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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 oncogenetic 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 oncogenetic 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/cs1210437add.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.

Table 1 Characteristics of subjects with ESCC and normal controls

P values were determined using a two-sided χ^2 test.

	Tissue samples	Serum samples in the discovery group			Serum samples in the validation group		
Variable	Cases ($n=45$)	Controls ($n=121$)	Cases ($n=120$)	*P* value	Controls ($n=81$)	Cases ($n=81$)	*P* value
Gender (*n*)				0.624			0.341
Male	34 (75.6%)	76 (62.8%)	79 (65.8%)		43 (53.1%)	49 (60.5%)	
Female	11 (24.4%)	45 (37.1%)	41 (34.2%)		38 (46.9%)	32 (39.5%)	
Age (years)				0.136			0.064
$\leqslant 45$	1 (2.2%)	20 (16.5%)	10 (8.3%)		15 (18.5%)	7 (8.7%)	
46–55	8 (17.8%)	28 (23.1%)	23 (19.2%)		25 (30.8%)	21 (25.9%)	
56–65	21 (46.7%)	46 (38.1%)	50 (41.2%)		29 (35.8%)	29 (35.8%)	
>65	15 (33.3%)	27 (22.3%)	37 (30.8%)		12 (14.8%)	24 (29.6%)	
Stage (*n*)							
I	7 (15.6%)	–	28 (23.2%)		–	–	
II	17 (37.8%)	–	31 (25.8%)		–	–	
III	21 (46.7%)	–	33 (27.5%)		–	–	
IV	0 (0%)	–	28 (23.2%)		–	–	

Isolation of miRs and quantification by real-time RT (reverse transcription)–PCR

miRs from tissues and serum were extracted using an miRNeasy Mini kit (Qiagen). The RNA extracted from the serum ranged from 20 to 100 ng/μl, and the RNA extracted from tissues ranged from 310 to 590 ng/μl. miRs were polyadenylated by *Escherichia coli* Poly (A) Polymerase (NEB) before RT to cDNA using aPrimeScript™ RT kit and 5′-GCGAGCACAGA-ATTAATACGACTCACTATAGGTTTTTTTTTTTTT-CG-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′-GCGAGCACAGAATTAATACGAC-3′; and the forward primers were 5′-ACGCAAA-TTCGTGAAGCGTT-3′ for *RNU6B* (U6 small nuclear RNA); 5′-TAGCAGCACGTAAATATTGGCG-3′ for *miR-16*; and 5′-AGGCAAGATGCTGGCATAGCT-3′ for *miR-31*. miR sequences were obtained from the miRBase database (http://microrna.sanger.ac.uk/). Each sample was run in triplicate. The expression of *miR-31* was calculated using the equation $2^{-\Delta C_t}$, where $\Delta C_t = (C_{t\,miR\text{-}31} - C_{t\,miR\text{-}16})$ in serum analysis and $\Delta C_t = (C_{t\,miR\text{-}31} - C_{t\,RNU6B})$ in tissue analysis. The relative expression of *miR-31* in tissues was calculated by $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (\Delta C_{t\,\text{tumour tissue}} - \Delta C_{t\,\text{normal tissue}})$.

Cell culture and miR transient transfection

Three ESCC cell lines, EC9706 (established in our laboratory) [25], KYSE150 and KYSE510 (gifts from Dr Y. Shimada, First Department of Surgery, Kyoto University, Kyoto, Japan) [26], were used in our present study. *miR-31* mimics and antisense inhibitors that contain 2′-OMe (*O*-methyl) modifications were synthesized and purified by Shanghai Gene-Pharma) siRNA (small interfering RNA) duplexes with non-specific sequences were used as a negative control. Cells were seeded on to 24-well plates at a density of 5×10^4 cells/well and were then transfected with 50 nM of the following: *miR-31* mimics, inhibitors (anti-*miR-31*) or non-specific sequences (NCs), using Lipofectamine™ 2000 (Invitrogen).

Colony 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 2×10^4 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 analysed under a microscope by counting the cells in ten random fields per insert. For the invasion assay, 8-μm Transwell^{TM} inserts were coated with 30 μg of Matrigel (Becton Dickinson) and air-dried. After rehydration, 2×10^4 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 (*EMP1*), TCAAGTT (*KSR2*) and TCGGGTT (*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 10^4 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, lysates 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 software (http://cbcsrv.watson.ibm.com/rna22.html). Statistical 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 TNM 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 relative 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 S2 at http://www.clinsci.org/cs/121/cs1210437add.htm). 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 significantly 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

