INTRODUCTION

Esophageal cancer (EC) is a common malignancy of the digestive tract, with high incidence. According to Zheng et al., its incidence rate in China has reached 19.9%. Furthermore, recent studies have reported that EC tends to affect the young population. Moreover, EC is one of the leading malignancies in terms of lethal rate. In previous studies, the 5-year mortality rate of EC patients has been reported to be as high as 33.51%. EC in its early stage does not cause any distinct clinical symptoms and is thus easily overlooked by patients. At diagnosis, tumors are found to have already reached the middle and late stages. Thus, the tumor must be detected and
treated early to effectively prevent EC. Due to the limited available methods for EC examination, its diagnosis needs to be confirmed via biopsy, and low specificity of tumor marker detection is not reliable for early EC screening. Thus, researchers worldwide are constantly working to identify new EC markers; however, significant results have not yet been achieved.

With the advancement of research, increasing number of studies have pointed out that microRNAs (mRNAs) may be the key to tumorigenesis. mRNAs are 22-nt-long noncoding short-chain RNAs. The function of mRNA is to combine the untranslated region at the 3′ end of its downstream target gene mRNA, causing changes in the translation and transcription processes of the target gene. miR-630 is a member of the mRNA family. In previous studies, miR-630 has been confirmed to be closely related to the occurrence and development of gastric cancer, breast cancer, and other tumors, but its role in EC remains unclear. Feng et al. reported that miR-630 may be the key factor involved in gastric cancer; this led us to speculate that miR-630 has the same effect in EC, which is also a digestive tract tumor, and to perform an experimental analysis to verify our conjecture and provide reference and guidance for future clinical diagnosis and treatment of EC.

2 | MATERIALS AND METHODS

2.1 | General data

In total, 58 EC patients admitted to our hospital and 60 healthy people visiting the hospital from April 2014 to 2016 were selected as study participants. EC patients constituted the study group, and healthy people constituted the control group. This study was conducted after obtaining approval from the First Hospital Affiliated to AMU's ethics committee and obtaining written informed consent from the participants.

2.2 | Cell data

Esophageal cancer cell lines KYSE-150, KYSE-450, and ECA109 and normal esophageal HEEC cell lines were purchased from ATCC company.

2.3 | Inclusion and exclusion criteria

Inclusion criteria were as follows: patients who exhibited clinical manifestations of EC and in whom EC was confirmed via biopsy performed at the pathology department of our hospital; those with tumor in the early and middle stages; those with complete case data; those who agreed to cooperate and participate in the investigations performed by medical staff at our hospital; and those aged 20–70 years. Exclusion criteria were as follows: patients with multiple tumors, other cardiovascular and cerebrovascular diseases, autoimmune deficiency diseases, infectious diseases, mental disorders, dysfunction of other organs, and physical disabilities, making them unable to take care of themselves. The characteristics of the control group patients were included in the exclusion criteria, that is, patients with normal physical examination findings and without any major previous medical history.

2.4 | Method

All the EC patients underwent surgery and postoperative chemotherapy at our hospital. Surgery was performed by senior surgeons. Pre and postoperatively, 4 ml of fasting venous blood was collected from the patients; the blood sample was kept at room temperature for 30 min and centrifuged for 10 min (4,000 rpm/min). The supernatant serum was detected using polymerase chain reaction (PCR). Total RNA was extracted, and its purity, concentration, and integrity were detected using an ultraviolet spectrophotometer and agarose gel electrophoresis. TransScript Green miRNA Two-Step qRT-PCR SuperMix was used for the reverse transcription of the extracted total RNA. The steps were performed following the manufacturer’s instructions, and complementary DNA was collected for PCR amplification. Each sample was provided with three repeated wells, and the experiment was performed three times. U6 was used as internal reference, and $2^{-\Delta\Delta C_T}$ was used to analyze the data.

2.5 | Cell culture

Esophageal cancer cells were cultured at 37°C. When the adherent growth and fusion of the cells reached 85%, 25% pancreatin was added for digestion. After digestion, the cells were added to the medium for continuous culture and passage. After passage, the cells were used for detecting miR-630 expression through PCR. miR-630 expression in the ECA109 cell line was found to be considerably high; thus, this cell line was selected for transfection and subsequent experiments. miR-630-mimics (overexpression sequence), miR-negative control (miR-NC), and miR-630-inhibit (inhibition sequence) were used to transfect the cells using Lipofectamine™ 2000
kit, and the operation steps were carefully completed following the manufacturer’s instructions.

