Original Article

Evaluating the miR-302b and miR-145 Expression in Formalin-Fixed Paraffin-Embedded Samples of Esophageal Squamous Cell Carcinoma

Mojtaba Tabrizi MSc[1], Mitra Khalili PhD[2], Mohammad Vasei MD[3], Nazila Nouraee PhD[1], Nader Mansour Samaei MD[4], Ali Khavanin MD[5], Mehrdad Khajehei MD[6], Seyed Javad Mowla PhD•[1]

Abstract

Background: MicroRNAs are involved in key cellular processes regulating, and their misregulation is linked to cancer. The miR-302-367 cluster is exclusively expressed in embryonic stem and carcinoma cells. This cluster also promotes cell reprogramming and stemness process. In contrast, miR-145 is mostly regarded as a tumor suppressor, where it regulates cellular functions such as cell division, differentiation, and apoptosis. By suppressing the main pluripotency factors (OCT4, SOX2, MYC and KLF4), miR-145 silences the self-renewal program in ESCs. Therefore, the main aim of this study is to find a potential link between the expression level of hsa-miR-302b and hsa-miR-145 with tumor vs. non-tumor as well as high-grade vs. low-grade states of the esophageal tissue samples.

Methods: A total number of 40 formalin-fixed, paraffin-embedded (FFPE) samples of esophageal squamous-cell carcinoma (ESCC) were obtained, and the tumor and marginal non-tumor areas delineated and punched off by an expert pathologist. Total RNA was extracted with Trizol, and cDNA synthesized using the miRCURY LNA™ Universal RT microRNA PCR Kit. Real-time reverse transcription polymerase chain reaction (RT-PCR) assays were performed using specific LNA-primers and SYBR Green master mix.

Results: The expression level of miR-302b failed to show any significant difference, neither between tumor and their non-tumor counterparts, nor among tumors with different grades of malignancies ($p > 0.05$). In contrast, miR-145 was significantly down regulated in all grades of tumor samples ($p < 0.001$). However, its expression level could not discriminate between different grades of malignancy ($p > 0.05$).

Conclusion: Our data revealed a significant down-regulation of miR-145 in ESCC tissue samples. Based on our ROC curve analysis data (AUC = 0.74, $p < 0.001$) miR-145 could be regarded as a potential tumor marker for diagnosis of esophageal cancer.

Keywords: Esophageal cancer, FFPE, hsa-miR-302b, hsa-miR-145, molecular marker

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Introduction

Esophageal cancer (EC) is the eighth most common cancer and the sixth leading cause of cancer-related death worldwide.[1–3] EC frequency is higher in men and its occurrence increases with age, with the highest incidence rate between the ages of 50 – 70 years, where the mortality rate is about 90% of all cases.[1,2,4] Several epidemiological studies indicated that hot drinks, alcohol, tobacco and low consumption of fresh fruits and vegetables are the main risk factors for EC.[1,2,5–7] There are two main forms of EC, each with distinct etiologic and pathologic characteristics. Esophageal squamous cell carcinoma (ESCC) is the most frequent subtype of EC, whiles the other subtype, adenocarcinoma, is less common.[1] Geographical distribution of EC is variable. Golestan Province, located in the south-east of the Caspian Sea in northern Iran, has one of the highest incidence rates of EC in the world.[8] Despite advances in medical and surgical techniques, the EC prognosis remains poor and long-term survival is in the range of 18% – 25%.[9]

MicroRNAs (miRNAs) are a growing class of short (18 – 22 mer) non-coding RNAs which primarily act by complementary binding to the 3' UTR of their mRNA targets, therefore blocking their translation. By post-translational modulation of their targets, miRNAs play critical regulatory roles in cell growth, proliferation, differentiation, cell death, and etc. Accumulating evidences suggest that altered expression of miRNAs results in the initiation and/or progression of a variety of tumors, where they function either as oncogenes or tumor suppressors. Due to their high stability in clinical samples and tissue-specific expression profiles, miRNAs have been considered as potential biomarkers in cancer diagnosis and classification.[10–12]

