

**Keywords:** oesophageal squamous cell carcinoma; plasma; microRNA; biomarker; diagnosis; monitoring; liquid biopsy; micro array

# Plasma microRNA profiles: identification of miR-25 as a novel diagnostic and monitoring biomarker in oesophageal squamous cell carcinoma

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**Background:** Recent studies have demonstrated that microRNAs are stably detectable in plasma/serum because of their binding to specific proteins or being packaged in secretory particles. This study was designed to detect novel microRNAs in plasma for cancer detection and monitoring using microRNA array-based approaches in oesophageal squamous cell carcinoma (ESCC) patients.

**Methods:** Through the integration of two Toray 3D-Gene microRNA array-based approaches to compare plasma microRNA levels between ESCC patients and healthy volunteers and between preoperative and postoperative ESCC patients, we identified a novel plasma biomarker in ESCC.

**Results:** (1) Eight upregulated and common microRNAs (miR-15b, 16, 17, 25, 19b, 20a, 20b, and 106a) were selected using two high-resolution microRNA array approaches. (2) Test-scale analyses by quantitative RT-PCR validated a significant higher levels of plasma miR-19b ( $P=0.0020$ ) and miR-25 ( $P=0.0030$ ) in ESCC patients than controls. However, a significant correlation was observed between plasma miR-19b levels and concentrations of red blood cells ( $P=0.0073$ ) and haemoglobin ( $P=0.0072$ ). (3) miR-25 expression was found to be significantly higher in ESCC tissues ( $P=0.0157$ ) and ESCC cell lines ( $P=0.0093$ ) than in normal tissues and fibroblasts. (4) In a large-scale validation analysis, plasma miR-25 levels were significantly higher in 105 preoperative ( $P<0.0001$ ) ESCC patients who underwent curative oesophagectomy and 20 superficial ESCC patients who underwent endoscopic resection ( $P<0.0001$ ) than in 50 healthy volunteers. (5) Plasma miR-25 levels were significantly reduced in postoperative samples than in preoperative samples ( $P<0.0005$ ) and were significantly increased during ESCC recurrences ( $P=0.0145$ ).

**Conclusions:** Plasma miR-25 might be a clinically useful biomarker for cancer detection and the monitoring of tumour dynamics in ESCC patients.

Oesophageal carcinoma is the sixth leading cause of cancer-related deaths worldwide (Jemal *et al*, 2011). Although there are two histological types of oesophageal carcinoma, oesophageal squamous

cell carcinoma (ESCC) is the predominant histological type in Asian countries and accounts for approximately 90% of oesophageal carcinomas (Hiyama *et al*, 2007) and is one of the most

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Revised 7 July 2014; accepted 17 July 2014; published online 12 August 2014

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aggressive carcinomas of the gastrointestinal tract. Although surgical techniques, perioperative management, and perioperative chemo and/or radiotherapy regimens have greatly progressed, even now ESCC continues to present with an extremely poor prognosis. Therefore, primary tumours must be detected at an early stage and treated with curative intent to improve survival rates, and patients with far advanced disease must be diagnosed preoperatively in order to avoid impairments in their quality of life following unnecessary extended surgery. Moreover, recurrent disease must be diagnosed when it is still minimal or clinically occult to improve the prognosis of ESCC patients.

Because identifying molecular targets for ESCC treatment may contribute to the improvement of survival of patients with this lethal disease, several recent studies have clarified that certain molecules, such as *COX-2*, *BCL-2*, *TP53*, *p16*, *cyclin D1*, *FAS*, *EGFR*, *VEGF*, and *E-cadherin* (Hollstein *et al*, 1990; Adélaide *et al*, 1995; Gratas *et al*, 1998; Meng *et al*, 2011; Shi *et al*, 2011), have important roles in tumorigenesis and the development of ESCC. Moreover, various genetic and epigenetic alterations that contribute to the carcinogenesis of ESCC have been elucidated. In clinical settings, however, only a few molecules have been validated as diagnostic, therapeutic, and/or prognostic biomarkers for ESCC. Conventional serum tumour markers, such as carcinoembryonic antigen (CEA) and squamous cell carcinoma antigen (SCC), have been used in convenient diagnostic assays (Kosugi *et al*, 2004; Mroczko *et al*, 2008) for the early detection of ESCC and the monitoring of tumour dynamics. These serum tumour markers, however, lack sufficient sensitivity and specificity to enable the early detection of ESCC. Hence, development of novel molecular biomarkers using less invasive technology is necessary and could allow clinicians to detect early ESCC, monitor tumour dynamics, and predict treatment sensitivity and prognosis.

MicroRNAs (miRNAs), which are small non-coding RNAs, regulate the translation of specific protein-coding genes. Since their discovery in 1993 (Lee *et al*, 1993), miRNAs have been intensively studied in cancer research, and these molecules are predicted to control as much as 30% of all gene expression (Lewis *et al*, 2005). Thus altered miRNA expression has been associated with several diseases, and tumour miRNAs are involved in tumorigenesis and the development of various cancers (He *et al*, 2005; Lu *et al*, 2005; Calin and Croce, 2006; He *et al*, 2007). Recently, several studies have identified that miRNAs are detectable in plasma/serum (Calin and Croce, 2006; Chen *et al*, 2008; Filipowicz *et al*, 2008; Ichikawa *et al*, 2012). Tumour-derived miRNAs are resistant to endogenous ribonuclease activity, because these may bind to proteins, such as the Argonaute 2 protein and high-density lipoprotein (Arroyo *et al*, 2011; Vickers *et al*, 2011), or may be packaged by secretory particles, including apoptotic bodies and exosomes in plasma/serum (Hasselmann *et al*, 2001; Mitchell *et al*, 2008; Cocucci *et al*, 2009; Kosaka *et al*, 2010). Therefore, miRNAs can be present in a remarkably stable form (Mitchell *et al*, 2008; Zhu and Fan, 2011), and the expression levels of serum miRNAs are reproducible and consistent among individuals (Chen *et al*, 2008; Mitchell *et al*, 2008). Furthermore, secretory vesicles, which include specific miRNAs, can function as intercellular transmitters. Namely, secreted miRNAs from donor cells can be transferred to and function in recipient cells (Valadi *et al*, 2007; Skog *et al*, 2008; Rechavi *et al*, 2009).

