The oncogenetic role of microRNA-31 as a potential biomarker in oesophageal squamous cell carcinoma

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ABSTRACT

miR-31 (microRNA-31) is frequently altered in numerous cancers. The aim of the present study was to investigate the role of miR-31 in ESCC (oesophageal squamous cell carcinoma). We measured miR-31 in 45 paired ESCC tissues and 523 serum samples using real-time RT (reverse transcription)–PCR. The serum samples were divided into a discovery group (120 ESCCs and 121 normal controls), a validation group (81 ESCCs and 81 controls), and a final group comprising six other common tumours (colorectal, liver, cervical, breast, gastric and lung cancers; total n = 120). A Mann–Whitney U test and Wilcoxon matched-pairs test were used for the statistics. miR-31 was up-regulated in 77.8 % of the ESCC tissues. Serum miR-31 levels in ESCC patients were significantly higher than in normal controls (P < 0.001). It yielded an ROC (receiver operating characteristic) AUC (area under the curve) of 0.902 [95 % CI (confidence interval), 0.857–0.936] in the discovery group and a similar result in the validation group [ROC AUC, 0.888 (95 % CI, 0.819–0.939)]. Patients with high-levels of serum miR-31 also had a poorer prognosis in relapse-free survival (P = 0.001) and tumour-specific survival (P = 0.005). In vitro studies showed that miR-31 promoted ESCC colony formation, migration and invasion. Luciferase reporter and Western blot assays confirmed that three tumour suppressor genes, namely EMP1 (epithelial membrane protein 1), KSR2 (kinase suppressor of ras 2) and RGS4 (regulator of G-protein signalling 4), were targeted by miR-31. We conclude that miR-31 plays oncogenetic functions and can serve as a potential diagnostic and prognostic biomarker for ESCC.

Key words: diagnosis, oesophageal squamous cell carcinoma (ESCC), microRNA, miR-31, prognosis.

Abbreviations: AUC, area under the curve; BAP1, BRCA1-associated protein 1; CI, confidence interval; CREG1, cellular repressor of E1A-stimulated genes 1; DMEM, Dulbecco’s modified Eagle’s medium; DOCK1, dedicator of cytokinesis 1; EMP1, epithelial membrane protein 1; ESCC, oesophageal squamous cell carcinoma; FGF7, fibroblast growth factor 7; GLTSCR1, glioma tumour suppressor candidate region gene 1; KSR2, kinase suppressor of ras 2; miR, microRNA; NC, non-specific sequence; NPV, negative predictive value; PPV, positive predictive value; PTPN1, protein tyrosine phosphatase non-receptor type 1; RGS4, regulator of G-protein signalling 4; RNU6B, U6 small nuclear RNA; ROC, receiver operating characteristic; RT, reverse transcription; SCC, squamous cell carcinoma; siRNA, small interfering RNA; UTR, untranslated region; VEZT, vezatin.

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INTRODUCTION

Oesophageal cancer ranks seventh and sixth in cancer incidence and mortality rate worldwide respectively [1]. A total of 50% of all oesophageal cancer worldwide occurs in China, with the highest incidence being in Henan province [2]. ESCC [oesophageal SCC (squamous cell carcinoma)] accounts for approx. 90% of all oesophageal carcinomas diagnosed at an advanced stage. The difficulty in managing ESCC is due to its aggressive invasion and early metastasis to lymph nodes, adjacent tissue and organs. The majority of cases are diagnosed at a relatively late stage of the disease, when the chances of surgical intervention are lost. Even with surgery, the median survival rate of ESCC patients after R0 resection (the complete removal of all tumour with microscopic examination of margins showing no tumour cells) is less than 2 years [3,4]. Additionally, ESCC is relatively resistant to both chemotherapy and radiotherapy [5]. Many studies have shown that the development and metastasis of ESCC relates to the dysregulation of several oncogenes and tumour suppressor genes in multiple pathways. Understanding the molecular pathogenesis of ESCC, and especially the mechanisms of tumorigenesis and metastasis, is extremely important for developing novel biomarkers and treatment strategies.

miRs (microRNAs), which are endogenous small single-stranded non-coding RNAs ranging from 19 to 25 nt, play an important role in epigenetic and post-transcriptional regulation networks. miRs are able to target several genes in one or multiple different pathways and dramatically change the biological function of organisms. Their roles in cancer development and metastasis lead to an extensive exploration of oncogene or tumour-suppressive miRs in multiple cancers, of which some were potential diagnostic and prognostic markers for certain cancers [6–8]. Among them, expression changes of miR-31 is frequently reported in multiple cancers: it is expressed differently in adenocarcinomas, up-regulated in colorectal cancer [9], and down-regulation in gastric cancer [10], breast cancer [11], prostate carcinoma [12] and serous ovarian carcinomas [13]. Meanwhile, there is an interesting observation that miR-31 is widely up-regulated in most SCCs. Microarray studies have revealed that miR-31 is one of the most up-regulated miRs in oral and pharyngeal [14], laryngeal [15] SCCs, and real-time PCR analysis has revealed that miR-31 is particularly up-regulated in lung SCC [16] and overexpressed by more than 6-fold in tongue SCC [17]. However, the expression of miR-31 in ESCC is still unknown. Furthermore, the finding of miRs in peripheral blood implies that circulating miRs might serve as good candidates for non-invasive biomarkers for the diagnosis and prognosis of cancers [18–22].

