MicroRNA-1322 Regulates ECRG2 Allele Specifically and Acts as a Potential Biomarker in Patients With Esophageal Squamous Cell Carcinoma

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A short tandem repeat (STR) polymorphism in the 3'UTR region of esophageal cancer-related gene 2 (ECRG2, also known as SPINK7) has been widely reported to be associated with the incidence and the prognosis of esophageal squamous cell carcinoma (ESCC). This study explores how the microRNA binding to the STR region affects ECRG2 expression in ESCC. Dual-luciferase reporter assays were used to verify the effects of the four microRNAs (miR-580, miR-1182, miR-1272, and miR-1322) predicted to bind the STR region of the ECRG2 3' untranslated region (UTR). The expression of identified effective microRNA was then analyzed in 44 paired ESCC and adjacent normal tissues and 402 case–controlled serum samples (divided into a discovery group and an independent validation group) by real-time RT-PCR assay. We found that only miR-1322 could significantly down-regulate the ECRG2 with TCA3 allele (P < 0.01), but it could not down-regulate the ECRG2 with TCA4 allele significantly (P > 0.05). MiR-1322 was also expressed significantly higher in ESCC tissue and serum samples than in controls (both P < 0.01). Additionally, serum levels of miR-1322 yielded an under receiver operating characteristic (ROC) curve area of 0.847 (95% CI, 0.795–0.890) for discriminating ESCCs from healthy controls in the discovery group and a similar result was obtained in the validation group (under ROC area is 0.845; 95%CI, 0.780–0.897). We conclude that miR-1322 can regulate ECRG2 in an allele-specific manner and that serum levels of miR-1322 can serve as a potential diagnostic biomarker for patients with ESCC.
Accession Number AF268198) [5,6]. There is a triplet TCA short tandem repeat (STR) polymorphism located in the 3’UTR of ECRG2 with two specific alleles, TCA3 (TCATCATCA) and TCA4 (TCATCATCATCA) [7]. The TCA3 allele is proved to be a risk factor for ESCC by the recently reports that subjects who carried the TCA3/TCA3 genotype were at an increased risk of ESCC compared to those carrying the TCA4/TCA4 genotype [7,8]. Other reports also revealed that patients with TCA3/TCA3 genotype have a significantly poorer prognosis in ESCC [9] and oral squamous cell carcinoma [10]. However, the molecular mechanism of why TCA3 rather than TCA4 allele is a risk factor of ESCC is still unknown.

MicroRNAs, which are endogenous small single-stranded noncoding RNAs ranging from 19 to 25 nucleotides, usually can inhibit the expression of genes by binding at their 3’UTR regions and dramatically change the biological function of organisms [11]. Therefore, we wanted to check if there are certain microRNAs that regulating the expression of ECRG2 in an allele specific manner in ECRG2 gene and then have impacts on the carcinogenesis of ESCC. Additionally, the role of microRNAs in cancer development and progression has led to an extensive exploration of microRNAs as biomarkers and molecular targets for multiple cancers. In particular, circulating microRNAs are increasingly explored as the noninvasive diagnostic and prognostic markers for certain cancers [12,13].

In this study, we investigated all the microRNAs predicted to bind at the ECRG2 STR region and verified which microRNA that had effects on this region. Next, we analyzed the expression of the verified microRNA within ESCC tissue and serum samples, and finally evaluated its diagnostic potential as a serum biomarker for ESCC.

MATERIALS AND METHODS

Study Population and Sample Preparation

The study subjects we recruited were described in our previous study [14]. In brief, we collected 44 paired ESCC tissue and adjacent normal samples which were verified by postsurgical pathologic examination (Henan Tumor Hospital, China). We also collected serum samples from 201 ESCC patients and 201 healthy controls matched by age and gender from the same hospital. This case–control study with serum samples was consisted of two groups, the discovery group and the validation group. The discovery group included 120 ESCC hospitalized patients diagnosed from January 2008 to March 2010 (verified by postsurgical pathologic examination or imaging in the Henan Tumor Hospital, China) and 120 age and gender matched healthy subjects (by physical examination) (Table 1). The validation group included 81 ESCC outpatients (verified by endoscopic biopsy) and 81 normal controls (verified normal by endoscope) from December 2009 to November 2010 in the hospital. All patients involved in our study had not undergone chemotherapy or radiotherapy prior to sampling. Pathologic evaluation was based on the criteria set forth by the American Joint Committee on Cancer staging criteria [15]. TNM staging was used, where T is the extent of the tumor invasion, N is the extent of spread to the lymph nodes, and