Regarding ESCC, several research groups, including our own, have reported the potential utility of circulating miRNAs in plasma/serum in clinical application (Zhang *et al*, 2010; Komatsu *et al*, 2011, 2012; Kurashige *et al*, 2012; Hirajima *et al*, 2013; Takeshita *et al*, 2013). Each identified miRNA in the plasma/serum of ESCC patients could be valuable for detecting cancer, monitoring tumour, and predicting prognosis. However, these reported blood-based miRNAs do not always indicate all candidates for ESCC, and more sensitive and promising candidate

miRNAs for screening cancer, monitoring the tumour status, and predicting prognosis could be found in clinical settings. Therefore, we performed a genome-wide miRNA array-based approach to detect novel plasma miRNA candidates for detecting ESCC and monitoring the tumour status.

In this study, we selected eight upregulated and common plasma miRNA candidates (miR-15b, 16, 17, 25, 19b, 20a, 20b, and 106a) through the integration of two miRNA array-based approaches, which were used to compare plasma miRNA levels between ESCC patients and healthy volunteers and between preoperative and postoperative ESCC patients, respectively. We validated that plasma miR-25, which is located in the miR-106-25 cluster, is overexpressed in ESCC tissues (Guo *et al*, 2008; Zhu *et al*, 2011; Xu *et al*, 2012; Fang *et al*, 2013), and has an oncogenic function to negatively regulate tumour suppressive genes such as *PTEN*, *p21*, *TP53*, *Bim*, *CDH1*, and *DR4* (Petrocca *et al*, 2008; Kan *et al*, 2009; Li *et al*, 2009; Polisenio *et al*, 2010; Kumar *et al*, 2011; Razumilava *et al*, 2012; Xu *et al*, 2012), was upregulated in ESCC patients and preoperative ESCC patients. Moreover, we clearly demonstrated that plasma miR-25 levels are useful to detect ESCC and monitor tumour dynamics for tumour resection and recurrence. Our results provided evidence that plasma miR-25 levels contribute to clinical decision making in ESCC treatments to a clinically satisfactory degree.

## MATERIALS AND METHODS

**Patients and samples.** This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, and each subject provided written informed consent. Between November 2008 and June 2011, 105 consecutive preoperative plasma samples were collected from consecutive ESCC patients who underwent curative oesophagectomy at the Kyoto Prefectural University of Medicine. Patient characteristics with respect to age, sex, venous invasion, lymphatic invasion, T stage, N stage, disease stage, and resection status are described in Table 1. Moreover, additional 20 consecutive pretreatment plasma samples were collected from consecutive superficial ESCC patients who underwent curative endoscopic resection (m2-sm1, R0). All patients were pathologically diagnosed with ESCC using surgical specimens and biopsies. Ten ESCC specimens were collected from patients undergoing oesophagectomy, and seven normal oesophageal tissue specimens of the abdominal esophagus were collected from patients undergoing total gastrectomy for gastric cancer; these patients were selected for the normal specimens because the non-cancerous oesophageal tissues of ESCC patients may still exhibit dysplasia or potentially be cancerous tissue. As a control, plasma was collected from 50 healthy volunteers. These healthy volunteers included medical personnel and patients with benign diseases, such as cholelithiasis. These patients underwent medical examinations, including endoscopy, and were found not to have any oesophageal disease or other cancerous disease. Tumour stages were assessed according to the Union of International Control of Cancer (UICC) classification (Sobin *et al*, 2009). The details of plasma samples were summarised in Supplementary Figure S1.

Peripheral blood (7 ml) was obtained from each patient at the time before the oesophagectomy or endoscopic resection and from the healthy volunteers. Blood was collected from patients and controls in sodium heparin tubes (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and immediately subjected to the three-spin protocol (1500 r.p.m. for 30 min, 3000 r.p.m. for 5 min, and 4500 r.p.m. for 5 min) to prevent contamination by cellular nucleic acids. The plasma samples were stored at  $-80^{\circ}\text{C}$  until further processing. The resected specimens were fixed in formalin and embedded in paraffin for pathological diagnosis. Macroscopic and microscopic classification of tumours was based on the UICC/TMN staging system (Sobin *et al*, 2009).

Table 1. ESCC patients characteristics and plasma miR-25 concentrations

Variables	Patients (n = 105)	Plasma miR-25 (mean, amol $\mu\text{l}^{-1}$ ) <sup>a</sup>	P-value <sup>a</sup>
<b>Age, years</b>			
< 65	49 (47%)	0.5801	0.1176
65 ≤	56 (53%)	0.4479	
<b>Sex</b>			
Male	86 (82%)	0.5327	0.1887
Female	19 (18%)	0.4050	
<b>Venous invasion<sup>b</sup></b>			
v0	67 (64%)	0.5669	0.1857
v1–2	30 (29%)	0.3926	
v3	8 (8%)	0.4688	
<b>Lymphatic invasion<sup>b</sup></b>			
ly0	60 (57%)	0.6409	<b>0.0110</b>
ly1–2	30 (29%)	0.3301	
ly3	15 (14%)	0.3439	
<b>T-stage<sup>b</sup></b>			
Tis	5 (5%)	1.1039	<b>0.0115</b>
T1	37 (35%)	0.6035	
T2	13 (12%)	0.5216	
T3	41 (39%)	0.4285	
T4	9 (9%)	0.1454	
<b>N-stage<sup>b</sup></b>			
N0	43 (41%)	0.4827	0.2215
N1	33 (31%)	0.6256	
N2	19 (18%)	0.3607	
N3	11 (10%)	0.5109	
<b>Stage<sup>b</sup></b>			
0	2 (1%)	0.9245	0.0698
I	28 (27%)	0.4965	
II	28 (27%)	0.7079	
III	37 (35%)	0.3790	
IV	11 (10%)	0.3904	

Abbreviations: ESCC = oesophageal squamous cell carcinoma; miR-25 = microRNA 25. P-value was considered significant at 0.05. NOTE: Significant values are in boldface type.  
<sup>a</sup>The Mann–Whitney U-test and Kruskal–Wallis H-test were performed to compare plasma miRNA concentrations.  
<sup>b</sup>TNM classification.