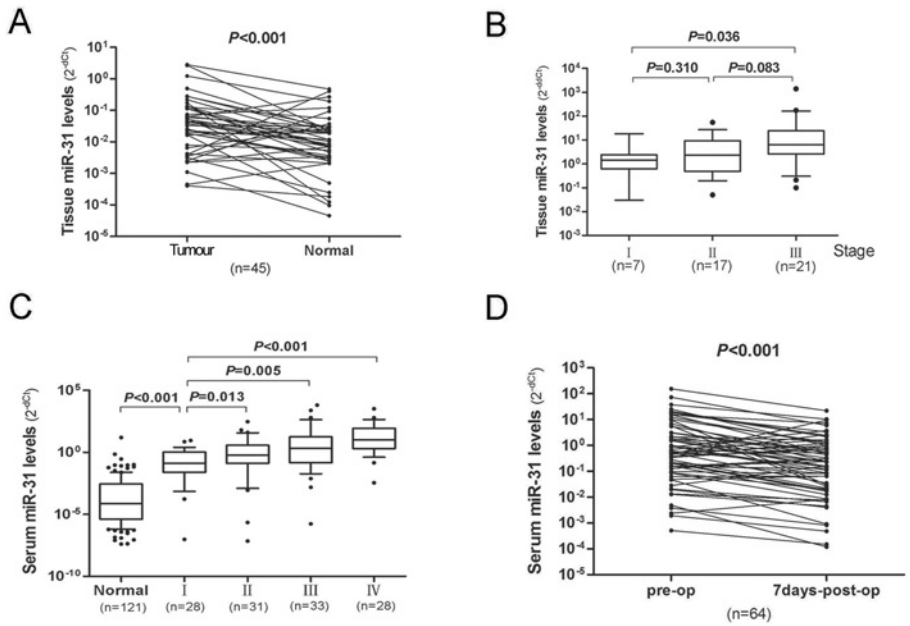

Figure 1 ***miR-31* expression in ESCC tissue and serum samples**

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 $2^{-\Delta C_t}$. (B) Relative expression of *miR-31* in tissues grouped by total TNM stage, normalized by $2^{-\Delta\Delta C_t}$. (C) Expressions of serum *miR-31* in healthy subjects and ESCC patients grouped by TNM 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

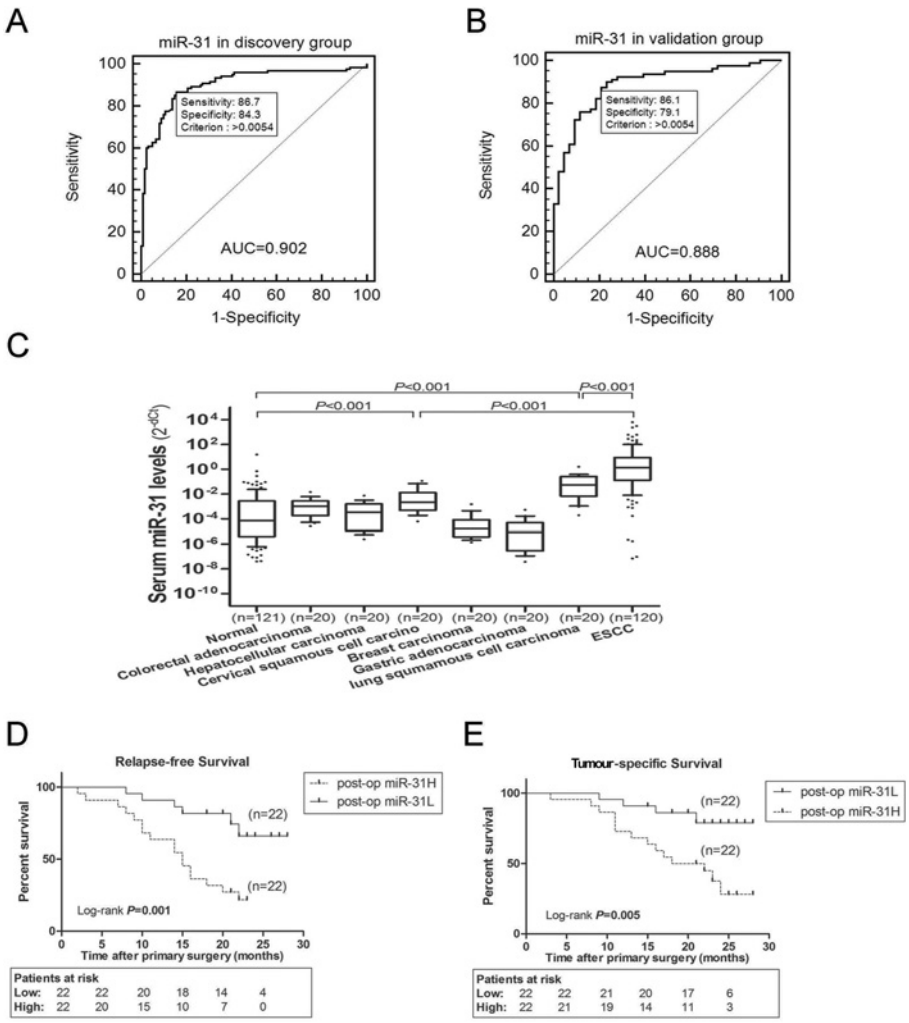

Figure 2 The role of serum *miR-31* in the diagnosis and prognosis of ESCC

(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 tumours. 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-31*L, *miR-31* low expression; *miR-31*H, *miR-31* high expression; post-op, post-operative.

low-level expression (post-op *miR-31*L) and 22 cases with high-level expression (post-op *miR-31*H). Strikingly, there was a significant difference between the relapse-free survival ($P = 0.001$, log-rank test) and tumour-specific survival ($P = 0.005$, log-rank test) (Figures 2D and 2E). 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).