2.6 | Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from tissues or cultured cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Total RNA (5 µg) was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) and supplemented with Oligo(dT)18 RT primers (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was subsequently performed using SYBR Premix Ex Taq (Takara Bio, Inc.) using a Bio-Rad CFX96 real-time PCR System (Bio-Rad Laboratories, Inc.). The conditions of qPCR were as follows: 94°C for 15 min, followed by 45 cycles of 94°C for 10 sec, 60°C for 30 sec, and 72°C for 30 sec. Each sample assayed in triplicate in three independent experiments. The mRNA levels were quantified using the 2−∆∆Cq method and normalized to internal reference gene U6.

2.7 | Cell scratch test

Transfected cells were inoculated into 6-well plates at a density of 2 × 10⁵ cells per well. After 24 h, a line along the diameter of the 6-hole plate was scratched using the small tip of a pipette. The floating cells were lightly washed with phosphate-buffered saline (PBS). Under a 20-fold microscope, five fields of view were randomly selected for each hole and images obtained. The cells were then cultured in a serum-free culture medium. After another 24 and 48 h, images were obtained in the same manner.

2.8 | Transwell test

Cells transfected for 24 h were collected, adjusted to a density of 5 × 10⁴ cells, inoculated on a 6-well plate, washed twice with PBS, and inoculated in the upper chamber. A total of 200 µl Dulbecco’s Modified Eagle Medium (DMEM) culture solution was added to the upper chamber and 500 ml of DMEM (containing 20% FBS) added to the lower chamber. Cells were fixed in paraformaldehyde for 10 min, washed thrice with double-distilled water, and stained with 0.5% crystal violet after it was dried; following this, cell invasion was observed under a microscope.

2.9 | Observation index

miR-630 expression levels were compared between the two groups, and a receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of miR-630 for EC. The relationship between miR-630 expression and EC clinical pathology was analyzed. The patients were followed up for 3 years; patient survival was recorded, and the influence of miR-630 expression on prognosis was assessed. miR-630 expression in the EC cell line was observed to determine its influence on EC cell migration and invasion.

2.10 | Statistical method

SPSS22.0 was used to analyze and process the data and Graphpad8 to visualize the graph results. Countable data were expressed as numbers and percentages. Chi-square test was used for between-group comparisons. Measurement data were expressed as mean ± standard deviation, and t-test was adopted for within-group comparisons. Comparison at multiple time points was performed using repeated-measures analysis of variance and Bonferroni correction. The diagnostic predictive value was analyzed using the ROC curve analysis. Moreover, survival rate was calculated using Kaplan–Meier method and compared using log-rank test. A p-value of < 0.05 was considered significant.

3 | RESULTS

3.1 | General data comparison

There were no significant between-group differences in age, body mass index (BMI), sex, living environment, smoking, digestive tract disease history, and nationality (p > 0.050) (Table 2).

3.2 | Comparison of miR-630 expression levels

Serum miR-630 expression levels were significantly lower in the study group than that in the control group (p < 0.05) (Figure 1).

3.3 | Diagnostic value of miR-630 for EC

The ROC curve analysis showed that when cutoff value was 5.38, the diagnostic sensitivity and specificity of miR-630 for EC were 73.33% and 76.67%, respectively, area under the ROC curve was 0.778 (95%CI 0.695–0.861) (Figure 2).

3.4 | Correlation between miR-630 expression and EC clinicopathology

miR-630 expression showed no association with age, sex, BMI, living environment, smoking, digestive tract disease history, and nationality of EC patients (p > 0.05); however, it was closely related to disease course, pathological stage, differentiation degree, and tumor metastasis (p < 0.05) (Table 3).
Influence of miR-630 expression on prognosis

The 54 EC patients were successfully followed up, with a 93.10% follow-up success rate. On the basis of the median miR-630 expression level, 82 patients were divided into the miR-630 low-expression (miR-630 < 4.56; n = 29) and miR-630 high-expression (miR-630 ≥ 4.56; n = 31) groups. A comparison of the prognosis of the two groups for 3 years showed that the prognosis of the miR-630 low-expression group was significantly worse than that of the miR-630 high-expression group (p < 0.05) (Figure 3).

miR-630 expression in EC cell lines

Among the EC cell lines, the lowest miR-630 expression level was detected in the ECA109 cell line (p < 0.05); thus, this cell line was selected for subsequent experiments. miR-630-mimics (overexpression sequence), miR-NC, and miR-630-inhibit (inhibition sequence) were transfected. The cell scratch test showed that the cell migration ability in the miR-630-mimics group was significantly reduced (p < 0.05); moreover, the Transwell experiment showed that the cell invasion ability in this group was significantly reduced (p < 0.05) (Figure 4).

