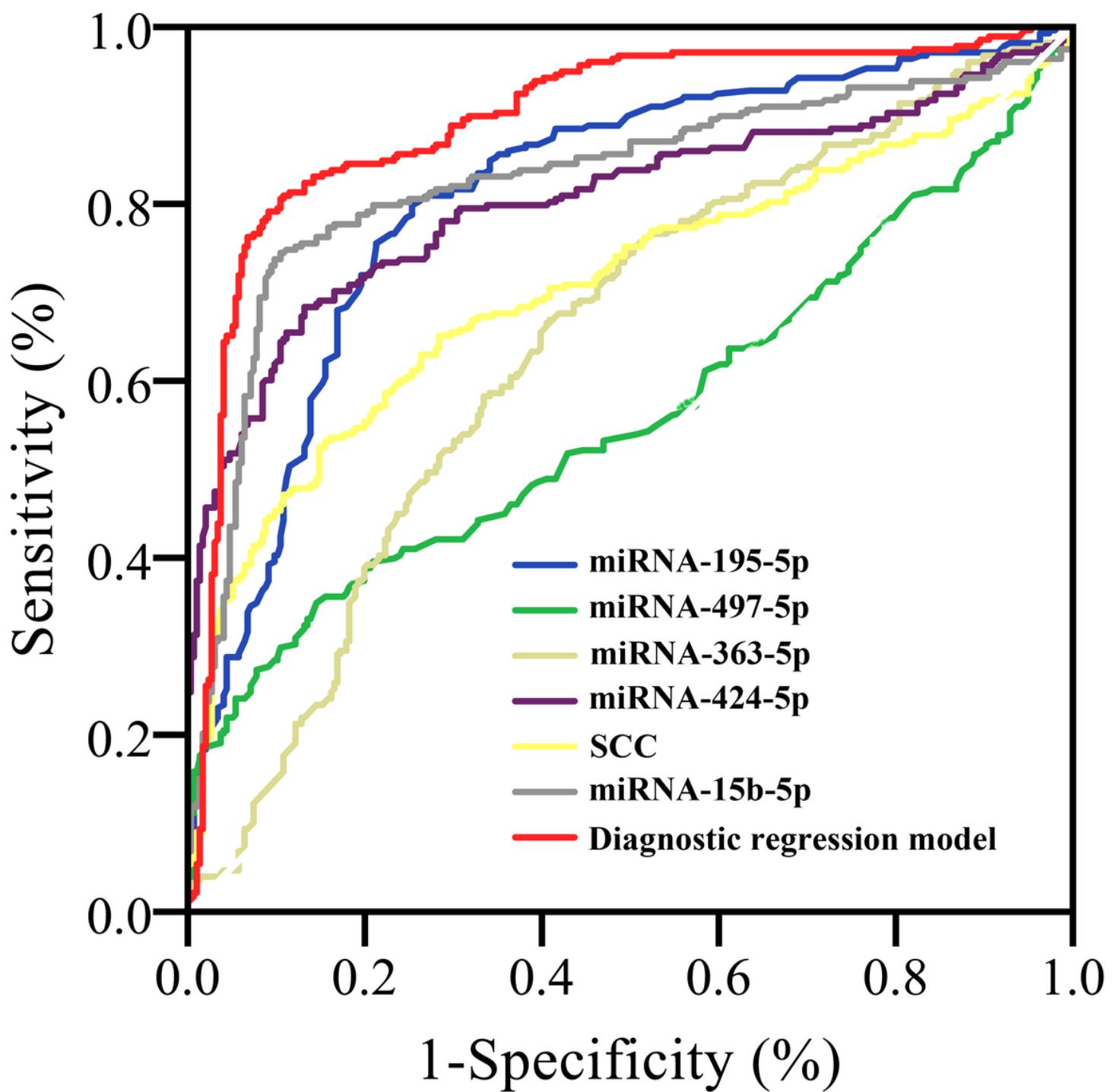


Figure 7

Establishment of early and mid-stage ESCC diagnostic regression model. SCC: squamous cell carcinoma antigen.

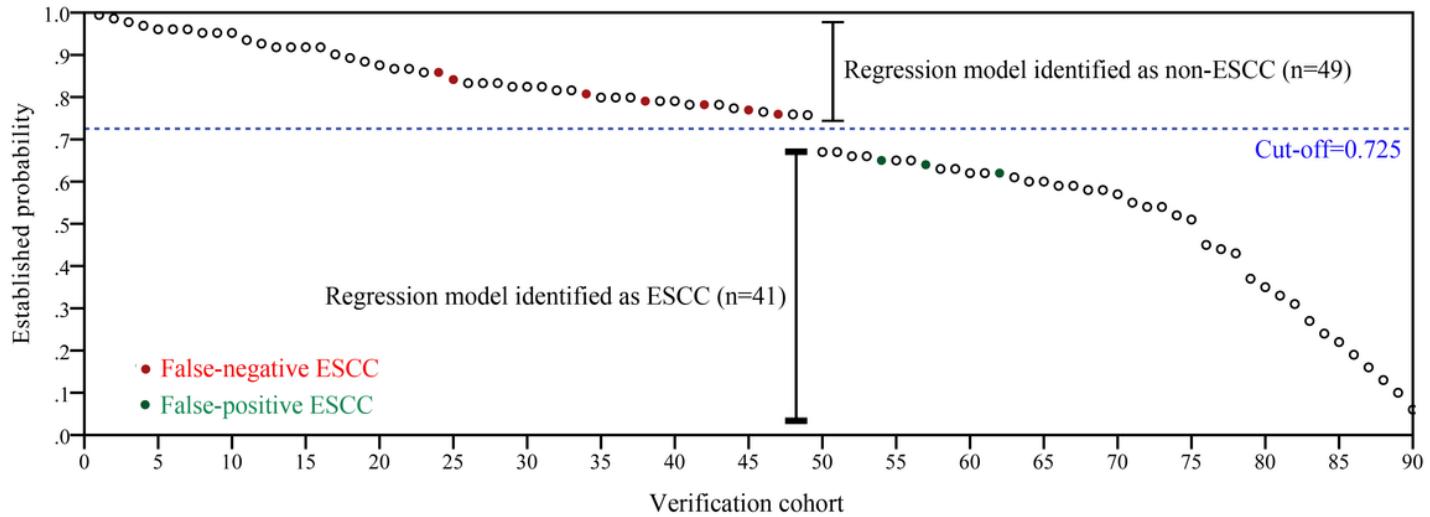


Figure 8

The scatter diagram of the predictive model in an external validation cohort.