**RNA extraction.** Total RNA was extracted from 400  $\mu\text{l}$  of plasma using the mirVana PARIS Kit (Ambion, Austin, TX, USA) and finally eluted into 100  $\mu\text{l}$  of preheated (95 °C) Elution Solution according to the manufacturer's protocol. Using the formalin-fixed paraffin-embedded tissues, total RNA was extracted from four 15- $\mu\text{m}$ -thick slices of tissue (total 60  $\mu\text{m}$  in thickness) using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion) and then eluted into 60  $\mu\text{l}$  of Elution Solution according to the manufacturer's protocol.

**miRNA microarray analysis.** Microarray analyses of the plasma samples were performed using the 3D-Gene miRNA microarray platform (Toray, Kamakura, Japan) (Nagino *et al*, 2006; Giovannetti *et al*, 2012; Konishi *et al*, 2012). RNA extraction was performed according to the manufacturer's instructions described previously (Konishi *et al*, 2012). Briefly, the amount of total RNA

in plasma was too small, and so 2 of the 4  $\mu\text{l}$  of extracted total RNA from 300  $\mu\text{l}$  of plasma samples were used in the microarray experiments. The reason why the volume of 300  $\mu\text{l}$  plasma was used as the common denominator in each microarray analysis is that there was no definite internal control in plasma miRNA analyses as shown in our previous studies (Tsujiura *et al*, 2010; Komatsu *et al*, 2011; Morimura *et al*, 2011; Komatsu *et al*, 2012; Konishi *et al*, 2012; Hirajima *et al*, 2013; Kawaguchi *et al*, 2013). This RNA was labelled with Hy5 using the Label IT miRNA labeling kit (Takara Bio, Otsu, Japan) and hybridised at 32 °C for 16 h on the 3D-Gene chip. The 3D-Gene miRNA microarray (Human\_miRNA\_17v1.0.0, Toray Industries, Kamakura, Japan) can mount >1500 miRNAs based on the Human miRNA Version17 of MirBase (<http://microrna.sanger.ac.uk/>). Microarray was scanned, and the obtained images were numerated using the 3D-GeneH scanner 3000 (Toray Industries). The expression level of each miRNA was globally normalised using the background-subtracted signal intensity of the entire miRNAs in each microarray. The obtained microarray images were analysed using GenePix Pro TM (Molecular Device, Sunnyvale, CA, USA).

In these miRNA array-based analyses, two different approaches were performed. One approach was used to compare plasma miRNA levels derived from 300  $\mu\text{l}$  of the plasma sample between ESCC patients and healthy volunteers. Namely, each 100  $\mu\text{l}$  plasma sample from three ESCC patients was equally mixed, and totally 300  $\mu\text{l}$  of plasma sample was used as a sample of ESCC patients. On the other hand, each 60  $\mu\text{l}$  plasma sample from five healthy volunteers was equally mixed, and totally 300  $\mu\text{l}$  of plasma sample was used as a healthy volunteers' sample. The second approach was used to compare plasma miRNA levels between paired preoperative and postoperative ESCC patients. Each 100  $\mu\text{l}$  plasma sample from three preoperative or postoperative ESCC patients was equally mixed, respectively, and totally 300  $\mu\text{l}$  of a plasma sample was used as a sample of preoperative or postoperative ESCC patients.

**Protocol for quantification of miRNA by quantitative RT-PCR (qRT-PCR).** Test-scale analyses were performed using qRT-PCR in order to validate the utility of selected candidates from miRNA array-based approaches. The amounts of miRNAs in the plasma were quantified by qRT-PCR using human TaqMan MicroRNA Assay Kits (Applied Biosystems, Foster City, CA, USA). The reverse transcription reaction was carried out using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) in 15  $\mu\text{l}$  solution containing 5  $\mu\text{l}$  of RNA extract, 0.15  $\mu\text{l}$  of 100 mM dNTPs, 1  $\mu\text{l}$  of MultiScribe reverse transcriptase (50 U  $\mu\text{l}^{-1}$ ), 1.5  $\mu\text{l}$  of 10 × reverse transcription buffer, 0.19  $\mu\text{l}$  of RNase inhibitor (20 U  $\mu\text{l}^{-1}$ ), 1  $\mu\text{l}$  of gene-specific primer (has-miR-25, Assay ID: 000403; has-miR-16, Assay ID: 000391; has-miR-20b, Assay ID: 001014; has-miR-106a, Assay ID: 002169; has-miR-17, Assay ID: 002308; has-miR-20a, Assay ID: 000580; has-miR-15b, Assay ID: 000390; has-miR-19b, Assay ID: 000396; and RNU6B, Assay ID: 001093), and 4.16  $\mu\text{l}$  of nuclease-free water. For cDNA synthesis, the reaction mixtures were incubated at 16 °C for 30 min, at 42 °C for 30 min, and at 85 °C for 5 min and then held at 4 °C. Next, 1.33  $\mu\text{l}$  of cDNA solution was amplified using 10  $\mu\text{l}$  of TaqMan 2 × Universal PCR Master Mix with no AmpErase UNG reagent (Applied Biosystems), 1  $\mu\text{l}$  of gene-specific primer/probe, and 7.67  $\mu\text{l}$  of nuclease-free water in a final volume of 20  $\mu\text{l}$ . Quantitative PCR was run on a 7300 Real-time PCR system (Applied Biosystems), and the reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Cycle threshold (Ct) values were calculated using the SDS 1.4 software (Applied Biosystems).

Plasma miRNA levels were calculated on a standard curve constructed using synthetic miRNAs from the mirVana miRNA Reference Panel (Ambion). Standard reference miRNAs were

amplified by RT-PCR of a 10-fold serial dilution of the mirVana miRNA Reference Panel. The linearity of quantitative RT-PCR was confirmed between the logarithm of the amount of input miRNA and Ct values for a range of concentrations (1–0.0001 fmol) of each synthetic miRNA.

Although some investigators have determined plasma miRNAs levels by comparing with internal control miRNAs (Ng *et al*, 2009), it remains controversial as to which miRNAs are suitable as internal controls for plasma assays. Therefore we confirmed a linear correlation between the logarithm of the amount of input synthetic miRNA and the Ct value using real-time PCR, as well as the feasibility of extracting total RNA and amplifying specific miRNA in plasma samples. On the basis of these findings, we utilised the absolute concentration for measuring plasma miRNA in this study.