Table 2 Univariate and multivariate Cox proportional hazard modelling of factors associated with relapse-free survival in 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.

		Univariate analysis		Multivariate analysis	
Variable	Subset	HR of recurrence (95 % CI)	*P* value	HR of recurrence (95 % CI)	*P* value
Age (years)	⩾55/<55	0.866 (0.356–2.109)	0.752	0.715 (0.206–2.479)	0.854
Gender	Male/female	1.029 (0.435–2.431)	0.948	1.091 (0.433–2.749)	0.597
Smoking status	Yes/no	1.555 (0.669–3.613)	0.305	1.384 (0.523–3.633)	0.513
Drinking status	Yes/no	1.314 (0.579–2.983)	0.513	0.807 (0.290–2.249)	0.682
Family history of cancer	Yes/no	1.132 (0.419–3.054)	0.807	0.603 (0.216–1.682)	0.334
T stage	T3/T1 + T2	4.650 (1.377–15.697)	0.013	2.573 (0.722–9.169)	0.145
Lymph node metastasis	Yes/no	4.769 (1.867–12.185)	0.001	3.882 (1.500–10.044)	0.005
miR-31	High/low	4.127 (1.620–10.514)	0.003	3.260 (1.264–8.421)	0.015

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 oncogenetic 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

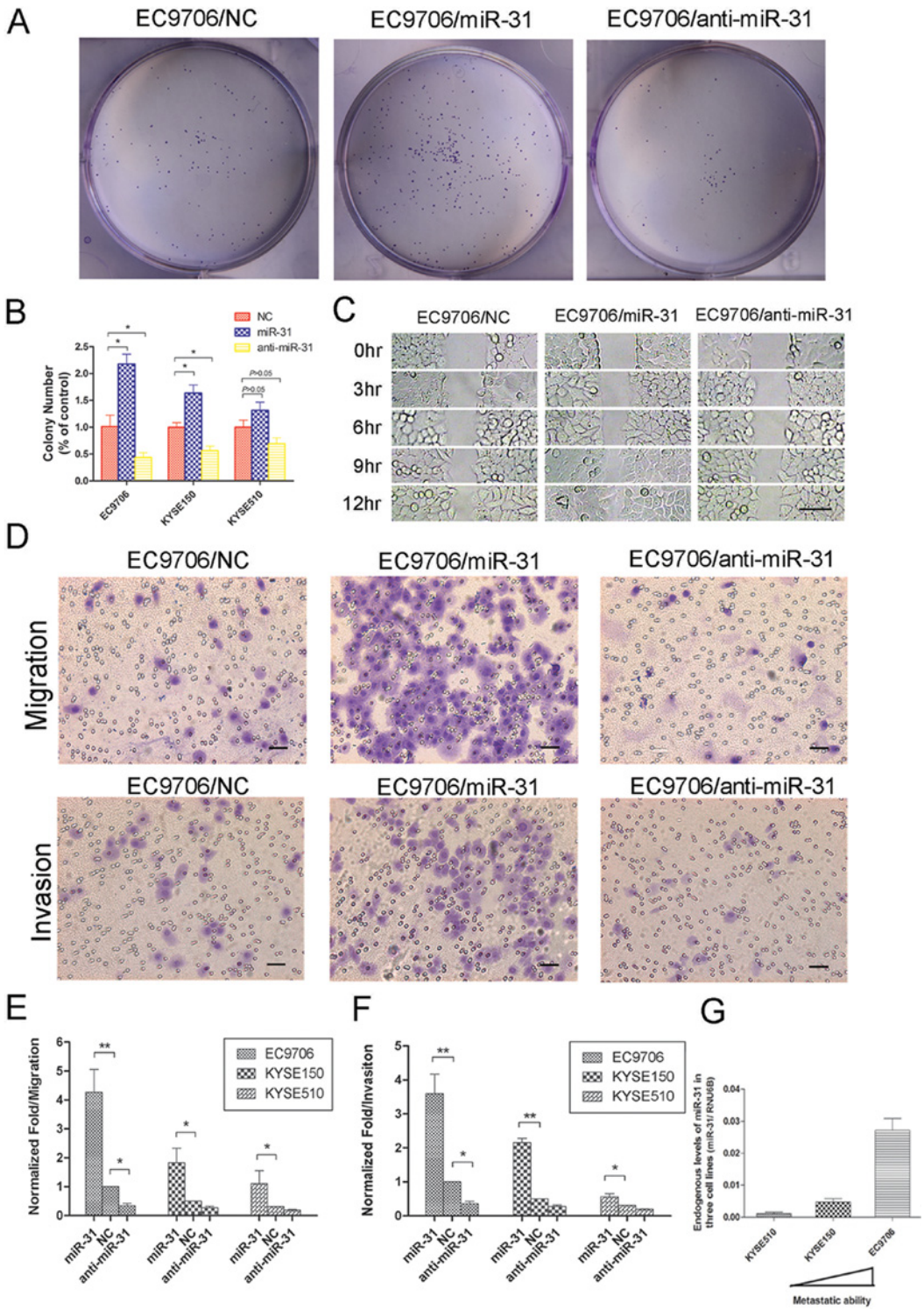

Figure 3 Colony formation, migration and invasion analyses of three ESCC cell lines after up-regulation or down-regulation of *miR-31*