In the present study, we first analysed the expression of miR-31 in ESCC tissue and serum samples. Then we evaluated the diagnostic value of serum miR-31 and explored its prognostic value in a follow-up study. Finally, we confirmed the oncogenic role of miR-31 in ESCC in vitro and identified important miR-31-targeted tumour-suppressor genes that affected SCC.

MATERIALS AND METHODS

Study population and sample preparation

We collected 45 paired ESCC tissue samples (cancerous and surrounding normal tissues), which were verified by post-surgical pathological examination (Henan Tumor Hospital, Henan, China). The carcinoma tissues obtained were poorly, moderately and well differentiated. The corresponding normal tissues were obtained at least 5 cm away from the primary tumour.

For serum samples, we recruited 241 subjects in the discovery group and 162 subjects in the validation group. The discovery group included 120 ESCC patients diagnosed between January 2008 to March 2010 (at the Henan Tumor Hospital, Henan, China) and 121 age- and gender-matched healthy subjects (by physical examination) from the same domestic area (Table 1). The validation group included 81 ESCC outpatients (verified by gastroscopy biopsy examination) and 81 normal controls (normal by gastroscopy) in the hospital from December 2009 to November 2010. We also recruited an additional 120 cases with colorectal adenocarcinoma (n = 20), cervical SCC (n = 20), breast carcinoma (n = 20), gastric adenocarcinoma (n = 20) and lung SCC (n = 20), as diagnosed by post-surgical pathological examination (see Supplementary Table S1 at http://www.clinsci.org/cs/121/csl210437add.htm).

Additionally, serum samples were collected from 64 out of the 120 patients in the discovery group at 7 days post-surgery. A total of 44 out of the 64 patients had follow-up data regarding their smoking status, alcohol consumption, familial history of cancer and survival (average, 19.4 months; range, 3–28 months). Survival data were obtained from medical records and the study ends were recurrence as determined by imaging and death from tumour-specific causes. Deaths from other causes were treated as uncensored cases.

None of the cases involved in our present study had undergone chemotherapy or radiotherapy prior to sampling. The pathological evaluation was based on the criteria outlined by the American Joint Committee on Cancer staging criteria [23]. TNM staging was used, where T is the extent of the tumour, N is the extent of spread to the lymph nodes and M is the presence of distant metastasis.

Informed consent was obtained from all participants for the use of their blood or tissue samples in the present study. This project was approved by the Ethics Committee of Henan Tumor Hospital.
Isolation of miRs and quantification by real-time RT (reverse transcription)–PCR

miRs from tissues and serum were extracted using an miRNAeasy Mini kit (Qiagen). The RNA extracted from the serum ranged from 20 to 100 ng/μl, and the RNA extracted from tissue ranged from 310 to 590 ng/μl. miRs were polyadenylated by *Escherichia coli* Poly (A) Polymerase (NEB) before RT to cDNA using a PrimeScript™ RT kit and 5′-AGCAAGATGCTGGCATAGCT-3′ for *miR-31* in tissues was calculated by the equation 2^(-ΔΔCt) in tissue analysis. The universal reverse primer was 5′-GGAGGCACAGAATTAATCAGACTCAGTATATGTTTTTTTTTTTCG-3′ [24] as the RT primer. Real-time PCR was performed on an ABI PRISM 7300 Real-time PCR system (Applied Biosystems), using an SYBRR Premix Ex Taq™ PCR kit (Takara), according to the manufacturer’s instructions. The universal reverse primer was 5′-GGAGGCAGCAGAATTAATCAGACTCAGTATATGTTTTTTTTTTTCG-3′ and the forward primers were 5′-ACGCACCGTGAAGCGTT-3′ for 5′-GCGAGCACAGAATTAATACGACTCAGTATATGTTTTTTTTTTTCG-3′ [24] as the RT primer. Real-time PCR was performed on an ABI PRISM 7300 Real-time PCR system (Applied Biosystems), using an SYBRR Premix Ex Taq™ PCR kit (Takara), according to the manufacturer’s instructions. The universal reverse primer was 5′-GGAGGCACAGAATTAATCAGACTCAGTATATGTTTTTTTTTTTCG-3′ and the forward primers were 5′-ACGCACCGTGAAGCGTT-3′ for *miR-31*.

