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

Tengfei Zhang,1 Dan Zhao,1 Qiming Wang,2 Xiying Yu,1 Yaling Cui,3 Liping Guo,1 and Shih Hsin Lu1*

1State Key Laboratory of Molecular Oncology, Department of Etiology and Carcinogenesis, Cancer Hospital and Institute, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
2Department of Internal Medicine, Henan Tumor Hospital, Zhengzhou University, Zhengzhou, China
3Medical Record Library of Medical Services, Henan Tumor Hospital, Zhengzhou University, Zhengzhou, China

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.

INTRODUCTION

Esophageal cancer is one of the most common fatal cancers worldwide with median survival from 1 to 2.2 yr [1]. Fifty percent of all esophageal cancers in the world occur in China, of which approximately ~90% are esophageal squamous cell carcinoma (ESCC) [2]. ESCC is difficult to be controlled due to its early metastasis and lack of early diagnostic tools. Esophageal cancer-related gene 2 (ECRG2, also known as SPINK7) is regarded as a tumor suppressor gene in esophageal cancer [3,4]. It was first cloned and identified by our laboratory (Genbank additional supporting information may be found in the online version of this article.

Additional supporting information may be found in the online version of this article.

Abbreviations: ESCC, esophageal squamous cell carcinoma; ECRG2, esophageal cancer-related gene 2; STR, short tandem repeat; NC, negative control; ROC, receiver operating characteristic; AUC, area under the curve.

Grant sponsor: National Natural Science Foundation Grant (China); Grant number: 30971448; Grant sponsor: State Key Basic Research Program Grant (China); Grant number: 2009CB521803; Grant sponsor: Chinese High-Tech R&D program Grant (China); Grant number: 2006AA024403.

Additional supporting information may be found in the online version of this article.

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Authorship: Tengfei Zhang and Dan Zhao performed microRNA-1322 related cell experiments, statistical and bioinformatics analysis and drafting of the manuscript; Qiming Wang helped with ESCC tissue and serum sample collection and processing; Xiying Yu provided quantitative RT-PCR of microRNA technical support; Yaling Cui in sample and clinical pathological data collection; Liping Guo in study supervision; Shih Hsin Lu conducted the study design.

Each author approved the final version of the manuscript.

This project was approved by the Ethics Committee of Henan Tumor Hospital (Henan, China) on January 9th, 2008.

This work had taken a poster presentation (Poster session A23) in the “Ninth Annual International Conference on Frontiers in Cancer Prevention Research” (November 7–10, 2010, Philadelphia, USA) and got the Scholar-in-Training Award supported by Susan G. Komen for the Cure®.

Dan Zhao present address is Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute-Frederick, Frederick, Maryland, USA.

*Correspondence to: Department of Etiology and Carcinogenesis, Cancer Hospital and Institute, Chinese Academy of Medical Sciences, Beijing 100021, China.

Received 4 August 2011; Revised 29 December 2011; Accepted 10 January 2012

DOI 10.1002/mc.21880

Published online in Wiley Online Library (wileyonlinelibrary.com).

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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 (TCATCATCATA) [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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<tr>
<th>Table 1. Characteristics of Subjects With ESCC and Normal Controls</th>
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<tr>
<td><strong>Tissue samples</strong></td>
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*Two-sided \( \chi^2 \) test.

Molecular Carcinogenesis
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.

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'-GCGAGCACAGAATTAATACGAC-3' as the universal reverse primer, and the forward primers were 5'-ACGGAAATCGTGAGCGTT-3' for RNU6B; 5'-TAGCAGCAGTAGTTAATATTGGCG-3' for miR-16; 5'-GATGATCTGTGCTGTAGTGCTG-3' for miR-1322; and 5'-TTGAGAATTCTAGAATCATTAGG-3' for miR-580. MicroRNA sequences were obtained from the miRBase database (http://microrna.sanger.ac.uk/). Each sample was run in triplicates. The expression of each microRNA was calculated using the formula

\[
    \text{sample} = \frac{C_{\text{t,miR-X}}}{C_{\text{t,normal tissue}}} \times \frac{C_{\text{t,U6}}}{C_{0}}
\]

where \(C_{\text{t}}\) is the fluorescence intensity at time \(t\), and \(C_{0}\) is the fluorescence intensity at time 0.