(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 $\pm$ S.E.M. $^{*}P < 0.05$ and $^{**}P < 0.01$ using a Student's t test.

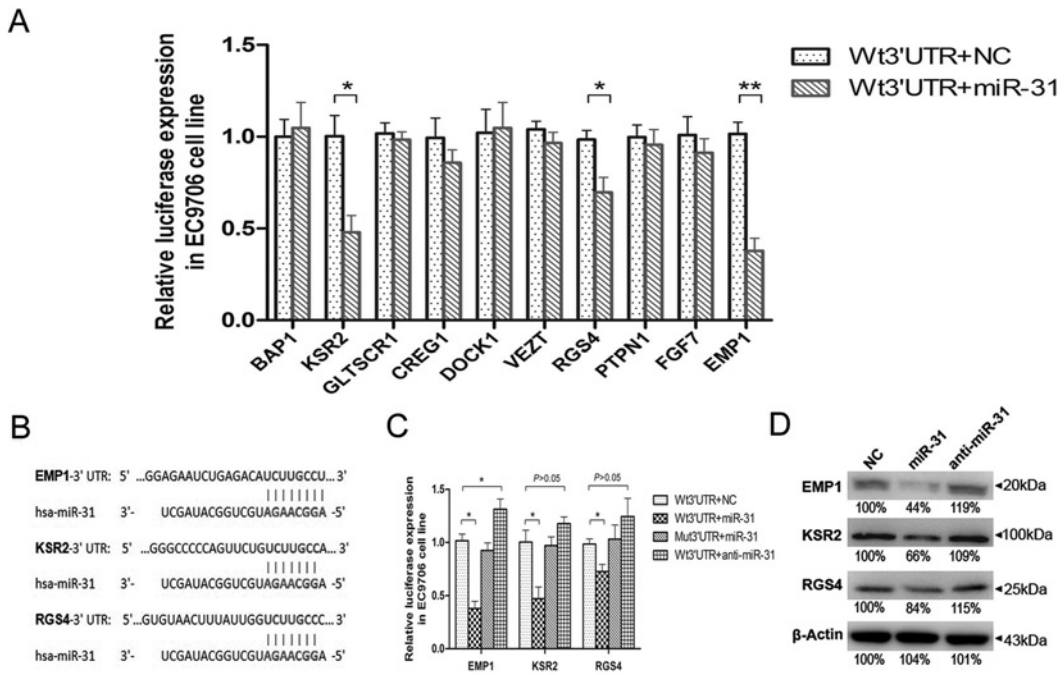

Figure 4 Detection of target genes regulated by *miR-31* in the EC9706 cell line

(A) Luciferase activities after transfection with the ten wild-type 3′-UTR-clone reporter constructs (Wt3′UTR) and *miR-31* or NC. (B) *miR-31*-binding sites in the 3′-UTR region of *EMP1*, *KSR2* and *RGS4*. (C) Luciferase activities after transfection of binding-site mutant 3′-UTR-clone reporter constructs (Mut3′UTR) and the effect of anti-*miR-31*. (D) Western blots for endogenous EMP1, KSR2 and RGS4 levels 48 h after transient transfection, with the NC group as a control.

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 oncogenetic 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 carcinogenetic 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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REFERENCES