**Table 1** Characteristics of subjects with ESCC and normal controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Tissue samples</th>
<th>Serum samples in the discovery group</th>
<th>Serum samples in the validation group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>34 (75.6 %)</td>
<td>76 (62.8 %)</td>
<td>43 (53.1 %)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (24.4 %)</td>
<td>45 (37.1 %)</td>
<td>38 (46.9 %)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤45</td>
<td>1 (2.2 %)</td>
<td>20 (16.5 %)</td>
<td>15 (18.5 %)</td>
</tr>
<tr>
<td>46–55</td>
<td>8 (17.8 %)</td>
<td>28 (23.1 %)</td>
<td>25 (30.8 %)</td>
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<tr>
<td>56–65</td>
<td>21 (46.7 %)</td>
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<tr>
<td>&gt;65</td>
<td>15 (33.3 %)</td>
<td>27 (22.3 %)</td>
<td>12 (14.8 %)</td>
</tr>
<tr>
<td>Stage (n)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7 (15.6 %)</td>
<td>28 (23.2 %)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>17 (37.8 %)</td>
<td>31 (25.8 %)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>21 (46.7 %)</td>
<td>33 (27.5 %)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>0 (0 %)</td>
<td>28 (23.2 %)</td>
<td></td>
</tr>
</tbody>
</table>

P values were determined using a two-sided χ² test.

**Colonies formation assay**

At 24 h after transfection, ESCC cell lines were seeded on to six-well plates (300 cells/well). At 12 days later, visible colonies were fixed with methanol and stained with 0.4 % Crystal Violet solution. Colonies were counted and the number of visible colonies in each well was determined. Each assay was performed in triplicate.

**Cell wound healing assay**

The migration ability of the EC9706 cells was determined using a wound healing assay. At 24 h post-transfection, a monolayer of cells was scratched with the angularity of a small X-ray film. Wound closures were photographed every 3 h.

**Cell migration and invasion assay**

Cell migration and invasion were examined using Transwell™ permeable supports (Corning). At 24 h post-transfection, cells were dissociated to 2×10⁴ cells in 100 μl of serum-free DMEM (Dulbecco’s modified Eagle’s medium) and added to the upper well of the Transwell™ inserts (8 μm pore size), while the lower chamber was filled with 600 μl of DMEM with 10 % FBS (fetal bovine serum). After 24 h, cells that had migrated to
the membrane of the insert were fixed, stained and then
alysed under a microscope by counting the cells in ten
random fields per insert. For the invasion assay, 8-μm
Transwell™ inserts were coated with 30 μg of Matrigel
(Becton Dickinson) and air-dried. After rehydration,
2×10⁴ cells were added to each insert. Cells that had
migrated through the matrix to the other side of the insert
were counted. Each assay was performed in triplicate.

Construction of reporter plasmids and
site-directed mutagenesis
The 3′-UTRs (untranslated regions) in predicted target
genes of miR-31 were amplified from a healthy donor.
The PCR products were digested with SacI (or NaeI)
and HindIII and inserted into the pMIR-REPORT
Luciferase vector (Applied Biosystems) using the T4
ligase kit (Takara). The predicted miR-31-binding site,
TCTTGTT, was found in EMP1 (epithelial membrane
protein 1), KSR2 (kinase suppressor of ras) and RGS4
(regulator of G-protein signalling 4). This sequence
was mutated (underlined) to TCGCGTT (EMPI),
TCAAGTT (KSR2) and TCGGTT (RGS4) using the
Site-Directed Mutagenesis kit (SBS Genetech),
according to the manufacturer’s instructions. All
mutations were verified by sequencing. The PCR primer
sequences are listed in Supplementary Table S2 (at
http://www.clinsci.org/cs/121/cs1210437add.htm).

Luciferase activity assay
Cultured cells were seeded on to 48-well plates at a
density of 10⁴ cells/well at 1 day prior to transfection.
Cells were then co-transfected with 150 ng of the
constructed plasmid, 1.0 ng of the Renilla luciferase
reporter plasmid pRL-SV40 (Promega) and 50 nM
miR-31, NC or anti-miR-31 for each well. At 24 h
later, luciferase activity was measured using the Dual-
Luciferase Reporter Assay System (Promega) on a
TD-20/20n luminometer (Turner Designs; Promega),
according to the manufacturer’s protocol. Results were
normalized to Renilla activity, and data are expressed as
relative luciferase activity. Experiments were performed
in triplicate on three separate occasions.

Western blot analysis
At 48 h after transient transfection of EC9706 cells, lys-
ates were obtained and Western blotting was performed.
Rabbit anti-EMP1 and anti-RGS4 polyclonal antibodies
were obtained from Santa Cruz Biotechnology, and a
mouse anti-KSR2 monoclonal antibody was obtained
from Novus. The resulting images were scanned
(LAS4000; Fuji Film) and the intensity of each blot band
was quantified using Multi Gauge version 3.0 (Fuji Film).

Bioinformatics and statistical analysis
The predicted target genes of miR-31 were
determined by Targetscan5.1 (http://www.targetscan.org/),
PicTar (http://pictar.mdc-berlin.de/) and RNA22 soft-
ware (http://cbsrv.watson.ibm.com/rna22.html). Stat-
istical analyses were carried out using SPSS software
(version 13.0). ROC (receiver operating characteristic)
curves, sensitivity, specificity, PPVs (positive predictive
values) and NPVs (negative predictive values) were
calculated using Medcalc software (version 11.2).