As the expression of miRNAs from tissue samples was normalised using the  $2^{-\Delta\Delta Ct}$  method relative to U6 small nuclear RNA (RNU6B),  $\Delta Ct$  was calculated by subtracting Ct values of RNU6B from those of the miRNAs of interest.  $\Delta\Delta Ct$  was then calculated by subtracting  $\Delta Ct$  of normal tissue from  $\Delta Ct$  of ESCC tissues. The change in gene expression was calculated using the equation  $2^{-\Delta\Delta Ct}$  (Livak and Schmittgen, 2001).

**ESCC cell lines and culture.** ESCC cell lines such as TE2, TE9, TE13, and KYSE70 and the fibroblast cell line WI-38 were purchased from RIKEN Cell Bank (Tsukuba, Japan). TE2, TE9, TE13, and KYSE70 cells were cultured in Roswell Park Memorial Institute-1640 medium (Sigma, St Louis, MO, USA). WI-38 cells were cultured in Dulbecco’s Minimum Essential Medium. All media were purchased from Sigma and supplemented with  $100\text{ ml}^{-1}$  fetal bovine serum (Trace Scientific, Melbourne, Victoria, Australia). All cell lines were cultured in  $50\text{ ml}^{-1}$  carbon dioxide at  $37^\circ\text{C}$  in a humidified chamber.

**Statistical analysis.** In miRNA array-based analyses, the signal intensity ratio and  $\log_2$  ratio of each plasma miRNA were calculated by the ratio of ESCC patients/healthy volunteers or pretreatment ESCC patients/postoperative ESCC patients. The Mann-Whitney test was used to compare differences in plasma miRNA levels between the cancer and healthy groups, and the Wilcoxon’s test was used to compare plasma levels between the paired samples before and 1 month after oesophagectomy or between the samples after oesophagectomy and at recurrences. A *P*-value of 0.05 was considered significant. The area under the receiver operating characteristic (ROC) curve (AUC) was used to assess the feasibility of using plasma miRNA levels as a diagnostic tool for detecting ESCC. The Youden index was used to determine the cutoff value for plasma miRNA levels (Akobeng, 2007).

**RESULTS**

**Study design to detect novel plasma miRNA biomarkers for ESCC.** This study was divided into several parts: (1) Selection of miRs that were among the top 15 upregulated miRNAs from two Toray 3D-Gene microRNA array-based approaches in comparisons of ESCC case and control plasmas and preoperative and post-ESCC plasmas, respectively. Of the top 15 miRNAs from each of these comparisons, there were a common set of eight miRNAs that were selected; (2) test-scale analyses using qRT-PCR in order to validate the utility of selected candidates from miRNA array-based approaches, comparing the plasma levels of the eight common miRNAs selected in 20 ESCC patients and 10 healthy controls; (3) evaluation of the correlation between each plasma miRNA level and peripheral blood cells in ESCC patients; (4) large-scale analysis of validation of plasma miR-25 levels by comparing 105 preoperative and 20 superficial ESCC patients who underwent curative oesophagectomy and endoscopic resection, respectively,

with 50 healthy volunteers; and (5) evaluation of whether plasma miR-25 levels reflect tumour dynamics in the treatment course of ESCC patients (Figure 1).

**Selection of plasma miRNA candidates through the integration of two comprehensive miRNA array-based approaches.** Novel miRNA candidates in the plasma for cancer detection and tumour monitoring were selected while using two miRNA array-based approaches to compare plasma miRNA levels between ESCC patients and healthy volunteers (Figure 2A) and between preoperative and postoperative ESCC patients (Figure 2B). Of the 1719 candidate miRNAs analysed, 219 plasma miRNAs were more upregulated in ESCC patients than in healthy volunteers with two-fold changes (Supplementary Table S1), whereas 156 plasma miRNAs were more upregulated in preoperative ESCC patients than in postoperative ESCC patients with two-fold changes (Supplementary Table S2). In order to detect more sensitive biomarkers, we focused on the top 15 miRs in this study (Figure 2). All data were summarised in Supplementary Tables S1 and S2. Of the 15 highly upregulated miRNAs from each group, eight common miRNAs (miR-15b, 16, 17, 25, 19b, 20a, 20b, and 106a) were selected. Concerning miR-451, which we previously reported in gastric cancer detection and monitoring (Konishi *et al*, 2012), the origin of high miR-451 level in plasma is currently uncertain in cancers, because miR-451 is known as a tumour-suppressive

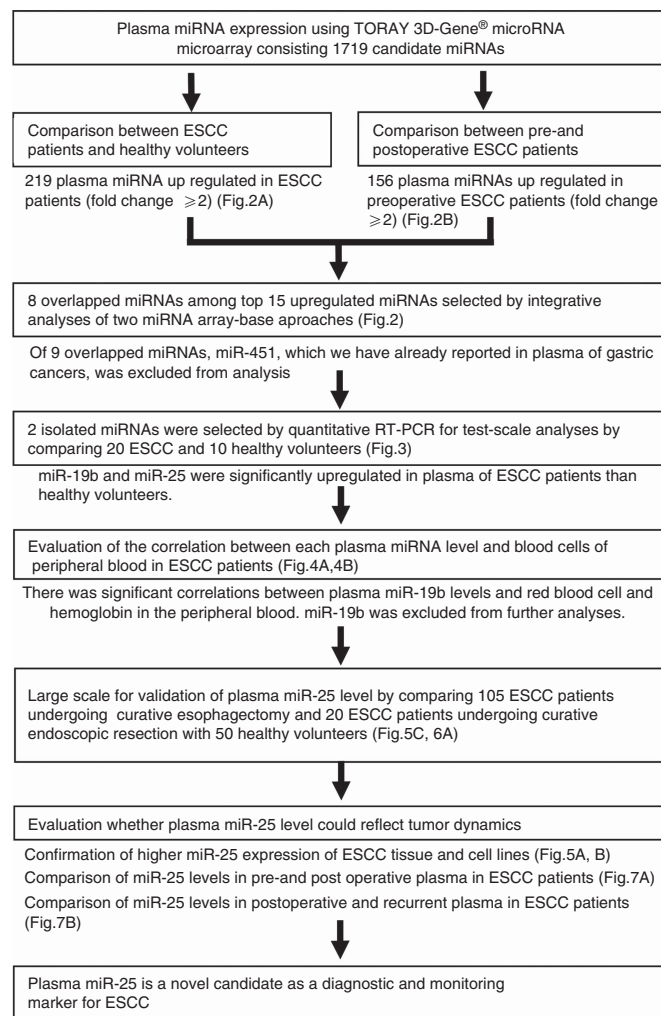


Figure 1. Study design to detect novel plasma miRNA biomarkers for ESCC.