1 Parkin, D. M., Bray, F., Ferlay, J. and Pisani, P. (2005) Global cancer statistics, 2002. CA Cancer J. Clin. **55**, 74–108
2 Holmes, R. S. and Vaughan, T. L. (2007) Epidemiology and pathogenesis of esophageal cancer. Semin. Radiat. Oncol. **17**, 2–9
3 Urba, S. G., Orringer, M. B., Turrisi, A., Iannettoni, M., Forastiere, A. and Strawderman, M. (2001) Randomized trial of preoperative chemoradiation versus surgery alone in patients with locoregional esophageal carcinoma. J. Clin. Oncol. **19**, 305–313
4 Kelsen, D. P., Ginsberg, R., Pajak, T. F., Sheahan, D. G., Gunderson, L., Mortimer, J., Estes, N., Haller, D. G., Ajani, J., Kocha, W. et al. (1998) Chemotherapy followed by surgery compared with surgery alone for localized esophageal cancer. N. Engl. J. Med. **339**, 1979–1984
5 Bonnetain, F., Bouche, O., Michel, P., Mariette, C., Conroy, T., Pezet, D., Roullet, B., Seitz, J. F., Paillot, B., Arveux, P. et al. (2006) A comparative longitudinal quality of life study using the Spitzer quality of life index in a randomized multicenter phase III trial (FFCD 9102): chemoradiation followed by surgery compared with chemoradiation alone in locally advanced squamous resectable thoracic esophageal cancer. Ann. Oncol. **17**, 827–834
6 Yanaihara, N., Caplen, N., Bowman, E., Seike, M., Kumamoto, K., Yi, M., Stephens, R. M., Okamoto, A., Yokota, J., Tanaka, T. et al. (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell **9**, 189–198
7 Ng, E. K., Chong, W. W., Jin, H., Lam, E. K., Shin, V. Y., Yu, J., Poon, T. C., Ng, S. S. and Sung, J. J. (2009) Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. Gut **58**, 1375–1381
8 Ventura, A. and Jacks, T. (2009) MicroRNAs and cancer: short RNAs go a long way. Cell **136**, 586–591
9 Slaby, O., Svoboda, M., Fabian, P., Smerdova, T., Knoflickova, D., Bednarikova, M., Nenutil, R. and Vyzula, R. (2007) Altered expression of miR-21,miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. Oncology **72**, 397–402
10 Zhang, Y., Guo, J., Li, D., Xiao, B., Miao, Y., Jiang, Z. and Zhuo, H. (2009) Down-regulation of miR-31 expression in gastric cancer tissues and its clinical significance. Med. Oncol. **16**, 360–364
11 Yan, L. X., Huang, X. F., Shao, Q., Huang, M. Y., Deng, L., Wu, Q. L., Zeng, Y. X. and Shao, J. Y. (2008) MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA **14**, 2348–2360
12 Schaefer, A., Jung, M., Mollenkopf, H. J., Wagner, I., Stephan, C., Jentzmik, F., Miller, K., Lein, M., Kristiansen, G. and Jung, K. (2010) Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. Int. J. Cancer **126**, 1166–1176
13 Creighton, C. J., Fountain, M. D., Yu, Z., Nagaraja, A. K., Zhu, H., Khan, M., Olokpa, E., Zariff, A., Gunaratne, P. H., Matzuk, M. M. and Anderson, M. L. (2010) Molecular profiling uncovers a p53-associated role for microRNA-31 in inhibiting the proliferation of serous ovarian carcinomas and other cancers. Cancer Res. **70**, 1906–1915
14 Lajer, C. B., Nielsen, F. C., Friis-Hansen, L., Norrild, B., Borup, R., Garnaes, E., Rossing, M., Specht, L., Therkildsen, M. H., Nauntofte, B. et al. (2011) Different miRNA signatures of oral and pharyngeal squamous cell carcinomas: a prospective translational study. Br. J. Cancer **104**, 830–840
15 Wang, P., Fu, T., Wang, X. and Zhu, W. (2010) Primary, study of miRNA expression pattens in laryngeal carcinoma by microarray. Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi **24**, 535–538
16 Liu, X., Sempere, L. F., Ouyang, H., Memoli, V. A., Andrew, A. S., Luo, Y., Demidenko, E., Korc, M., Shi, W., Preis, M. et al. (2010) MicroRNA-31 functions as an oncogenic microRNA in mouse and human lung cancer cells by repressing specific tumor suppressors. J. Clin. Invest. **120**, 1298–1309

17 Wong, T. S., Liu, X. B., Wong, B. Y., Ng, R. W., Yuen, A. P. and Wei, W. I. (2008) Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue. Clin. Cancer Res. **14**, 2588–2592

18 Hu, Z., Chen, X., Zhao, Y., Tian, T., Jin, G., Shu, Y., Chen, Y., Xu, L., Zen, K., Zhang, C. and Shen, H. (2010) Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. J. Clin. Oncol. **28**, 1721–1726

19 Chen, X., Ba, Y., Ma, L., Cai, X., Yin, Y., Wang, K., Guo, J., Zhang, Y., Chen, J., Guo, X. et al. (2008) Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell. Res. **18**, 997–1006

20 Gilad, S., Meiri, E., Yogev, Y., Benjamin, S., Lebanony, D., Yerushalmi, N., Benjamin, H., Kushnir, M., Cholakh, H., Melamed, N. et al. (2008) Serum microRNAs are promising novel biomarkers. PLoS ONE **3**, e3148

21 Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'Briant, K. C., Allen, A. et al. (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc. Natl. Acad. Sci. U.S.A. **105**, 10513–10518

22 Lawrie, C. H., Gal, S., Dunlop, H. M., Pushkaran, B., Liggins, A. P., Pulford, K., Banham, A. H., Pezzella, F., Boultwood, J., Wainscoat, J. S. et al. (2008) Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br. J. Haematol. **141**, 672–675

23 Edge, S. B., Byrd, D. R., Compton, C. C., Fritz, A. G., Greene, F. L. and Trotti, A. (2010) American Joint Committee on Cancer: AJCC Cancer Staging Manual, 7th edn, p. 646, Springer, New York