RESULTS

**miR-31 is overexpressed in ESCC tissues**
We assessed the expression of miR-31 in ESCC
tissue samples (tissue characteristics are in Table 1).
Using RNU6B as an endogenous control in 45
pairs of ESCC tissues, we found that miR-31 was
overexpressed in 77.8% of cancer tissues compared
with their corresponding normal tissues. Additionally,
66.7% of the 45 cases showed at least a 2-fold up-
regulation (Figure 1A). Of the 45 pairs, miR-31 levels
varied across the TMN staging (P = 0.050, Kruskal–
Wallis test). Expression of miR-31 in stage III was
higher than that in stage I (Figure 1B). Relative
expression of miR-31 was also influenced by N stage
classification (P = 0.024; Supplementary Figure S1A at
http://www.clinsci.org/cs/121/cs1210437add.htm), but
no difference was found in T stage classification
(P = 0.181; Supplementary Figure S1B).

**miR-31 levels are elevated in serum
samples of ESCC**
To determine levels of miR-31 in serum, a relat-
ive quantification was applied for sample-to-sample
variations. To select a better normalization control,
expression of RNU6B and miR-16 in serum were
evaluated. Of the 86 serum samples collected (43
ESCC and 43 healthy subjects), miR-16 was detected
in all samples; however, RNU6B was not detected in
five out of the 86 samples (Supplementary Figure
Therefore we selected miR-16 as the internal
normalization control, as it displayed higher expression
levels and more stability than RNU6B in serum samples.
Other reports have also shown miR-16 is a good
endogenous control in serum or plasma miR analyses
[18,19,21,27]. Serum from 241 subjects were used as a
discovery group for testing (Table 1). We detected
increased levels of miR-31 in the serum of ESCC patients
compared with healthy controls. The serum miR-31 levels
varied by TNM staging (Figure 1C) and were positively
correlated with TNM staging as revealed by Spearman
bivariate correlation analysis (P < 0.001, r = 0.519).
When stratified by metastasis, miR-31 expression was also signi-
ificantly different (P < 0.001; Supplementary Figure 3A at
http://www.clinsci.org/cs/121/cs1210437add.htm), and
had an increasing trend with the progression of
Serum miR-31 in the diagnosis and prognosis of ESCC

Figure 1  miR-31 expression in ESCC tissue and serum samples
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) miR-31 expression in paired ESCC cancerous and surrounding normal tissues, normalized by $2^{-\Delta\Delta Ct}$. (B) Relative expression of miR-31 in tissues grouped by total T/N/M stage, normalized by $2^{-\Delta\Delta Ct}$. (C) Expressions of serum miR-31 in healthy subjects and ESCC patients grouped by T/N/M stage. (D) Levels of miR-31 in serum of ESCC patients before (pre-op) and 7 days after (7 days post-op) surgical resection. Statistical analyses were performed using a Wilcoxon test for paired samples (A and D) or a Mann–Whitney U test for relative expression test (B and C).

metastasis stage ($P < 0.001$, $r = 0.476$; Spearman bivariate correlation). Data on the depth of tumour invasion were available in 92 cases (who had surgery) out of the 120 ESCC patients, among which the expression of miR-31 in the T3 stage group was significantly higher than that in T1 and T2 group ($P = 0.017$; Supplementary Figure 3B).

**Serum levels of miR-31 decrease after surgical removal of ESCC**
Among the 92 patients who had surgery in the discovery group, we collected serum from 64 of those patients 7 days after surgery and compared the miR-31 levels with those prior to surgery. Strikingly, serum levels of miR-31 were significantly reduced in the post-operative samples when compared with their pre-operative samples ($P < 0.001$; Figure 1D). Among the 64 patients, 49 (75.4 %) had a significant decrease after surgery with 45 (69.2 %) falling more than 2-fold.

**Serum level of miR-31 as a potential diagnosis marker in ESCC**
In the discovery group, we found that serum levels of miR-31 differentiated ESCC patients from healthy subjects, with a ROC AUC (area under the curve) of 0.902 [95% CI (confidence interval), 0.857–0.936]. At the cut-off value of 0.0054, the sensitivity was 86.7 % and the specificity was 84.3 % (Figure 2A). In order to verify this finding, we recruited 81 ESCC outpatients and 81 normal controls into the validation group (Table 1). This biomarker yielded a ROC AUC of 0.888 (95% CI, 0.819–0.939) and, at the previously identified cut-off value 0.0054, the sensitivity, specificity, PPV and NPV were 86.1, 79.1, 80.5 and 85.1 % respectively (Figure 2B). To explore the diagnostic potential of miR-31 in discriminating ESCC from other cancers, we collected serum from 120 individuals prior to surgery. This included six common tumours in addition to ESCC (Supplementary Table S1). Quantitative real-time PCR revealed that serum miR-31 levels in ESCC were higher than in the six other cancers tested (Figure 2C). We also found that, when compared with normal controls, serum miR-31 was expressed at higher levels in lung SCC ($P < 0.001$), cervical SCC ($P < 0.001$) and colorectal adenocarcinoma ($P = 0.013$), and at lower levels in gastric adenocarcinoma ($P < 0.001$). There was no significant difference in levels of miR-31 in hepatocellular carcinoma ($P = 0.346$) or breast carcinoma ($P = 0.135$).