Members of the miR-302 cluster (miR-302a, miR-302b, miR-302c, miR-302d, and miR-367) are the most existing miRNAs in human embryonic stem cells (hESCs), transcribed from a ~700-bp region on chromosome 4, and forms a polycistronic transcript. Its expression is claimed to be restricted to ES and embryonic carcinoma (EC) cells, where they are quickly down-regulated upon differentiation. This cluster is one of the main stemness regulators, where its ectopic expression is enough to transform cancer cells to stem cells.[13] Interestingly, the ESC-specific transcription factors including OCT4, SOX2, Nanog and Rex-1 have binding sites on the miR-302 promoter and hence regulating its expres-

Authors' affiliations: [1]Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran, [2]Department of Medical Genetics and Molecular Medicine, Zanjan University of Medical Sciences, Zanjan, Iran, [3]Department of Pathology and Digestive Disease Research Institute, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran, [4]Human Genetics Department, Golestan University of Medical Sciences, Gorgan, Iran, [5]Emergency Medicine Department, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, [6]Student Research Committee, Shiraz Medical School, Shiraz University of Medical Sciences, Shiraz, Iran.
•Corresponding author and reprints: Seyed Javad Mowla PhD, Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, P. O. Box: 14115-145, Tehran, Iran. Tel: +98-21-82883464, Fax: +98-21-82884717, E-mail: sjmowla@modares.ac.ir.
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sion.[13–14]

MiR-145 is another important microRNA widely regarded as a tumor suppressor, where its expression is down-regulated in many cancers. This specific pattern of expression highlights its importance as a molecular marker for diagnosis and therapy of cancers.[15–17] MiR-145 is transcribed from a 4.09 kb region on chromosome 5 (5q32-33), which is one of the known chromosomal fragile sites. This can partly explain miR-145 down-regulation in many cancers. MiR-145 silences the self-renewal program in ESCs and facilitates their differentiation.[18,19]

While the ES-specific transcription factors (OCT4, SOX2, Nanog and Rex-1) bind to miR-302 promoter and induce its expression in pluripotent cells, the same factors suppress the expression of miR-145 and induce cell differentiation.[20,21]According to previous studies, the expressions of some embryonic miRNAs are re-expressed in cancers. In this study, we aimed to evaluate the expression of miR302b and miR-145 in tumor and non-tumor samples of EC.

Materials and Methods

Clinical ESCC specimens

We performed a matched case-control study in which 40 randomly selected formalin-fixed paraffin-embedded (FFPE) tissue samples of patients with esophageal SCC and their adjacent non-tumor tissue samples were obtained from Namazi hospital (Shiraz University of Medical Sciences, Shiraz/Iran). For each patient, the clinico-pathological information including gender, age, as well as the grade and stage of tumors were gathered. The experimental procedures were approved by the ethics committees of Namazi hospital and Tarbiat Modares University. Representative sections of FFPE samples were stained with hematoxylin and eosin (H&E) dyes, and tumor/non-tumor are as delineated by an expert pathologist (Dr. Mohammad Vasei).

RNA isolation

From FFPE blocks of each patient, the tumor and apparently normal areas were carefully punched off and cut into thin sections. Sections were then deparaffinized by xylene, treated with15 µg/ml of proteinase K (Fermentas, Lithuania) in PK buffer (1 mM EDTA, 1 mM NaCl, 5 mM Tris-HCl, PH 7.4) and incubated at 54ºC for 3 hours. Using Trizol reagent (Invitrogen, USA), total RNA was extracted and dissolved in 20 to 50 µl of RNase-free water, according to the manufacturer's instructions.The concentrationand purity of RNA were determined by spectrophotometer. RNase-free DNase (Fermentas, Lithuania) treatment of total RNA was performed to eliminate any potential contamination with genomic DNA.