DISCUSSION

Esophageal cancer is one of the most common digestive tract tumors, and the mortality rate of EC patients remains high. Recent research has been focused on finding a new diagnostic method and treatment for EC. Arnold et al. have proposed that EC can be effectively predicted through histological subtyping in the future. Tian et al. have reported circulating tumor DNA 5-hydroxymethylcytosine to be a new diagnostic marker for EC. However, the use of this marker is not clinically applicable due to high testing requirements and high cost. However, as a major research hotspot in clinical practice, miRNAs have the advantages...
of convenient detection and low testing requirements. Once the exact mechanism of action of mRNA in tumor is detected, it can not only be used as a diagnostic marker in future clinical practice but also as an auxiliary index to evaluate patient rehabilitation and prognosis, indicating its importance in clinical application. However, the present study aimed to provide a new reference direction for clinical diagnosis and treatment of EC by determining the role of miR-630 in EC and its influence on EC cell migration and invasion.

The experimental results showed low miR-630 expression in EC, suggesting that miR-630 is involved in EC occurrence and development; this is consistent with the findings reported by Bertoli et al. on the role of miR-630 in prostate cancer. miR-630 has been proved to play a major role as a tumor suppressor gene in other tumors, and the present study found that miR-630 is closely related to disease course, differentiation degree, pathological staging, and metastasis of EC, further confirming the relationship between miR-630 and EC development. Furthermore, in the ROC curve analysis, miR-630 showed good diagnostic value for predicting EC occurrence, suggesting that miR-630 is an excellent indicator for future clinical diagnosis of EC. The recent in-depth studies on mRNAs have led increasing number of scholars to propose that mRNA detection may be a key to tumor screening in the future. Compared with traditional cancer markers, mRNAs can also be detected in the peripheral blood, and the detection sample can be conveniently obtained. Moreover, different mRNAs exhibit different reactions to various tumors and exhibit a higher clinical differential value than cancer markers that possess high sensitivity but lack specificity. In addition,
compared with detection using imaging, the detection results obtained using miRNAs are more intuitive and do not require reliance on human experience for assessment, thus reducing the rates of misdiagnosis and missed diagnosis caused due to human factors to a certain extent. The prognostic follow-up of EC patients showed that those with low miR-630 expression have the worse prognosis, confirming that miR-630 is significantly associated with EC prognosis. This finding is consistent with the study results of Chen et al., 24 supporting our point of view.

To further clarify the effect of miR-630 on EC, the biological behavior changes in EC cells were analyzed by transflecting miR-630 into EC cell lines. The results showed low miR-630 expression in EC cell lines, proving the accuracy of earlier detection results. The ECA109 cell line, with the largest expression difference, was selected for transfection analysis. It was found that miR-630 overexpression can inhibit EC cell invasion and migration, suggesting that miR-630 acts as a tumor suppressor gene in EC, consistent with a previous study findings.25 However, Zhang et al.26 showed that miR-630 can promote epithelial ovarian cancer proliferation and invasion by targeting KLF6, indicating that miR-630 exerts different biological effects in different tumors; however, this needs to be confirmed in further experimental analyses. In addition, some studies have
reported that miR-630 can affect the biological behavior of nasopharyngeal carcinoma by targeting EZH2. EZH2 has been proven to be closely related to the poor prognosis of EC. Therefore, the influence of miR-630 on EZH2 cells may also be related to EZH2. However, due to the limited experimental conditions, no follow-up research could be performed. This will be the focus of our future research for further analysis and discussion.

Because we also failed to perform tumor-forming experiments in nude mice, the effect of miR-630 on actual tumor formation could not be determined. Moreover, the gene, protein, and signal pathways in the human body are extremely complex; therefore, the exact mechanism through which miR-630 affects EC remains unclear. In the future, we will perform more sound experimental analysis to overcome the abovementioned limitations.

To sum up, miR-630 expression is low in EC and has good diagnostic value for this tumor. miR-630 overexpression can decrease EC cell invasion and migration, indicating that miR-630 will be the key to the diagnosis and treatment of EC in the future.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

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

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How to cite this article: Liu X, Wu W, Zhang S, et al. Effect of miR-630 expression on esophageal cancer cell invasion and migration. J Clin Lab Anal. 2021;35:e23815. https://doi.org/10.1002/jcla.23815