**Serum miR-31 levels correlate with the prognosis of ESCC**
Follow-up data were acquired for 44 patients who had undergone surgical resection. According to median serum levels of miR-31 7 days after surgery, we classified the 44 patients into two groups: 22 cases with
Figure 2 The role of serum miR-31 in the diagnosis and prognosis of ESCC

(A) ROC curve analysis using serum miR-31 levels for discriminating ESCC in discovery group [AUC, 0.902 (95% CI, 0.857–0.936); cut-off value is 0.0054; sensitivity, 86.7%; specificity, 84.3%]. (B) ROC curve in the validation group at a cut-off value of 0.0054 [AUC, 0.888 (95% CI, 0.819–0.939), sensitivity, 86.1%; specificity, 79.1%]. (C) Serum miR-31 expression in other malignant neoplasms, including six kinds of common tumors. In the box plots, the lines denote 10th, 25th, median, 75th and 90th percentiles for each, using the Mann–Whitney U test. (D and E) Relapse-free survival and tumour-specific survival curves grouped by the median levels of serum miR-31 in patients 7 days after surgical resection. Values below the graphs are the number of patients at risk at that time point of each group. miR-31L, miR-31 low expression; miR-31H, miR-31 high expression; post-op, post-operative.

We performed a univariate Cox regression analysis on the patients’ relapse-free survival and found that the depth of tumour invasion (T3 compared with T1+T2, \( P = 0.013 \)), regional lymph node metastasis (‘yes’ compared with ‘no’, \( P = 0.001 \)) and serum levels of miR-31 (high compared with low, \( P = 0.003 \)) were significantly correlated (Table 2). However, age, gender, smoking, alcohol consumption and family history of cancer showed no correlation (Table 2). A multivariable Cox regression analysis also revealed that serum levels of miR-31 after surgery was an independent factor contributing to prognosis after correction for all of these clinicopathological factors [hazard ratio of recurrence, 3.260 (95% CI, 1.264–8.421); \( P = 0.015 \)] (Table 2). Cox regression analysis on tumour-specific survival was also performed and showed similar results (Supplementary Table S3 at http://www.clinsci.org/cs/121/cs1210437add.htm). A log-rank test on patients grouped by pre-operative serum levels of miR-31 also suggested that patients with higher serum miR-31 expression had a trend to poorer prognosis, but the \( P \) value was not significant (relapse-free survival, \( P = 0.067 \); tumour-specific survival, \( P = 0.197 \)) (Supplementary Figure S2).
miR-31 promotes colony formation, migration and invasion in vitro

To determine whether miR-31 had any effects on ESCC cell lines, we analysed colony formation, migration and invasion. miR-31 significantly promoted colony formation by more than 2-fold compared with the NC control, whereas anti-miR-31 reduced colony formation by antagonizing endogenous miR-31 in EC9706 cells (both \( P < 0.05 \); Figure 3A). Similar results were obtained in KYSE150 and KYSE510 cells, two additional ESCC cell lines (Figure 3B). We also found that miR-31 increased EC9706 cell motility in a cell wound-healing assay (Figure 3C). Transwell™ assays revealed that exogenous miR-31 enhanced migration 4.26-fold (\( P < 0.01 \)) and invasion 3.6-fold (\( P < 0.01 \)) in EC9706 cells (Figure 3D). This effect was specifically attributed to the biological activities of miR-31, as anti-miR-31 reduced cell migration and invasion by 64 and 66% respectively (both \( P < 0.05 \)). Other ESCC cell lines also showed similar results (Figures 3E and 3F). Interestingly, the endogenous levels of miR-31 in three cell lines, shown in Figure 3(G), was consistent with the inherently migratory or invasive ability of the three cell lines: EC9706(NC) > KYSE150(NC) > KYSE510(NC) (Figures 3E and 3F). Taken together, these results suggest an oncogenic role for miR-31 in regulating ESCC cell proliferation and motility.

Detection of the target genes of miR-31 by luciferase activity and Western blot analysis