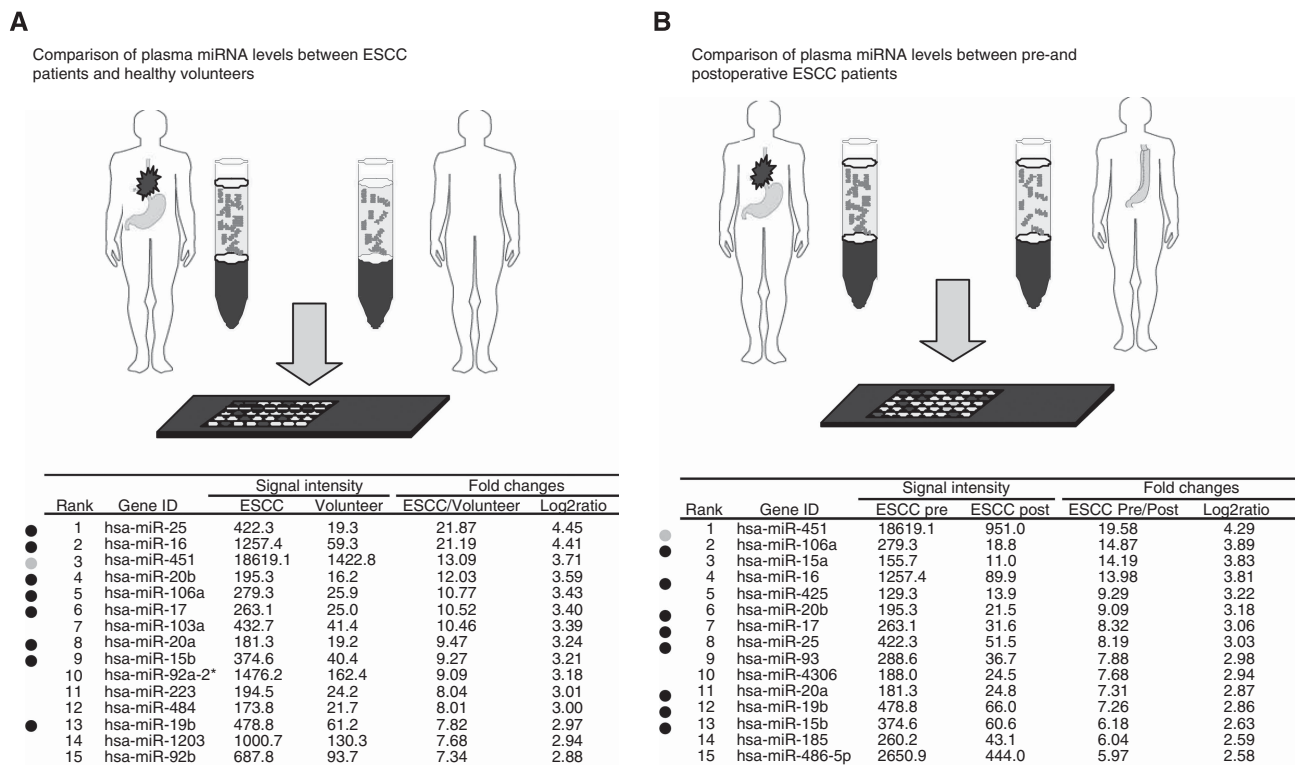


Figure 2. Selection of plasma miRNA candidates through the integration of two comprehensive miRNA array-based approaches. While using two miRNA array-based approaches to compare plasma miRNA levels between (A) ESCC patients and healthy volunteers and (B) between preoperative and postoperative ESCC patients, novel miRNA candidates for cancer detection and tumour monitoring were selected. Of the 15 highly upregulated miRNAs from each group, eight common miRNAs were selected.

microRNA and downexpressed in primary cancers. (Konishi *et al*, 2012). Therefore, in this study, we excluded miR-451 for further analyses, because plasma miR-451 needs considerable investigations and is not still suitable for biomarker in cancer detection and tumour monitoring.

**Test-scale analyses comparing plasma levels of eight common miRNAs in ESCC patients and healthy controls.** For test-scale analyses, we investigated the plasma levels of the eight miRNAs selected in 20 ESCC patients and 10 health volunteers by quantitative RT-PCR. miR-25 ( $P=0.0030$ ) and miR-19b ( $P=0.0020$ ) were validated to be more significantly upregulated in ESCC patients than in healthy volunteers (Figure 3). miR-106a ( $P=0.0172$ ) and miR-15b ( $P=0.0109$ ) were significantly lower in the plasma of ESCC patients than of healthy volunteers. These miRNAs were excluded from further analyses, because their expression patterns were inversely different from those reflected in the array-based results (Figure 2).

**Evaluation of the correlation between plasma miRNA levels and peripheral blood cells.** Recent reports demonstrated that some circulating miRNAs may be derived from peripheral blood cells (Pritchard *et al*, 2012; Cheng *et al*, 2013). We determined the correlation between the plasma levels of the miRNAs selected and peripheral blood cells in 20 ESCC patients. We found a significant correlation between the plasma miR-19b levels and concentrations of red blood cells and haemoglobin in the peripheral blood (Figure 4A). Considering the influence of secretion of miRNAs from blood cells or haemolysis on the results of clinical application, we excluded miR-19b from further analyses in this study. As no significant correlation was observed between plasma miR-25 levels and any type of blood cell in the peripheral blood of 20 ESCC patients, we did not eliminate this miRNA from further analyses (Figure 4B).