Real-time RT-PCR assay

The optimal concentration of total RNA for cDNA synthesis was determined empirically in the maximum range of RNA concentrations suggested by the manufacturer (200ng of total RNA). Synthesis of cDNA was performed using the mercury LNA™ Universal RT microRNA PCR Kit (Exiqon, Denmark). Briefly, the tubes were incubated for 60 minutes at 42°C, followed by heat-inactivation of the reverse transcriptase (RT) enzyme for 5 minutes at 95°C. Real-time RT-PCR was performed using miR-302b and miR-145 LNA™ primers (Exiqon, Denmark), as well as SYBR Green master mix (Exiqon, Denmark). 5S rRNA gene was also used as a housekeeping internal control. PCR reactions were conducted at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, and 60°C for 1 minute in an ABI 7500 real-time quantitative PCR system (Applied Biosystems, USA). Human embryonic carcinoma cell line NTERA2 (NT2; kindly provided by Dr Peter Andrews at Sheffield University) was employed to check the specificity of target primers to amplify hsa-miR-302b and hsa-miR-145. Human embryonic carcinoma cell line NTERA2 was also considered as a positive control. To determine the reaction efficiencies for each primer pair, LinReg PCR (12.x) software program (AMC, Amsterdam, http://LinRegPCR.nl) was applied, and real-time PCR data were adjusted based on the exact PCR efficiency. For calculation of miRNAs expression fold change, the expression level in each sample was normalized to that of 5S rRNA. Then, miRNAs expression in tumor samples was adjusted to their matched non-tumor samples ($2^{-\Delta\Delta CT}$). For comparing the expression of miRNAs in tumor and non-tumor groups, the expression level of each sample was calibrated to that of the least expressed sample. Furthermore, for each sample a no-RT control was included to detect any potential genomic DNA contamination.

Statistical analysis

According to the Kolmogorov–Smirnov normality test (KS-test), statistical differences between well, moderately and poorly differentiated tumors and their matched non-tumor esophageal samples were determined either by paired t-test or Wilcoxon non-parametric test. All tests were performed as two-tailed. $p < 0.05$ was considered statistically significant. Correlations between miRNA expressions were estimated using Spearman correlation coefficient. Receiver operating characteristic (ROC) curve analysis was employed to determine whether the expressions of the aforementioned miRNAs have the sensitivity and specificity to discriminate between tumor and non-tumor samples, as well as between early and advanced stages of tumors. GenEX software (MultiD Analyses AB, Goteborg, Sweden), and Statistical Program for Social Sciences (SPSS) software version 17 (SPSS Inc., Chicago, IL, USA) were utilized for statistical analysis of real-time PCR data.

Results

Optimizing miRNAs amplification

FFPE samples of 40 patients with ESCC were used in this study. The pathologic subtypes of tumors were: 9 poorly, 6 moderately and 25 well differentiated. Using spectrophotometer, the result of quality control determined acceptable ratios for purity and concentration of RNA extractedfrom FFPE samples.

The melt curve analysis of PCR products demonstrated a predicted single melt curve pick, and hence the authenticity of the PCR products (Figure1). There were no amplification product in the negative and "no RT" controls. Using real-time RT-PCR, the mean CTs for miR-302b and miR-145 amplification in all samples (both tumor and non-tumor) were determined as 34.4 ± 1.5 and 22.5 ± 3.2, respectively. We initially employed both U6 SnRNA and 5S rRNA as internal controls. However, due to its high Ct values and wide variation of its expression level, the U6 SnRNA quantification was stopped in later experiments.