We next explored the functional target genes of miR-31. There were 235 and 285 potential miR-31 target genes as predicted by TargetScan 5.1 and Pictar software respectively. We initially used RNA22 software to verify these and selected ten genes based on their 3’-UTR to test by luciferase assay. These genes, which appeared most likely to be associated with the functions of miR-31 in ESCC, included four potential tumour suppressor genes: BAP1 (BRCA1-associated protein-1), KSR2, GLTSCR1 (glioma tumour suppressor candidate region gene 1) and CREG1 (cellular repressor of E1A-stimulated genes 1); four cell-motility-related genes: DOCK1 (dedicator of cytokinesis 1), VEZT (vezatin), RGS4 and PTPN1 (protein tyrosine phosphatase non-receptor type 1); and two epithelial-cell-growth-related genes: FGF7 (fibroblast growth factor 7) and EMP1. We found that miR-31 only repressed the luciferase activity of three of these genes: EMP1, KSR2 and RGS4 (Figure 4A). The putative binding sites for miR-31 in the three genes are shown in Figure 4(B). Mutating the miR-31-binding sites in those genes abolished the repressive effect of miR-31, whereas anti-miR-31 significantly enhanced the luciferase activity (Figure 4C). Additionally, overexpression of miR-31 decreased the expression levels of EMP1, KSR2 and RGS4 proteins by 56, 34 and 16% respectively (Figure 4D). Anti-miR-31 enhanced the expression of these three proteins by 9–19% (Figure 4D). These results showed that EMP1, KSR2 and RGS4 are three genes targeted by miR-31.

DISCUSSION

The present study is the first in-depth investigation into serum levels of an miR in ESCC. We analysed the expression of miR-31 in ESCC tissues and sera, explored the effects of miR-31 on ESCC cell lines, and determined the target genes regulated by miR-31. We have discovered that serum levels of miR-31 were elevated with an increase in the grade of TNM stage, consistent with the results observed in the tissue samples. Importantly, we also assessed the value of miR-31 as a diagnostic and prognostic indicator in ESCC.

First, we found that patients with ESCC have a much higher level of miR-31 in serum compared with healthy controls, and this may serve as a promising biomarker for
Figure 3  Colony formation, migration and invasion analyses of three ESCC cell lines after up-regulation or down-regulation of miR-31

(A) Colony formation of the EC9706 cell line. (B) Number of colonies counted in each cell line normalized by NC. (C) Ability of EC9706 cells to migrate, recorded every 3 h, in the cell-wound-healing assay. Scale bar, 100 μm. (D) Transwell™ cell migration and invasion assay in the EC9706 cell line. Scale bar, 50 μm. (E) Relative number of migrating cells normalized by NC-migrated cells in EC9706. (F) Number of relative invading cells. (G) Real-time PCR for endogenous miR-31 expression in the three cell lines, normalized to RNU6B. Each well was examined in duplicate with at least three experiments, and the results are means ± S.E.M. *P < 0.05 and **P < 0.01 using a Student’s t test.
a differential diagnosis of ESCC. In fact, the diagnostic potential of miR-31 was confirmed in two separate groups, the discovery and validation groups (Figures 2A and 2B). Since miRs have been shown to be stable in serum [21] and an increasing number of reports have revealed that circulating miRs could serve as a diagnostic marker for various cancers [7,19,28], our present study on serum miR-31 provides a novel, reliable and effective approach that may be helpful in the diagnosis and monitoring of oesophageal cancer. Furthermore, we have explored the serum levels of miR-31 in six other common tumours. The results indicate that serum miR-31 levels are higher in ESCC patients than in patients with any of the other tumours studies (each $P < 0.001$ compared with each tumour group), which implies that high levels of miR-31 may serve as a specific diagnostic marker for ESCC. However, these need to be validated using a screening test for oesophageal cancer in further studies.

Survival analyses showed that post-operative patients with higher levels of serum miR-31 had a poorer prognosis in both relapse-free and tumour-specific survival compared with those with low miR-31 levels (Figures 2D and 2E). Multivariate Cox analysis also revealed that the post-operative level of serum miR-31 is an independent prognostic factor for ESCC, indicating that miR-31 is a promising biomarker for monitoring the progression of ESCC. Our present results show that the level of miR-31 in serum significantly decreased after surgical resection. This indicates that detectable levels of serum miRs may arise from tumour cells, which is consistent with other reports [19,21]. Interestingly, the fact that post-operative levels of serum miR-31 could serve as a prognostic indicator other than pre-operative levels led us to hypothesize that serum miR-31 after surgery itself has a role in the development of ESCC (Figures 2D and 2E, and Supplementary Figures S3A and S3B). However, this needs to be explored further, as our sample size and the duration of follow-up are limited. In addition, our samples included several relapse-surviving cases, which could have skewed the final results.

Considerable evidence has revealed the importance of miR-31 in cancer, but its role as a tumour suppressor or oncogenic factor is still controversial: it inhibits cell proliferation of serious ovarian carcinomas [13] and cell metastasis in breast cancer [29], and impairs migration in endometrial cancer cells [30]. In contrast, miR-31 promotes cell tumorigenesis in lung cancer [16], and cell migration and invasion in Kaposi’s sarcoma [31]. In our present study, we confirmed the oncogenetic role of miR-31 in ESCC via its ability to promote cell colony formation, migration and invasion in vitro. We identified three tumour suppressor genes targeted by miR-31: EMP1 (a tumour-associated epithelial membrane protein [32]), KSR2 (an important negative regulator of MAPK (mitogen-activated protein kinase) pathways [33–35]) and RGS4 (which has inhibitive functions on
cell motility and metastasis [36–39]). Out of these genes, EMP1 could have a specific suppressive impact on SCCs as it suppresses cell proliferation and metastasis in ESCC [40–42], regulates cell tumorigenesis and metastases in head and neck SCC [43], regulates cell differentiation in squamous-differentiated bronchial epithelial cells [32], correlates with cell apoptosis and proliferation in nasopharyngeal SCC [44], and is associated with lymph node metastasis in oral SCC [45]. However, little is known about the role of EMP1 in other kinds of carcinomas. Thus we conclude that abnormally high expression of miR-31 in squamous epithelium that specifically suppresses the function of EMP1 could be a common carcinogenic mechanism in various SCCs. This may also help to explain why miR-31 is highly expressed in most SCC tissue [14–17] and serum (Figure 2C and [28]) samples. However, the mechanisms of miR regulation in cancer are complex. Comprehensive studies on the carcinogenic effect of miR-31 are needed to establish it as a biomarker for use in both clinical diagnosis and prognosis of patients with ESCC.