**Large-scale analysis of validation of plasma miR-25 levels by comparing ESCC patients with healthy volunteers.** Next, plasma miR-25 levels were examined on a large scale for our validation study using plasma samples of ESCC patients and healthy volunteers in qRT-PCR assays. The linearity of qRT-PCR was confirmed from concentrations of 1 fmol to 0.0001 fmol of each synthetic miRNA, such as miR-25 ( $R^2=0.9934$ ) between the logarithm of the amount of input miRNA and Ct values (Supplementary Figure S2). Plasma miR-25 was detectable in all samples from 105 consecutive ESCC patients and 50 healthy volunteers. The differential expression of plasma miR-25 in the ESCC patients was compared with that in normal healthy volunteers using a waterfall plot (Figure 5C). Plasma miR-25 levels were significantly higher in the ESCC patients than in the healthy volunteers ( $P<0.0001$ ; ESCC patients vs healthy volunteers (mean  $\pm$  s.d.):  $0.51 \pm 0.57$  vs  $0.19 \pm 0.23$  amol  $\mu\text{l}^{-1}$ ) (Figure 5C). No significant correlation was observed between the plasma miR-25 levels and any type of blood cell in the peripheral blood of the 105 ESCC patients (Supplementary Figure S3). Table 1 shows the association between the plasma miR-25 levels and clinicopathological factors in the 105 consecutive ESCC patients. A high plasma miR-25 level was shown to be significantly associated with the absence of lymphatic invasion ( $P=0.0110$ ) and early tumour stage in ESCC ( $P=0.0115$ ).

**Further study on clinical application of plasma miR-25 in superficial ESCC patients undergoing endoscopic resection.** To further evaluate the possibility of clinical application of plasma miR-25, plasma miR-25 levels in 20 consecutive superficial ESCC patients who underwent curative endoscopic resection (m2-sm1, R0) and 50 healthy volunteers were examined by real-time PCR (Figure 6A). The plasma miR-25 levels were significantly higher in the superficial ESCC patients than in the healthy volunteers (mean  $\pm$  s.d.:  $1.82 \pm 0.54$  vs  $0.19 \pm 0.23$ ,  $P<0.0001$ ). Figure 6B

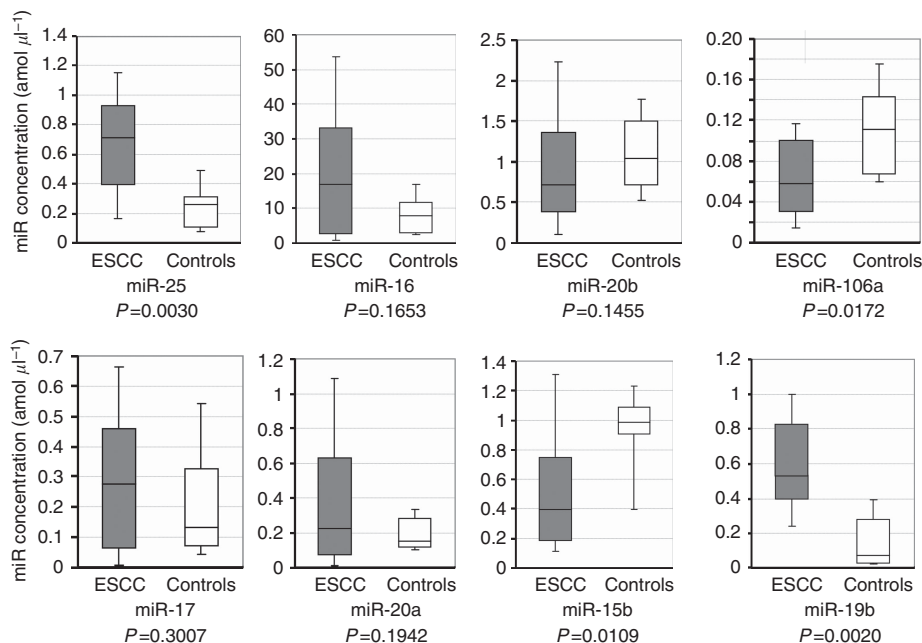


Figure 3. Test-scale analyses comparing plasma levels of eight common miRNAs in ESCC patients and healthy controls. For test-scale analyses, we investigated the plasma levels of eight selected miRNAs in 20 ESCC patients and 10 health volunteers by qRT-PCR. miR-25 ( $P=0.0030$ ) and miR-19b ( $P=0.0020$ ) were validated to be significantly upregulated in ESCC patients than in healthy volunteers.

shows that the value for the AUC for the plasma miR-25 analysis was 0.8555. To detect any cutoff points that could differentiate cancer patients from healthy volunteers, we utilised the AUC with the Youden index (Akobeng, 2007). An optimal cutoff point was indicated at  $0.32 \text{ amol } \mu\text{l}^{-1}$ , with a sensitivity of 85.0% and a specificity of 86.0%. Indeed, the sensitivity of plasma miR-25 for superficial ESCC detection was much higher than that of conventional tumour markers, including SCC, CEA, and CYFRA, at our institute (pT0-1; SCC, CEA, and CYFRA: 25.0, 21.1, and 12.5%, respectively) (details not shown). Our results provided evidence that plasma miR-25 levels can be used to distinguish early ESCC patients from healthy volunteers to a clinically satisfactory degree in comparison with conventional tumour markers.