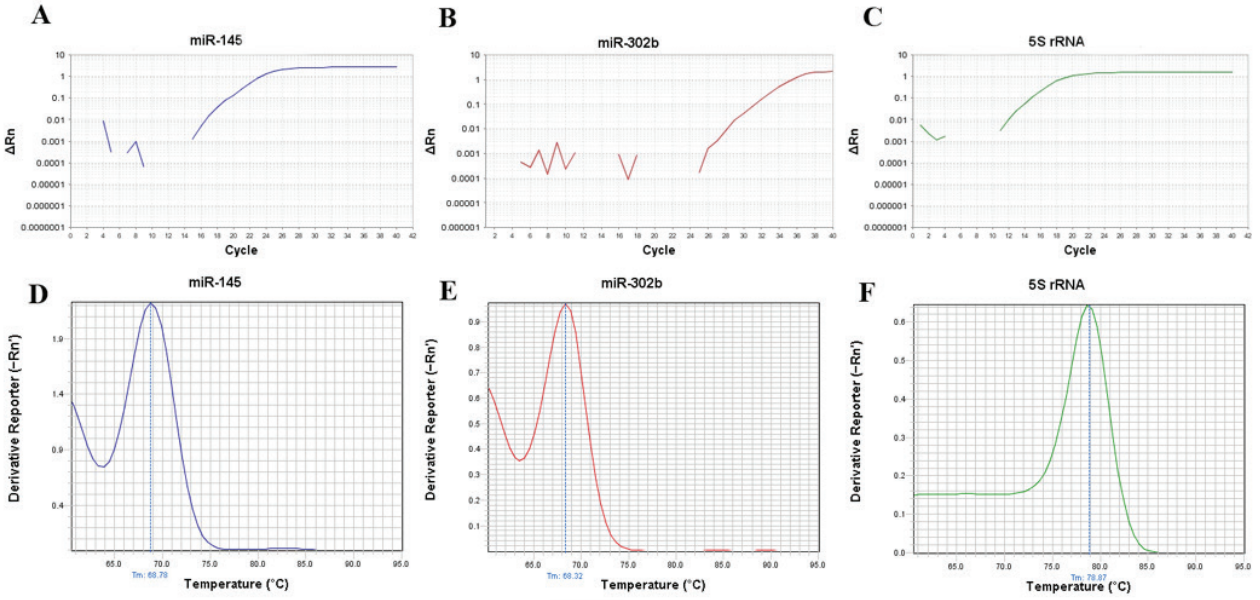

Figure 1. Representative amplification and melt-curve plots for different primers. **(A)** miR-145, **(B)** miR-302b and **(C)** 5S rRNA amplifications with specific primers.**(D-F)** the corresponding melt curves of A-C graphs. The single melt curve peak of PCR products confirmed the specificity of amplifications with related primers.