**AUTHOR CONTRIBUTION**

Tengfei Zhang participated in the miR-31-related cell experiments, statistical and bioinformatics analysis, and writing the paper; Qiming Wang participated in ESCC tissue and serum sample collection and processing, and analysis and interpretation of the data; Dan Zhao performed the quantitative real-time PCR of miR and provided technical support, and was involved in the revising of the paper; Yaling Cu performed the clinical pathological analysis and collected the follow-up data; Bangrong Cao performed the quantitative real-time PCR test of miR-31; Liping Guo supervised the study; and Shih-Hsin Lu provided the study concept and designed the experiments.

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Serum miR-31 in the diagnosis and prognosis of ESCC

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The oncogenetic role of microRNA-31 as a potential biomarker in oesophageal squamous cell carcinoma

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Figure S1  Expression of miR-31 in ESCC tissue samples

(A) Relative expression of miR-31 in ESCC tissues classified according to N stage (N0/N1 + N2; n = 45). (B) Relative expression of miR-31 in ESCC tissues classified according to T stage (T1 + T2/T3; n = 45). The number of cases in each group is indicated below the x-axis. Statistically significant differences were determined using Mann–Whitney U tests. The horizontal lines denote the medians.

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Correspondence: Professor Shih Hsin Lu (email shlu1212@gmail.com).
Figure S2  Evaluation to determine the better normalization control for the serum miR-31 test
(A) Scatter plot of Ct values of the two candidate normalization controls RNU6B and miR-16 in the serum from healthy controls (N) and ESCC patients. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in quantitative real-time PCR. The horizontal lines denote the medians. Statistically significant differences were determined using Mann–Whitney U tests.

Figure S3  Expression of miR-31 in ESCC serum samples
(A) Expression of serum miR-31 classified according to metastasis (N0/N1/N2 + N3/M1); M1 represents patients with distant metastasis regardless of lymph node metastasis. In the box plots, the lines denote the 10th, 25th, median, 75th and 90th percentiles for each. (B) Expression of serum miR-31 classified according to T stage (T1+ T2/T3). The horizontal lines denote the medians. The number of cases for each group is indicated below the x-axis. Statistically significant differences were determined using Mann–Whitney U tests.

Figure S4  Role of serum miR-31 in the prognosis of ESCC by Kaplan–Meier analysis (n = 44)
Relapse-free survival (A) and tumour-specific survival (B) curves grouped by the median serum levels of miR-31 in patients before surgery. The numbers of patients at risk at each time point is indicated below. pre-op miR-31L, low expression of miR-31 pre-operation; pre-op miR-31H, high expression of miR-31 pre-operation.
Table S1  Characteristics of the subjects with six types of malignant tumour (n = 120) in addition to ESCC and normal subjects

<table>
<thead>
<tr>
<th>Pathological feature</th>
<th>Normal</th>
<th>Colorectal adenocarcinoma</th>
<th>Hepatocellular carcinoma</th>
<th>Cervical SCC</th>
<th>Breast carcinoma</th>
<th>Gastric adenocarcinoma</th>
<th>Lung SCC</th>
<th>ESCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases (n)</td>
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<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>120</td>
</tr>
<tr>
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<td></td>
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<td>Male</td>
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<td>16</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>14</td>
<td>79</td>
</tr>
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<td>20</td>
<td>20</td>
<td>8</td>
<td>6</td>
<td>41</td>
</tr>
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<td>9</td>
<td>9</td>
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<td>6</td>
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<td>3</td>
<td>7</td>
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<td>3</td>
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<td>7</td>
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<td>&gt;65</td>
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<td>3</td>
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<td>2</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td>37</td>
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<td>Stage (n)</td>
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<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>5</td>
<td>8</td>
<td>7</td>
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<tr>
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<td>5</td>
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<tr>
<td>IV</td>
<td>9</td>
<td>9</td>
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<td>4</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>28</td>
</tr>
</tbody>
</table>

Table S2  Primers used in the luciferase activity assay and site-directed mutagenesis

In (a), SacI/HindIII were used as the incision enzymes, T4 was the ligase and pMIR-REPORT™ Luciferase was the plasmid.