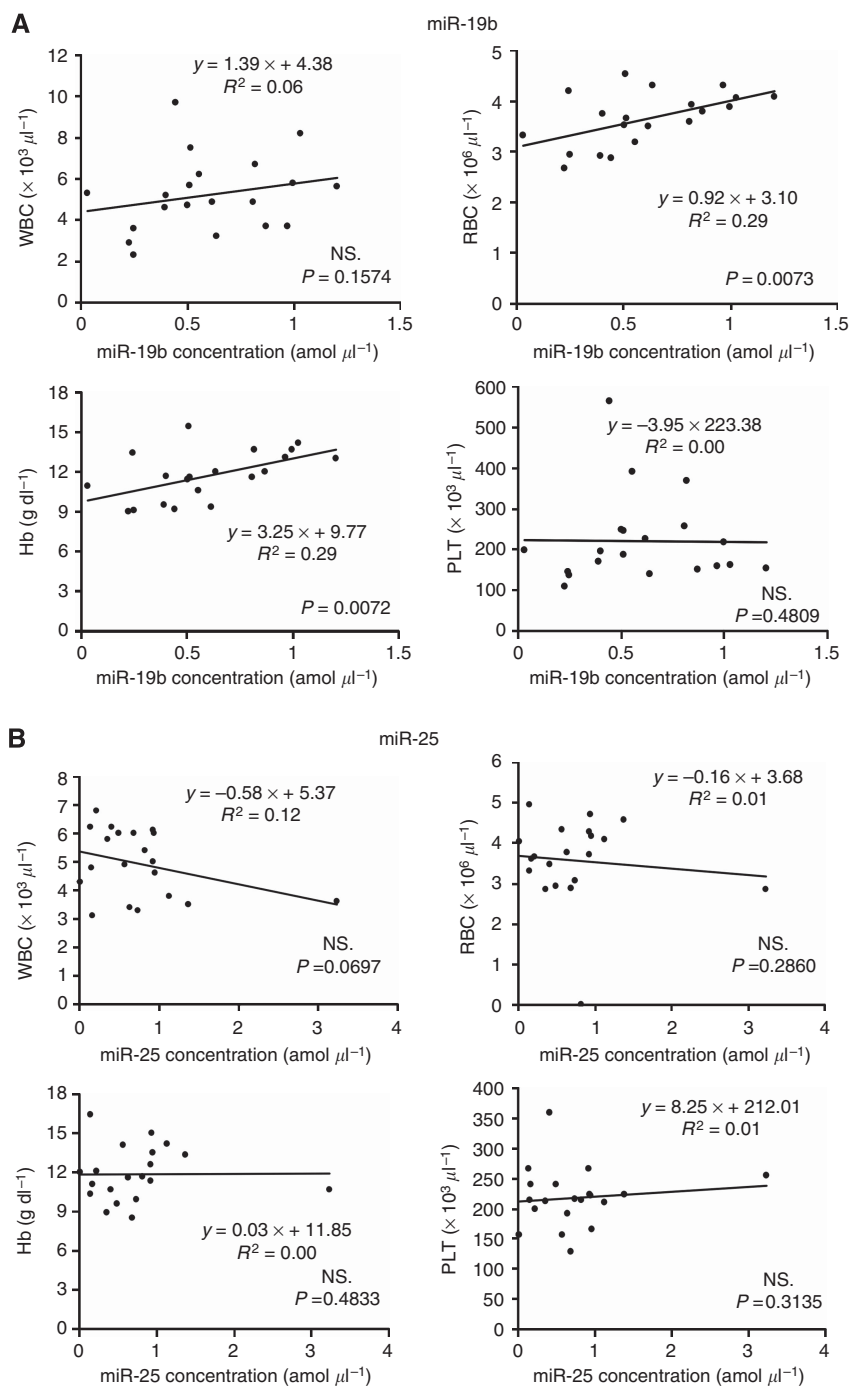
**Evaluation to determine whether plasma miR-25 levels reflect tumour dynamics in the treatment course of ESCC patients.** To validate whether plasma miR-25 levels reflect tumour dynamics, we first confirmed the higher miR-25 expression in primary ESCC tissues and ESCC cell lines. miR-25 expression in 10 ESCC tissues and 7 normal oesophageal tissues was determined by qRT-PCR. Moreover, the human ESCC cell lines TE-2, TE-9, TE-13, and KYSE70, and a human fibroblast cell line WI-38 were evaluated by qRT-PCR. The results are shown in Figure 5A and B after normalisation to control U6 expression. miR-25 levels were significantly higher in ESCC tissues than in normal ESCC tissues ( $P=0.0157$ ). Furthermore, the miR-25 levels were significantly higher in the ESCC cell lines than in the fibroblast cell line and the normal oesophageal tissues ( $P=0.0093$ ). Consequently, miR-18a expression was shown to be significantly increased in most ESCC tissues and cell lines but not in normal tissues and fibroblasts. Second, the miR-25 level was analysed in paired preoperative and postoperative plasma samples from ESCC patients who underwent curative oesophagectomy and endoscopic resection. As shown in the array-based approach (Figure 2B), plasma miR-25 levels were shown to be significantly reduced in samples after oesophagectomy ( $P=0.0001$ ) (Figure 7A). In some patients from whom paired samples were collected after oesophagectomy and after recurrences, a significant re-elevation in the plasma miR-25 levels was found at recurrence ( $P=0.0145$ ) (Figure 7B). These findings indicated that

the plasma miR-25 level might reflect tumour dynamics in the treatment course of ESCC patients.

## DISCUSSION

During the course of a programme of genome-wide miRNA profiling in the plasma of ESCC patients using high-resolution miRNA arrays, we identified a novel plasma miR-25. The plasma expression of this miRNA was significantly higher in preoperative ESCC patients and in pretreatment superficial ESCC patients undergoing endoscopic resection than in postoperative ESCC patients and healthy volunteers. Moreover, plasma miR-25 expression was independent of the blood cell status and reflects the tumour dynamics of preoperation and postoperation and recurrences. Our results provide evidence that plasma miR-25 levels contribute to cancer detection and can be used to monitor ESCC patients to a clinically satisfactory degree in comparison with conventional tumour markers.

During the past decade, non-coding RNAs, so-called miRNAs, have been demonstrated to regulate gene expression by targeting mRNAs for translational repression or cleavage. Moreover, miRNAs have emerged as integral components of the oncogenic and tumour-suppressor network, regulating almost all cellular processes altered during tumour development and may provide new therapeutic strategies such as biomarkers and therapeutic targets for cancers (He *et al*, 2005; Lu *et al*, 2005; Calin and Croce, 2006; He *et al*, 2007). Furthermore, miRNAs have been shown to be present in a remarkably stable form in plasma/serum, and the expression level of serum/plasma miRNAs is reproducible and consistent among individuals (Chen *et al*, 2008; Mitchell *et al*, 2008). In fact, a number of reports have suggested the presence of circulating miRNAs and their potential use as novel biomarkers for cancers, such as prostate cancer (Mitchell *et al*, 2008), leukaemia (Lawrie *et al*, 2008), oral cancer (Wong *et al*, 2008), pancreatic cancer (Wang *et al*, 2009; Morimura *et al*, 2011; Kawaguchi *et al*, 2013), colorectal cancer (Ng *et al*, 2009), lung cancer (Hu *et al*, 2010), breast cancer (Heneghan *et al*, 2010), and gastric cancer (Tsujiura *et al*, 2010; Konishi *et al*, 2012).