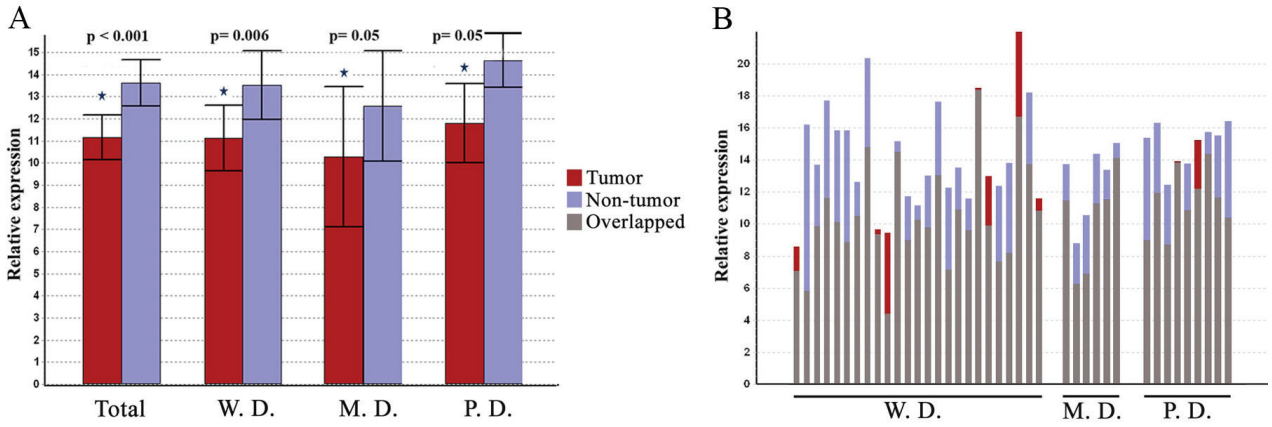

Figure 2. A comparison of miR-145 gene expression in well (W.D.), moderately (M.D.) and poorly (P.D.) differentiated of esophageal tumor samples vs. their matched non-tumor samples. The red and blue boxes represent tumor and non-tumor samples, respectivily, whereas the gray boxes represent the overlapped data from both tumor and non-tumor samples. In each sample, the expression level of miR-145 gene is normalized to its related internal control (5S rRNA), and then calibrated to that of the least expressed sample. Next, the log2 scale for the individual samples was calculated. **A)** the bar plots show the mean value of miR-145 expression in tumor and non-tumor samples, with the 95% confidence interval as error bars. Note that the gene expression of miR-145 is significantly down-regulated in tumor vs. non-tumor samples, and also in different grades of malignancies. **B)** The expression of miR-145 in individual samples, distributed in well, moderately and poorly differentiated groups.

Differential expression of miR-302b and miR-145 in esophageal tumors and their matched non-tumor tissues

The relative expression of miR-302b and miR-145 in 40 paired of tumor/non-tumor ESCC surgical specimens was determined by real-time PCR. A significant down-regulation of miR-145 in tumor samples was detected, compared to their non-tumor counterparts from the same patients ($p < 0.001$,). Further analysis of miR-145 gene expression in tumors with different grades of malignancies revealed its significant down-regulation in different grades of malignancy: poorly ($p = 0.05$), moderately ($p = 0.005$) and well ($p = 0.006$) differentiated samples, compared to their non-tumor counterparts. In contrast to miR-145, we could not detect any significant difference of miR-302b expression neither between tumor vs. non-tumor states nor in different grades of malignancies (Figure3).

According to Spearman correlation coefficient, no significant and considerable correlation was observed between the expression levels of miR-302b and miR-145 (data not shown).

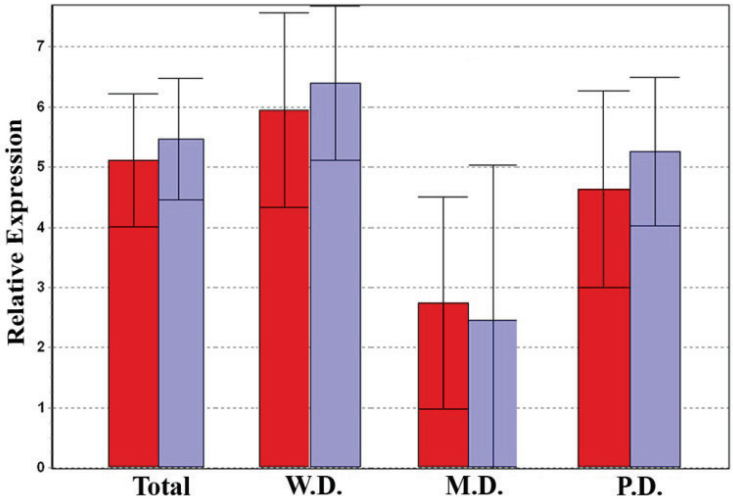

Figure 3. A comparison of miR-302b expression in well (W.D.), moderately (M.D.) and poorly (P.D.) differentiated of esophageal tumor samples vs. their matched non-tumor samples. The red and blue boxes represent tumor and non-tumor samples, respectivily. In each sample, the expression level of miR-302b gene is normalized to that of internal control (5S rRNA), and then calibrated to that of the least expressed sample. Finally the log2 scale for the individual samples was calculated. The columns show the mean value of miR-302b expression in tumor and non-tumor samples, with 95% confidence interval as error bar. As it is evident, there are no significant difference between miR-302b gene expression level in tumors vs. their matched non-tumor samples.