(a) Amplification of 3′-UTR region in the predicted target genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward sequence (5′→3′)</th>
<th>Reverse sequence (5′→3′)</th>
<th>PCR fragment size (bp)</th>
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<tbody>
<tr>
<td>BAP1</td>
<td>ACGTGAGCTCAGGACCCCAACTACGATG</td>
<td>TCGGAAGCTTAGGGCACGAGAAGGAAC</td>
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<tr>
<td>KSR2</td>
<td>ACGTGAGCTGCTGCTCTTCACTTCCCTCTAT</td>
<td>TCGGAAGCTTACATACCTTGTTCCAC</td>
<td>358</td>
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<tr>
<td>GLTSCR1</td>
<td>ACGTGAGCTCCAGCCGGACCTGAGGATAAC</td>
<td>TCGGAAGCTTCAGGAAATGGGAAACAGG</td>
<td>317</td>
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<tr>
<td>CREG1</td>
<td>ACGTGAGCTGCTGTGAGGATTGATTGCTTT</td>
<td>TCGGAAGCTTATCCAGGAGTTGGAC</td>
<td>299</td>
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<tr>
<td>DOCK1</td>
<td>TCGTGCCGGCGTGGGTCTGGGAGGTAGAT</td>
<td>TCGGAAGCTTCATGAGCTGGAGGAGG</td>
<td>265</td>
</tr>
<tr>
<td>VEZT</td>
<td>ACGTGAGCTCTGGAGAGGGATTGAAG</td>
<td>TCGGAAGCTTCGCCACTGTTGAACTA</td>
<td>504</td>
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<tr>
<td>RGS4</td>
<td>ACGTGAGCTCAATGGTCTTGGGCAACAG</td>
<td>TCGGAAGCTTACATACCTTGTTCCAC</td>
<td>339</td>
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<tr>
<td>PTPN1</td>
<td>ACGTGAGCTTCTGCGAGGAGGAGGAC</td>
<td>TCGGAAGCTTCACCAGCAGTTGGCAAG</td>
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<tr>
<td>FGF7</td>
<td>ACGTGAGCTC-AACCTGCTGTCCTGCCCCACCT</td>
<td>TCGGAAGCTTACATACCTTGTTCCAC</td>
<td>384</td>
</tr>
<tr>
<td>EMP1</td>
<td>ACGTGAGCTCT-AACCTGCTGTCCTGCCCCACCT</td>
<td>TCGGAAGCTTACATACCTTGTTCCAC</td>
<td>516</td>
</tr>
</tbody>
</table>

(b) PCR primer used in site-directed mutagenesis

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward sequence (5′→3′)</th>
<th>Reverse sequence (5′→3′)</th>
<th>GC content (%)</th>
<th>Tm (°C)</th>
</tr>
</thead>
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<td>EMP1</td>
<td>GAATCTGAGACATGCGCTATGATG</td>
<td>GTCATGACAAATTATATGCTGCACTTAC</td>
<td>40</td>
<td>78.6</td>
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<tr>
<td>KSR2</td>
<td>CCCAGGTCTCAGGCCCCCAAGACAAC</td>
<td>GGTTTTTTGCTGCAAAGGCACTGGGG</td>
<td>46.7</td>
<td>78.1</td>
</tr>
<tr>
<td>RGS4</td>
<td>CTTATTTGTGGCCTTATTAATTATAGCTGCACTTAC</td>
<td>GTCATGACAAATTATATGCTGCACTTAC</td>
<td>40</td>
<td>78.6</td>
</tr>
</tbody>
</table>

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Table S3  Univariate and multivariate Cox proportional hazards modelling of factors associated with tumour-specific survival in the ESCC patient group (n = 44)

P values are Cox proportional hazards. High, post-operative miR-31 high expression; low, post-operative miR-31 low expression; HR, hazard ratio.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Subset</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR of recurrence (95 % CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Age ⩾ 55/age &lt; 55</td>
<td>1.020 (0.363–2.867)</td>
<td>0.970</td>
</tr>
<tr>
<td>Gender</td>
<td>Male/female</td>
<td>1.092 (0.409–2.919)</td>
<td>0.772</td>
</tr>
<tr>
<td>Smoking status</td>
<td>Yes/no</td>
<td>1.863 (0.734–4.729)</td>
<td>0.190</td>
</tr>
<tr>
<td>Drinking status</td>
<td>Yes/no</td>
<td>1.915 (0.753–4.866)</td>
<td>0.272</td>
</tr>
<tr>
<td>Family history of cancer</td>
<td>Yes/no</td>
<td>1.252 (0.410–3.822)</td>
<td>0.693</td>
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<tr>
<td>T stage</td>
<td>T3/T1 + T2</td>
<td>5.079 (1.166–22.123)</td>
<td>0.030</td>
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<tr>
<td>Lymph node metastasis</td>
<td>Yes/no</td>
<td>5.247 (1.715–16.049)</td>
<td>0.004</td>
</tr>
<tr>
<td>miR-31</td>
<td>High/low</td>
<td>4.331 (1.422–13.188)</td>
<td>0.010</td>
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