24 Shi, R. and Chiang, V. L. (2005) Facile means for quantifying microRNA expression by real-time PCR. BioTechniques **39**, 519–525

25 Han, Y., Wei, F., Xu, X., Cai, Y., Chen, B., Wang, J., Xia, S., Hu, H., Huang, X., Wu, M. and Wang, M. (2002) Establishment and comparative genomic hybridization analysis of human esophageal carcinomas cell line EC9706. Zhonghua Yi Xue Yi Chuan Xue Za Zhi **19**, 455–457

26 Shimada, Y., Imamura, M., Wagata, T., Yamaguchi, N. and Tobe, T. (1992) Characterization of 21 newly established esophageal cancer cell lines. Cancer **69**, 277–284

27 Huang, Z., Huang, D., Ni, S., Peng, Z., Sheng, W. and Du, X. (2009) Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. Int. J. Cancer **127**, 118–126

28 Liu, C. J., Kao, S. Y., Tu, H. F., Tsai, M. M., Chang, K. W. and Lin, S. C. (2010) Increase of microRNA *miR-31* level in plasma could be a potential marker of oral cancer. Oral Dis. **70**, 1635–1644

29 Valastyan, S., Reinhardt, F., Benaich, N., Calogrias, D., Szasz, A. M., Wang, Z. C., Brock, J. E., Richardson, A. L. and Weinberg, R. A. (2009) A pleiotropically acting microRNA, *miR-31*, inhibits breast cancer metastasis. Cell **137**, 1032–1046

30 Aprelikova, O., Yu, X., Palla, J., Wei, B. R., John, S., Yi, M., Stephens, R., Simpson, R. M., Risinger, J. I., Jazaeri, A. and Niederhuber, J. (2010) The role of miR-31 and its target gene SATB2 in cancer-associated fibroblasts. Cell Cycle **9**, 4387–4398

31 Tsai, Y. H., Wu, M. F., Wu, Y. H., Chang, S. J., Lin, S. F., Sharp, T. V. and Wang, H. W. (2009) The M type K15 protein of Kaposi's sarcoma-associated herpesvirus regulates microRNA expression via its SH2-binding motif to induce cell migration and invasion. J. Virol. **83**, 622–632

32 Chen, Y., Medvedev, A., Ruzanov, P., Marvin, K. W. and Jetten, A. M. (1997) cDNA cloning, genomic structure, and chromosome mapping of the human epithelial membrane protein CL-20 gene (EMP1), a member of the PMP22 family. Genomics **41**, 40–48

33 Liu, L., Channavajhala, P. L., Rao, V. R., Moutsatsos, I., Wu, L., Zhang, Y., Lin, L. L. and Qiu, Y. (2009) Proteomic characterization of the dynamic KSR-2 interactome, a signaling scaffold complex in MAPK pathway. Biochim. Biophys. Acta **1794**, 1485–1495

34 Channavajhala, P. L., Rao, V. R., Spaulding, V., Lin, L. L. and Zhang, Y. G. (2005) hKSR-2 inhibits MEKK3-activated MAP kinase and NF-κB pathways in inflammation. Biochem. Biophys. Res. Commun. **334**, 1214–1218

35 Dougherty, M. K., Ritt, D. A., Zhou, M., Specht, S. I., Monson, D. M., Veenstra, T. D. and Morrison, D. K. (2009) KSR2 is a calcineurin substrate that promotes ERK cascade activation in response to calcium signals. Mol. Cell **34**, 652–662

36 Niedergethmann, M., Alves, F., Neff, J. K., Heidrich, B., Aramin, N., Li, L., Pilarsky, C., Grutzmann, R., Allgayer, H., Post, S. and Gretz, N. (2007) Gene expression profiling of liver metastases and tumour invasion in pancreatic cancer using an orthotopic SCID mouse model. Br. J. Cancer **97**, 1432–1440

37 Xie, Y., Wolff, D. W., Wei, T., Wang, B., Deng, C., Kirui, J. K., Jiang, H., Qin, J., Abel, P. W. and Tu, Y. (2009) Breast cancer migration and invasion depend on proteasome degradation of regulator of G-protein signaling 4. Cancer Res. **69**, 5743–5751

38 Puiffe, M. L., Le Page, C., Filali-Mouhim, A., Zietarska, M., Ouellet, V., Tonin, P. N., Chevrette, M., Provencher, D. M. and Mes-Masson, A. M. (2007) Characterization of ovarian cancer ascites on cell invasion, proliferation, spheroid formation, and gene expression in an *in vitro* model of epithelial ovarian cancer. Neoplasia **9**, 820–829

39 Tatenhorst, L., Senner, V., Puttmann, S. and Paulus, W. (2004) Regulators of G-protein signaling 3 and 4 (RGS3, RGS4) are associated with glioma cell motility. J. Neuropathol. Exp. Neurol. **63**, 210–222

40 Wang, H. T., Liu, Z. H., Wang, X. Q. and Wu, M. (2002) Effect of EMP-1 gene on human esophageal cancer cell line. Ai Zheng **21**, 229–232