<table>
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<th>Variable</th>
<th>Tissue samples</th>
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<th>Serum samples validation group</th>
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</tr>
<tr>
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<td>75 62.5</td>
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<tr>
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<td>28 23.3</td>
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*Two-sided $\chi^2$ test.
MIRCO RNA-1322 REGULATES ECRG2 AND SERVES AS A BIOMARKER IN ESCC

Isolation of MicroRNAs and Quantification by Real-Time RT-PCR

MicroRNAs from tissues and serum were extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany). The protocol of microRNAs Real-time RT-PCR were described previously [14]. For Real-time PCR of microRNAs, we used 5'-GGCGGACACAGAATTTAACGC-3' as the universal reverse primer, and the forward primers were 5'-ACGCTCTATGAGCTG-3' for RNU6B; 5'-TAGTACGCAATGATGGCG-3' for miR-16; and 5'-GATGCATTCTGAGTGCT-3' for miR-1322; and 5'-TTGAGAAATATGAGTATCCTAGG-3' for miR-580. MicroRNA sequences were obtained from the mirBase database (http://www.mirbase.org). Each sample was run in triplicates. The expression of each microRNA was calculated using the formula:

\[ C_t = \frac{C_{t,miR-X} - C_{t,miR-16}}{C_{t,miR-X} - C_{t,miR-16}} \]

and expressed as relative luciferase activity. Each experiment was performed in triplicates on three separate occasions.

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M is the presence of distant metastasis. Informed consents were obtained from all participants in this study. This project was approved by the Ethics Committee of Henan Tumor Hospital.

Construction and Site-Directed Mutagenesis of Reporter Plasmids

The 3'UTR regions of ECRG2 with the TCA3 or TCA4 allele were amplified by PCR from healthy donors. The PCR products were digested with SacI and HindIII and inserted into the pMIR-REPORT Luciferase vector (Applied Biosystems, Carlsbad, CA) using the T4 ligase kit (Takara, Dalian, China). The constructed plasmids were sequenced to verify the STR allele, and the two luciferase reporter plasmids (TCA3 allele and TCA4 allele wild type 3'UTR plasmids [Wt3'UTR]) were constructed. For the site-directed mutagenesis, the predicted microRNAs binding site “TCATCATCA” in the constructed plasmids (TCA3 and TCA4-Wt3'UTR) was mutated to “TCATCAAGC” for miR-1322 test and mutated to “AGTTCATCA” for miR-580 test, using the Site-Directed Mutagenesis Kit (SBS Genetics, Beijing, China) per manufacturer's instructions and been designated as Mut3'UTR. All the plasmids were verified by Sanger sequencing. (PCR primers used are listed in Supplementary Table 1).

Dual-Luciferase Reporter Assays

Cultured cells were seeded into 48-well plates at a density of 1 x 10^4 cells/well 1 d prior to transfection. Cells were then co-transfected with 150 ng of constructed reporter plasmid, 1.0 ng Renilla reporter control plasmid pRL-SV40 (Promega, Madison, WI) and 50 nM miR mimics (NC or anti-miR) for each well. Twenty-four hours later, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) on a Turner Designs TD-20/20n luminometer (Promega) according to the manufacturer’s protocol. Results were normalized to Renilla luciferase activity and data were expressed as relative luciferase activity. Each experiment was performed in triplicates on three separate occasions.

Western Blot

Forty-eight hours after transient transfection, cell lysates were acquired and Western blot was performed as described previously using mouse anti-ECRG2 monoclonal antibody generated by our laboratory [19]. The results were scanned by LAS4000 imaging system (Fuji Film, Tokyo, Japan).

MicroRNA In Situ Hybridization (ISH) Assay on Frozen Sections

Dissected ESCC tissues were first put into 4% Paraformaldehyde/PBS and 0.5 M Sucrose/PBS at 4°C for 8 h. Then tissues were frozen in OCT and cut into 8 μm-thick sections. The slides were then acetylated for 10 min in 200 mL volume mixture of triethanolamine (2.33 mL), acetic anhydride (500 μL), and DEPC water (197 mL). Fluorescence-labeled LNA probes against miR-580, miR-1322, and U6 were obtained from Exiqon (Exiqon, Vedbaek, Denmark). Hybridization solution was prepared by adding 1–2 μL of labeled probes per 200 μL of hybridization buffer (50% denonized formamide; 0.3 M NaCl; 20 mM Tris–HCL, pH 8.0;
shown in Figure 2A. This result suggested that difference in a non-ESCC cell line, HEK293, as lines (EC9706, EC109, KYSE150) and there was no activity of TCA4 reporter plasmid is two- to four-

TCA4 allele, and found that luciferase expressing reporter plasmids containing either the TCA3 or TCA4 STR polymorphism was located in the stem-loop structure (Figure 1B), which would have high melting temperature of 55°C in a chamber humidified with 50% formamide and 1× SSC. Then the slides was stringently washed: first wash slides two times for 30 min at 55°C in washing solution (50% formamide, 0.1% Tween-20, 1× SSC); then wash slides for 15 min in 0.2× SSC at room temperature; finally wash the slides for 15 min in PBS. Then the slides were photographed under the laser confocal microscopy (Leica Tcs SP2, Mannheim, Germany).