Cell Culture and Transient Transfection of MicroRNAs

Human ESCC cell lines EC9706 [16] and EC109 [17] were established in our laboratory. KYSE150 [18] was a gift from Dr. Y. Shimada (Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University, Kyoto, Japan). Human embryonic kidney cell line HEK293 was purchased from American Tissue Type Collection (Manassas, VA). All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum and incubated at 37°C in a 5% CO₂ humidified incubator. MicroRNA mimics (miR-580, miR-1322, and anti-miR-1322) that contain 29-Ome modifications were synthesized and purified by Shanghai Gene-Pharma Company (Shanghai, China). SIRNA duplexes with nonspecific sequences were used as a negative control (NC). Cells were seeded into 6-well plates at a density of 2 x 10⁵ cells per well and were then transfected with 50 nM of the following: miR mimics, inhibitors or nonspecific sequences (NC), using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA).

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 [W3’UTR]) were constructed. For the site-directed mutagenesis, the predicted microRNAs binding site “TCATCATCA” in the constructed plasmids (TCA3 and TCA4-W3’UTR) was mutated to “TCATGAAGC” for miR-1322 test and mutated to “AGTTCTAC” 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 10⁴ 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 of acetone mixture of triethanolamine (2.33 mL), acetic anhydride (500 μL), 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% denatured formamide; 0.3 M NaCl; 20 mM Tris–HCL, pH 8.0;
5 mM EDTA; 10 mM NaPO₄, pH 8.0; 10% Dextran Sulfate; 1× Denhardt’s solution; 0.5 mg/mL yeast RNA. For hybridization, add 50–100 μL of hybridization solution to slides, incubating 2 h at a temperature of 55°C in a chamber humidified with 50% formamide and 1× SSC. Then the slides were 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
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 compared to 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 phenome-
non and can be attributed to the fact that endoge-
nous 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 func-
tion in esophageal cancer development. It can
prevent chromosome instability through a p53-
dependent mechanism [4], cause ESCC cell apo-
psis by interaction with MT2A [6,20], and inhibit
ESCC cell migration and invasion by binding at
the uPA protein [3,21–23]. There are a lot of stud-
ies reported the TCA STR polymorphism in ECRG2
3’UTR region are associated with the risk and pro-
gnosis 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 peo-
ple 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 initia-
tion 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 biomark-
er, we also measured its expression in serum sam-
ples 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 discov-
ery group (AUC = 0.847) (Figure 4D) revealed se-
rum 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 poten-
tial 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 investi-
gate the function of this molecular in ESCC, we
need to perform in-depth studies from cell molecu-
lar pathways to animal models.

ACKNOWLEDGMENTS

We thank Dr. Jive Ma and Dr. Xinguang Cao
from the Department of Pathology and Endoscopy
of Henan Tumor Hospital for helping in the patho-
logical analysis and sample collection. We also
thank the Fellows Editorial Board of National Insti-
tute of Health in United States for editorial assis-
tance. This study was supported by National
Natural Science Foundation Grant 30971448
(China), State Key Basic Research Program Grant
2009CB521803 (China) and Chinese High-Tech
R&D Program Grant 2006AA02A403 (China).

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(ECRG2) implicates susceptibility to esophageal cancer in
ECRG2 TCA short tandem repeat polymorphism with the
risk of oesophageal cancer in a North Indian population.
polyorphism in exon 4 of esophageal cancer-related gene
Z detected in genomic DNA is a prognostic marker for


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