41 Wang, H. T., Kong, J. P., Ding, F., Wang, X. Q., Wang, M. R., Liu, L. X., Wu, M. and Liu, Z. H. (2003) Analysis of gene expression profile induced by EMP-1 in esophageal cancer cells using cDNA microarray. World J. Gastroenterol. **9**, 392–398

42 Zinovyeva, M. V., Monastyrskaya, G. S., Kopantzev, E. P., Vinogradova, T. V., Kostina, M. B., Sass, A. V., Filyukova, O. B., Uspenskaya, N. Y., Sukhikh, G. T. and Sverdlov, E. D. (2010) Identification of some human genes oppositely regulated during esophageal squamous cell carcinoma formation and human embryonic esophagus development. Dis. Esophagus **23**, 260–270

43 Yu, Y. H., Kuo, H. K. and Chang, K. W. (2008) The evolving transcriptome of head and neck squamous cell carcinoma: a systematic review. PLoS ONE **3**, e3215

44 Zhou, Y. B., Huang, Z. X., Ren, C. P., Zhu, B. and Yao, K. T. (2009) Screening and preliminary analysis of the apoptosis- and proliferation-related genes in nasopharyngeal carcinoma. Nan Fang Yi Ke Da Xue Xue Bao **29**, 645–647

45 Zhang, J., Cao, W., Xu, Q. and Chen, W. T. (2010) The expression of EMP1 is downregulated in oral squamous cell carcinoma and possibly associated with tumour metastasis. J. Clin. Pathol. **64**, 25–29

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SUPPLEMENTARY ONLINE DATA

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

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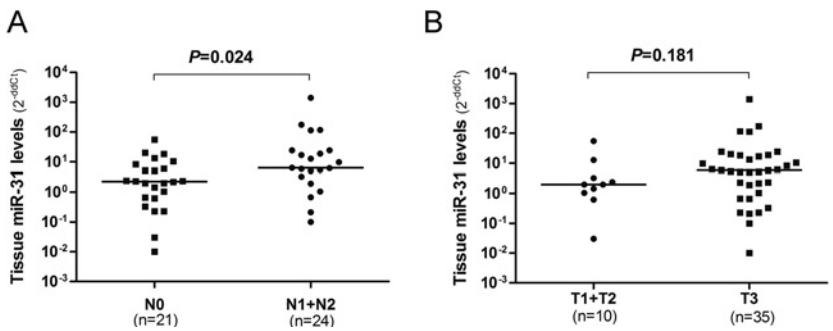

Figure S1 Expression of *miR-31* in ESCC tissue samples

(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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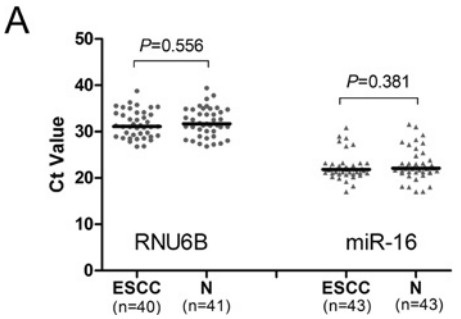

Figure S2 Evaluation to determine the better normalization control for the serum *miR-31* test

(A) Scatter plot of C_t values of the two candidate normalization controls *RNU6B* and *miR-16* in the serum from healthy controls (N) and ESCC patients. The cycle threshold (C_t) 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.

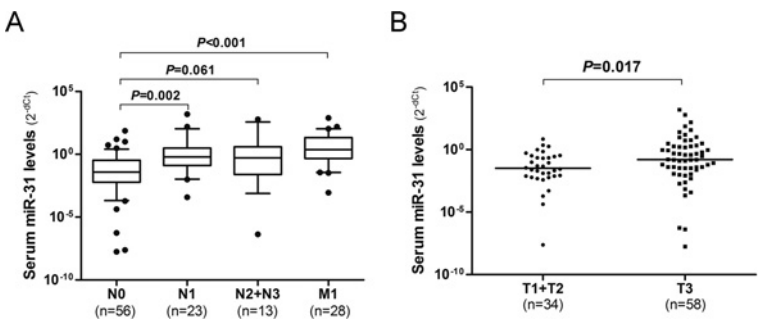

Figure S3 Expression of *miR-31* in ESCC serum samples

(A) Expression of serum *miR-31* classified according to metastasis (N0/N1/N2 + N3/M1); M1 represents patients with distant metastasis regardless of their 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.