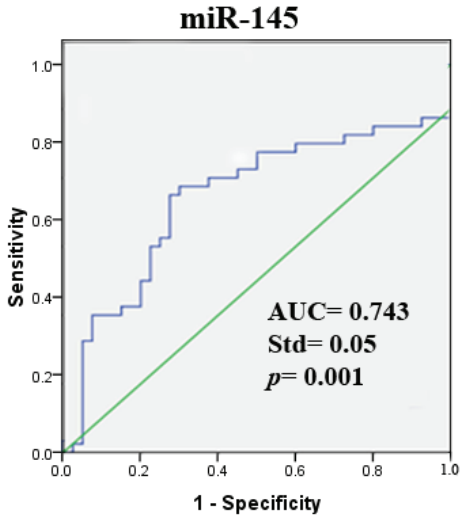

Figure 4. Receiver operating characteristic (ROC) curve analysis determineda good sensitivity and specificity for miR-145 expression level on discriminating tumor from non-tumor states of the samples. The calculated area under the curve (AUC = 0.74) demonstrated the suitability of miR-145 to correctly classify tumor and non-tumor groups of the esophageal samples (p < 0.001).

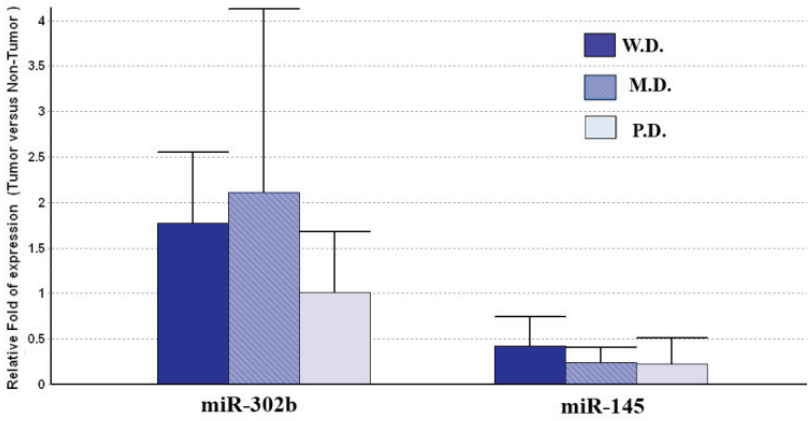

Figure 5. Relative gene expressions of miR-302b (A) and miR-145 (b) in well (W.D.), moderately (M.D.) and poorly (P.D.) differentiated esophageal tumors. In each sample, the expression level of genes is initially normalized to that of internal control, 5S rRNA, and then the fold change of miR-302b and miR-145 expression in tumor versus non-tumor samples was calculated by $2^{-\Delta\Delta CT}$ formula.The related error bars show the 95% confidence interval for each group. Note that there is no significant expression alteration among different grades of malignancies.

Association of miR-145 expression with patients' clinico-pathological characteristics:

Using ROC analysis, the suitability of miR-302b and miR-145 expression levels was evaluated to discriminate between tumor and non-tumor states of the samples. According to ROC curve analysis, miR-145 seems to be a good candidate for accurate discrimination of tumor from non-tumor samples (AUC = 0.74, P < 0.001; Figure 4). As expected, miR-302b was not able to distinguish tumor from non-tumor samples (AUC = 0.52, P = 0.75). Both miRNAs failed to accurately detect early from advanced tumor stages (p > 0.05). In agreement with the ROC data, there was no significant difference between fold changes of miRNAs' expression in well, moderately and poorly differentiated grades of malignancies (Figure 5).