Bioinformatics and Statistical Analysis

The secondary structure of ECRG2 3'UTR region was predicted by RNAcofold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAcofold.cgi). Predicted microRNAs targeted on ECRG2 STR region were determined by Targetscan5.1 (http://www.targetscan.org/) and MicroInspector (http://bioinfo.uni-plovdiv.bg/microinspector/) softwares. Statistical analysis was carried out using SPSS software (version 13.0; SPSS, Chicago, IL). Receiver operating characteristic (ROC) curves, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated by Medcalc software (version 11.2; Medcalc, Mariakerke, Belgium).

RESULTS

Four MicroRNAs Are Predicted to Bind the STR Region of ECRG2

The TCA STR polymorphism is located in the 3'UTR region of ECRG2. There are two alleles, TCA3 and TCA4, which could constitute into three kinds of genotypes, TCA3/TCA3 genotype, TCA3/ TCA4 genotype, and TCA4/TCA4. Figure 1A shows the site of the STR region and the sequencing data of three kinds of genotype. The predicted secondary structure of the STR region showed that the TCA STR polymorphism was located at the loop of the stem-loop structure (Figure 1B), which would subject for microRNAs binding at this region. Bioinformatics analysis with TargetScan5.1 and MicroInspector software showed four microRNAs (miR-580, miR-1182, miR-1272, and miR-1322) were predicted to bind in this region (Figure 1C).

MiR-580 and miR-1322 Regulate ECRG2

We first cloned the ECRG2 3’UTR luciferase reporter plasmids containing either the TCA3 or TCA4 allele, and found that luciferase expressing activity of TCA4 reporter plasmid is two- to fourfold higher than that TCA3 in the three ESCC cell lines (EC9706, EC109, KYSE150) and there was no difference in a non-ESCC cell line, HEK293, as shown in Figure 2A. This result suggested that certain molecules may bind to the TCA STR region in an allele-specific mechanism especially in ESCC cells. To test this hypothesis, we transfected mimics of the predicted microRNAs into the EC9706 cell line. We found that miR-1182 (Figure 2B) and miR-1272 (Figure 2C) did not regulate either the TCA3 or TCA4 plasmid in our assays. However, miR-1322 mimics and inhibitors dramatically regulated luciferase activity of TCA3 plasmid (P < 0.05) but not the TCA4 plasmid (P > 0.05) (Figure 2D). Over expression of miR-580 could dramatically down-regulated luciferase activity of both the TCA3 and TCA4 plasmid expression (P < 0.05), but its inhibitors had no effects on either of these two alleles (Figure 2E). Similar results were obtained in other two ESCC cell lines, EC109 (Supplementary Figure 1A and B) and KYSE150 (Supplementary Figure 1C and D). In addition, we confirmed TCA STR region (Figure 2F and G) is the binding site of miR-1322 and miR-580 by the facts that mutating this binding site could abolish the repressive effect of both miR-1322 and miR-580. Western blot assay also verified that exogenous miR-1322 and miR-580 could repress the levels of ECRG2 protein, but only anti-miR-1322 appeared to enhance the expression protein levels of ECRG2. These results indicated that only miR-1322 and miR-580 could regulate ECRG2 gene expression. Importantly, miR-1322 could allele specifically regulate ECRG2 gene. However, the fact that the antisense sequence of miR-1322, but not miR-580, could affect the expression of ECRG2 led us to detect the endogenous expression levels of the two microRNAs in vitro and in vivo.