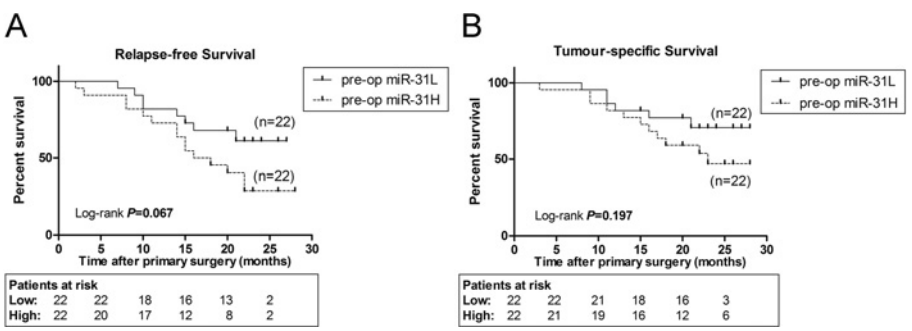

Figure S4 Role of serum *miR-31* in the prognosis of ESCC by Kaplan–Meier analysis ($n = 44$)

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-31*L, low expression of *miR-31* pre-operation; pre-op *miR-31*H, 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

Pathological feature	Normal	Colorectal adenocarcinoma	Hepatocellular carcinoma	Cervical SCC	Breast carcinoma	Gastric adenocarcinoma	Lung SCC	ESCC
Cases (*n*)	121	20	20	20	20	20	20	120
Gender (*n*)								
Male	76	9	16	0	0	12	14	79
Female	45	11	4	20	20	8	6	41
Age (years)								
$\leqslant 45$	20	1	3	9	9	4	1	10
46–55	28	4	5	6	6	3	7	23
56–65	46	12	9	3	4	7	7	50
>65	27	3	3	2	1	6	5	37
Stage (*n*)								
I		2	0	1	2	1	2	28
II		4	5	8	7	5	13	31
III		5	6	2	7	5	5	33
IV		9	9	9	4	9	0	28

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

Gene name	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	PCR fragment size (bp)
BAP1	ACGTGAGCTCAGGACCCACAACTACGATG	TCGGAAGCTTAGGGCACGATGGAAGGAAC	376
KSR2	ACGTGAGCTCTGCTTCACTTTCCCTCTAT	TCGGAAGCTTCTGATACATTCTTTCCCAC	358
GLTSCR1	ACGTGAGCTCCCAGGACGTTGACCAGATAAC	TCGGAAGCTTCAGGAAATGGGAAGAAACAGG	317
CREG1	ACGTGAGCTCGTTTCCAGTGGTGCTCTT	TCGGAAGCTTATACCCAGGATTGTGAGAC	299
DOCK1	TCGTGCCGGCGTGGGTCTGGGAGGTAGAT	TCGGAAGCTTCCATGACAGTGAGGAGGAT	265
VEZT	ACGTGAGCTCCTGGAAAGGGAATATGAG	TCGGAAGCTTCACGCACTGTTGAAACTA	504
RGS4	ACGTGAGCTCATTGGTCTTGCCCTATTAT	TCGGAAGCTTTCATGACAATTATAATAGGGCAAGC	339
PTPN1	ACGTGAGCTCTTAGGGAAGCAGGGACAC	TCGGAAGCTTACCACGAGAAAGGCAAAATG	387
FGF7	ACGT-GAGCTC-AACTGTCTGGCTCAACGG	TCGGAAGCTTCTTTCAGTGCTGCCACCT	384
EMP1	ACGT-GAGCTC-TAAGGTCATGCTGGTGGG	TCGGAAGCTTAGTGGGATAGGCAGGGTC	516

(b) PCR primer used in site-directed mutagenesis

Gene name	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	GC content (%)	T_m (°C)
EMP1	GAATCTGAGACATCGCGCCTACTTTTCTTTATTAG	CTAATAAAGAAAAGTAGGCGCGATGTCTCAGATTC	40	78.6
KSR2	CCCAGTTCTGTCAAGCCATACCAAAAAACC	GGTTTTTTGGTATGGCTTGACAGAACTGGG	46.7	78.1
RGS4	CTTTATTGGTCGGGCCCTATTATAATTGTCATGAC	GTCATGACAATTATAATAGGGCCCGACCAATAAAG	40	78.6

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.

		Univariate analysis		Multivariate analysis	
Variable	Subset	HR of recurrence (95 % CI)	*P* value	HR of recurrence (95 % CI)	*P* value
Age (years)	Age $\geqslant$ 55/age $<$ 55	1.020 (0.363–2.867)	0.970	0.767 (0.207–2.840)	0.692
Gender	Male/female	1.092 (0.409–2.919)	0.772	0.651 (0.208–2.042)	0.462
Smoking status	Yes/no	1.863 (0.734–4.729)	0.190	1.413 (0.519–3.847)	0.499
Drinking status	Yes/no	1.915 (0.753–4.866)	0.272	1.243 (0.379–4.076)	0.720
Family history of cancer	Yes/no	1.252 (0.410–3.822)	0.693	0.572 (0.178–1.839)	0.349
T stage	T3/T1 + T2	5.079 (1.166–22.123)	0.030	3.175 (0.706–14.282)	0.132
Lymph node metastasis	Yes/no	5.247 (1.715–16.049)	0.004	4.124 (1.332–12.767)	0.014
miR-31	High/low	4.331 (1.422–13.188)	0.010	2.787 (0.894–8.685)	0.077

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