Discussion

Accumulating reports have indicated the involvement of microRNAs in the initiation and/or progression of various types of tumors. Moreover, it is already showing that the miRNAs expression is cell- and tissue-specific.[22] The cluster of miR-302, which is the most abundantly expressed miRNA in undifferentiated ESCs is a good example of cell-specific expression. Accordingly, miR-302 expression is sharply turned off upon the induction of differ-

entiation.[13] To date, there are several conflicting reports on the role of the miR-302 in tumorigenesis. A coordinate over-expression of all members of the cluster has been just reported for malignant germ cell tumors (GCT), demonstrating the specificity of these markers for GCT.[23,24] In contrary, some other investigators have proposed a tumor suppressor activity for miR-302.[25,28] In our study, the detection of miR-302b in ESCC samples did not occur at a reliable level of expression (CT = 34.4 ± 1.5). Moreover, we couldn't find a significant expression alteration of miR-302b between tumor vs. non-tumor samples, and also among different grades of malignancies. It can be concluded that the expression of miR-302b is probably restricted to a rare subpopulation of cancer stem cells, which exists in almost all cancers including ESCC.[29,30] Accordingly, the expression of the miR-302 is already reported in a rare stem cell-like subpopulation of glioma cell line.[31] Nevertheless, ROC analysis on miR-302b expression data suggest its unreliable ability to discriminate tumor from non-tumor and also early from advanced tumor stages.

In contrast to miR-302b, the expression level of miR-145 was much higher in our samples and was detectable at reliable CT values (22.5 ± 3.2). The miR-145 is widely regarded as a tumor suppressor and its down-regulation was already reported for many cancers, including: breast,[32] colon,[33] prostate,[34] B- cell,[35] gastric[36] and bladder.[37] In accordance with the previous literature, our data revealed a very significant down-regulation of miR-145 in tumor samples compared to their non-tumor counterparts. This finding is in agreement with the inhibitory role of miR-145 in an undifferentiated state of pluripotent and induced pluripotent stem (ips) cells. MiR-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells.[18,21] In accordance with our previous study on miR-145 expression in gastric cancer,[38] a prominent down-regulation of miR-145 in early stages of esophageal tumorigenesis may point to its critical role in the prevention of tumor initiation. Profiling altered expression of microRNAs in ESCC, Wu and his colleagues reported a deregulation of miR-143 and miR-145 in tumor samples.[39] Our finding are consistent with those of other studies,[39,40] and suggest that a tumor suppressor activity for miR-145 in ESCC.

Furthermore, the data of ROC curve analysis demonstrated an AUC of 0.74 for miR-145. The high value of AUC reflects a good specificity and sensitivity for a potential diagnostic marker of esophageal cancer. However, ROC curve analysis failed to discriminate early from advanced stages of tumors. The latter finding might be due to the low number of samples in late stages. It also indicates that miR-145 down-regulation occurred at an early stage of ESCC. In our previous study on the same samples, we found that miR-21 is overexpressed in ESCCs, compared to their adjacent non-tumor tissues ($p < 0.001$).[41] MiR-21 is an oncomir and its frequent upregulationis already reported in different cancers, including ESCCs. Moreover, the serum concentration of miR-21 in ESCC patients was significantly higher than that in healthy controls, indicating that miR-21 has a potential to be used as a biomarker for early detection of ESCC.[42] Up-regulation of miR-21, an oncomiR, and down-regulation of miR-145, a tumor suppressor miRNA, in early stages of esophageal tumorigenesis points to their critical roles in promoting and prevention of tumor initiation, respectively.

In conclusion, considering the high stability and ease of miR-145 detection in FFPE samples, evaluation of miR-145 expression in tumor tissues, serum or other body fluids of patients, is a reliable biomarker for diagnosis of ESCC.

Authors Contribution

The first two authors are equally contributed to this work.

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