MiR-1322 Was Expressed in ESCC Tissues

We first detected the expression of miR-1322 and miR-580 in cell lines and found that miR-1322 expressed much higher in three ESCC cell lines than miR-580. (Supplementary Figure 2). Then we quantitated the two microRNAs in 44 paired ESCC tissue samples (characteristics are shown in Table 1), using U6 small nuclear RNA (RNU6B) as an endogenous control. The expression of miR-580 as determined by Real-time PCR was very low in both ESCC and adjacent normal tissues, and undetectable in 32 paired tissues (Figure 3A). In contrast, miR-1322 was highly expressed and was over-expressed in 73.3% (34 cases) of cancer tissues compared to normal controls, with 63.6% (28 cases) of samples showing at least a twofold up-regulation (Figure 3A). In addition to real-time PCR analysis, microRNA In situ Hybridization assay in the ESCC tissue also showed that there was miR-1322 but not miR-580 expressed (Figure 3B). Additionally, miR-1322 levels in ESCC tissues varied across the TNM stage (P = 0.045, Kruskal-Wallis test) with expression at stage III significantly higher than that in stage I (Figure 3C), which
also give us more interests for exploring the role of miR-1322 in serum samples.

The Levels of MiR-1322 in Serum Samples of Patients With ESCC

We then analyzed miR-1322 levels in ESCC patients' serum samples. For this test, a relative quantification was applied for minimize sample-to-sample variations. We chose miR-16 as the internal normalization control as described in our previous studies [14].

In the discovery group, we detected increased levels of miR-1322 in the sera of patients with ESCC compared to normal controls. The levels of miR-1322 in serum varied by TNM staging (Figure 4A) and were positively correlated with TNM staging as revealed by Spearman Bivariate

Figure 1. Short tandem repeat polymorphism in the 3’UTR region of ECRG2 and the putative microRNAs binding sites in this region. (A) The details of ECRG2 gene (also named SPINK7, rectangle in green, blue and red color represent DNA, mRNA and the coding region respectively) and the STR in its 3’UTR region, including TCA3/TCA3 genotype, TCA3/TCA4 genotype and TCA4/TCA4 genotype, showed by the DNA sequencing profile (Bases in green background prompt heterozygous). (B) The putative structure of part of the 3’UTR region in ECRG2 with two kinds of TCA alleles. The sequences in the red circle are the TCA STR regions. (C) The predicted microRNAs binding sites at the TCA STR site of the 3’UTR region. Bases in red color are the TCA STR site.

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Correlation analysis ($P < 0.001$, $r = 0.293$). The expressions of miR-1322 was also influenced by metastasis classification ($P = 0.002$, Kruskal–Wallis test, Supplementary Figure 3A), but no difference was found in T stage classification ($P > 0.05$, Kruskal–Wallis test, Supplementary Figure 3B).

We found that levels of miR-1322 in serum of patients with ESCC were significantly higher...
than normal controls in the discovery group (Figure 4B). ROC curves revealed that the level of miR-1322 in serum could serve as a useful biomarker for patients with ESCC differentiated, with under-ROC-curve area (area under the curve, AUC) of 0.847 (95% CI, 0.795–0.890). At the cut-off value of 0.0459, the sensitivity was 81.7% while the specificity was 82.5% (Figure 4D).

In order to verify this result, we recruited additional 81 ESCC outpatients and 81 normal controls into the independent validation group (Table 1). Analysis of miR-1322 also showed the similar results with significantly higher levels of miR-1322 in serum of patients with ESCC (Figure 4C). In the validation group, this biomarker yielded an under-ROC-curve area of 0.845 (95% CI, 0.780–0.897), and at the previously identified cut-off value 0.0459, the sensitivity, specificity, PPV, NPV were 83.7%, 80.5%, 81.1%, and 83.2%, respectively (Figure 4E).

**DISCUSSION**

In this study, we identified miR-1322 could bind allele specifically at the TCA3 allele at the STRs polymorphism site of 3’UTR of ECRG2 and suppress the expression of ECRG2. We also found miR-1322 was over expressed in the ESCC tumor tissues compared to their adjacent normal tissues and the serum levels of miR-1322 were significant higher in patients with ESCC than healthy controls. There are a lot of studies reported the TCA STR polymorphism in ECRG2 3’UTR region and associated with the risk and prognosis of ESCC [7–10]. Our research illuminated how this TCA STR polymorphism contributes to the risk and prognosis of ESCC.

By bioinformatics analysis with multiple approaches, we found four microRNAs (miR-580, miR-1182, miR-1272, and miR-1322) were predicted to bind at the STR region of ECRG2. Then we used dual-
luciferase report assay system to test the modulation effects of four microRNAs by using the synthesized microRNAs mimics and inhibitors on the expression of TCA3 and TCA4 allele specific ECRG2 3’UTR containing plasmids. We identified miR-580 could regulate both kinds of TCA alleles plasmids, while miR-1322 could regulate them in an allele specific manner, which can intensely down-regulate the luciferase activity with TCA3 allele and barely affected the TCA4 allele. These results were consistency in all three ESCC cell lines (Supplementary Figure 1). Besides, after we mutated the “TCATCATCA” sequences in the plasmids containing ECRG2 3’UTR, the regulatory functions of the two miRNAs were abolished, which further confirmed the TCA STR region was the binding site for microRNAs. Western blot assays showed that in EC9706 cell line, anti-miR-1322 significantly up-regulated the ECRG2 protein expression, while anti-miR-580 seemed not functional in our

Figure 4. The role of serum levels of miR-1322 in the diagnosis of ESCC. The number of cases for each group is indicated below the X-axis. In the box plots, the lines denote the 10th, 25th, median, 75th, and 90th percentiles for each. (A) Expressions of serum miR-31 in healthy subjects and ESCC patients in discovery group classified by TNM stage. (B, C) MiR-1322 expression in serum of ESCC patients and healthy subjects within the discovery or validation group. (D) ROC curve analysis using serum miR-1322 levels for discriminating ESCC in the discovery group (AUC = 0.847, 95%CI, 0.795–0.890; cut-off value is 0.0459, sensitivity = 81.7%, specificity = 82.5%). (E) ROC curve in the validation group (AUC = 0.845, 95%CI, 0.780–0.897), at previous cut-off value 0.0459, sensitivity = 83.7%, specificity = 80.5%. Statistical analyses were performed using Mann–Whitney test for relative expression test (A–C).
assays (Figure 2H). This is an interesting phenomenon and can be attributed to the fact that endogenous level of miR-580 is too low in ESCC cell lines (Supplementary Figure 2). This phenomenon has also been confirmed in vivo. By real-time PCR and microRNA in situ hybridization assays we found that miR-580 was not detectable in esophageal tissues while miR-1322 was found abundantly expressed. Additionally, we found that miR-1322 expressed significantly higher in ESCC tissues than adjacent normal tissues. Besides, the expression of miR-1322 was correlated with the TNM staging of tumor. So we concluded that it was miR-1322 rather than miR-580 that played the major role in ESCC by regulating ECRG2.

ECRG2 has important tumor suppressive function in esophageal cancer development. It can prevent chromosome instability through a p53-dependent mechanism [4], cause ESCC cell apoptosis by interaction with MT2A [6,20], and inhibit ESCC cell migration and invasion by binding at the uPA protein [3,21–23]. There is a lot of studies reported the TCA STR polymorphism in ECRG2 3’UTR region are associated with the risk and prognosis of ESCC. It has been reported that TCA3 allele carriers confer higher ESCC risk and patients with TCA3 allele have poorer prognosis compared with the TCA4 allele carriers [7–10]. Since ECRG2 is an esophageal cancer related tumor suppressor, the function of allele specific binding of miR-1322 to TCA3 may help explain many reports that people with TCA3 allele of ECRG2 have high risks and poor prognosis of ESCC. These data also suggested that miR-1322 plays an oncogenic role in initiation and progression of ESCC.

Since microRNAs have been shown to be stable in serum [24] and can serve as diagnostic markers for various cancers [25,26], and currently there are few serum markers available for ESCC, in order to probe the potential role of miR-1322 as a biomarker, we also measured its expression in serum samples collected from ESCC patients compared with healthy control subjects. We found that serum levels of miR-1322 varied across the TNM stages of ESCC patients and is significant higher than healthy controls. ROC curve analysis in the discovery group (AUC = 0.847) (Figure 4D) revealed serum miR-1322 is a potential diagnostic marker for ESCC and the result was also confirmed by an independent validation group (ROC AUC = 0.845) (Figure 4E). Our study on serum miR-1322 provides a potential novel, reliable, and effective approach for the discrimination of ESCC that may be much helpful for the early diagnosis and control of esophageal cancer if we have more population studies on miR-1322 and ESCC coming out in the future.

In conclusion, our study has identified a potential oncogenic microRNA, miR-1322, for ESCC, and illustrated the molecular mechanism for miR-1322 allele specific effects on ECRG2 expression which can explain the different ESCC risk and prognosis regarding to the STR polymorphism located in the 3’-UTR of ECRG2 gene. The higher expression of miR-1322 in ESCC tissues as well as serum of ESCC patients suggested that miR-1322 may serve as a potential target for ESCC treatment and a molecular marker for ESCC control. To our knowledge, this study is also the first exploration about the function of miR-1322. To further investigate the function of this molecular in ESCC, we need to perform in-depth studies from cell molecular pathways to animal models.

ACKNOWLEDGMENTS

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REFERENCES


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