**Figure 4.** Evaluation of the correlation between plasma miRNA levels and peripheral blood cells. The correlation between selected plasma miRNA levels and peripheral blood cells in 20 ESCC patients was determined. **(A)** A significant correlation was observed between plasma miR-19b levels and concentrations of red blood cells and haemoglobin in the peripheral blood, **(B)** whereas no significant correlation was observed between plasma miR-25 levels and any type of blood cells in the peripheral blood.

In ESCC, there have been only a few reports, including three reports from our research group, on the role of circulating miRNAs in plasma/serum patients with ESCC (Zhang *et al*, 2010; Komatsu *et al*, 2011, 2012; Kurashige *et al*, 2012; Hirajima *et al*, 2013; Takeshita *et al*, 2013). However, these reports did not always indicate that these reported miRNAs were the only candidate biomarkers for ESCC. Therefore more sensitive and promising candidate miRNAs for screening cancer, monitoring the tumour status, and predicting prognosis need to be found in clinical settings. There have been only two specific reports about serum miRNA using the comprehensive array-based approach. Namely,

Zhang *et al* (2010) investigated the serum miRNA profiles in ESCC patients using Solexa sequencing. Among the 25 selected candidate miRNAs analysed, they identified 7 serum miRNAs (miR-10a, 22, 100, 148b, 223, 133a, and 127-3p) as ESCC biomarkers (Zhang *et al*, 2010). Moreover, Takeshita *et al* (2013) examined serum miRNA profiles using Agilent human miRNA microarrays to compare the serum miRNA levels in ESCC patients with the levels in healthy controls. Thus miR-1246 was the most markedly elevated miRNA in ESCC patients and was proved to be an independent poor prognostic factor (Takeshita *et al*, 2013). However, there have been no reported miRNAs in the plasma of

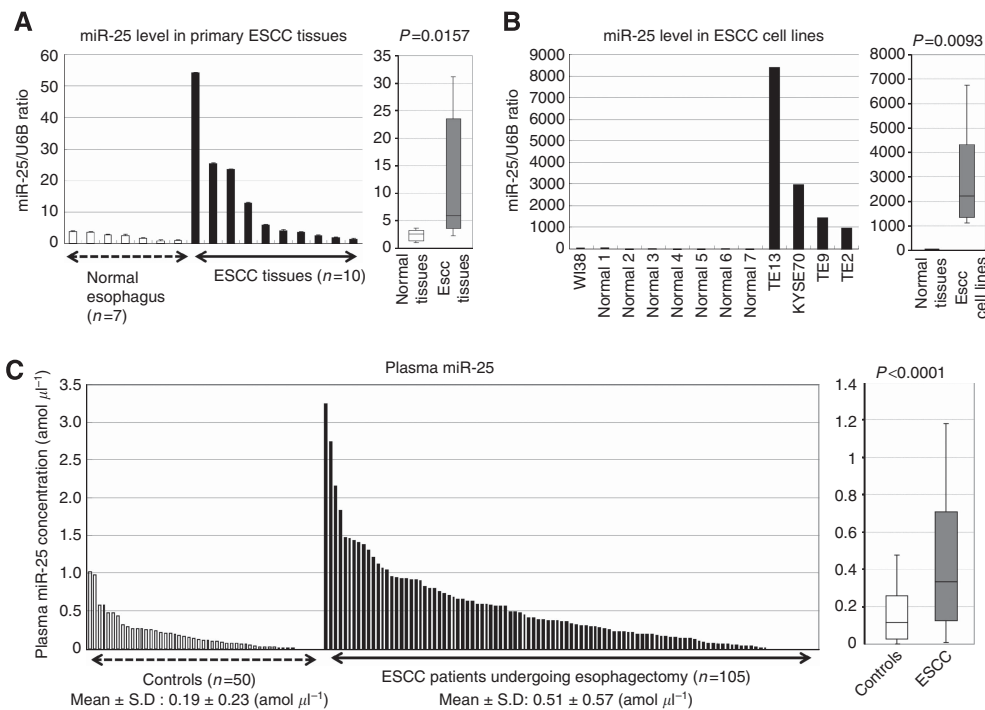


Figure 5. Evaluation of miR-25 levels in ESCC tissues, ESCC cell lines and plasma samples of patients with ESCC. (A) Difference in miR-25 expression between ESCC tissues and normal tissues determined by a waterfall plot. miR-25 expression levels were significantly higher in ESCC tissues than in normal oesophageal tissues ( $P=0.0157$ ). The upper and lower limits of the boxes and lines inside the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively. (B) Difference in miR-25 expression between ESCC cell lines, a fibroblast cell line WI-38, and normal tissues. miR-25 expression levels were significantly higher in ESCC cell lines than in both a fibroblast cell line and normal oesophageal tissues ( $P=0.0093$ ). (C) Plasma miR-25 levels in 105 consecutive ESCC patients and 50 healthy volunteers. Using a real-time RT-PCR assay, difference in plasma miR-25 expression between ESCC patients and normal healthy volunteers was determined by a waterfall plot. Plasma miR-25 levels were significantly higher in ESCC patients than in healthy volunteers ( $P<0.0001$ ).

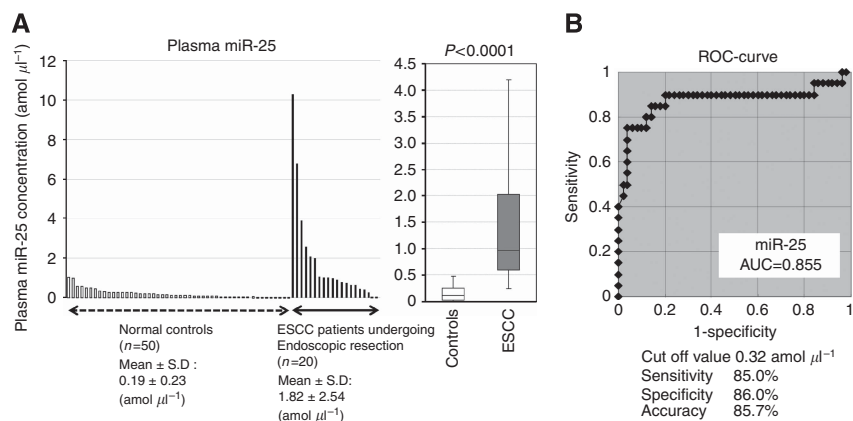


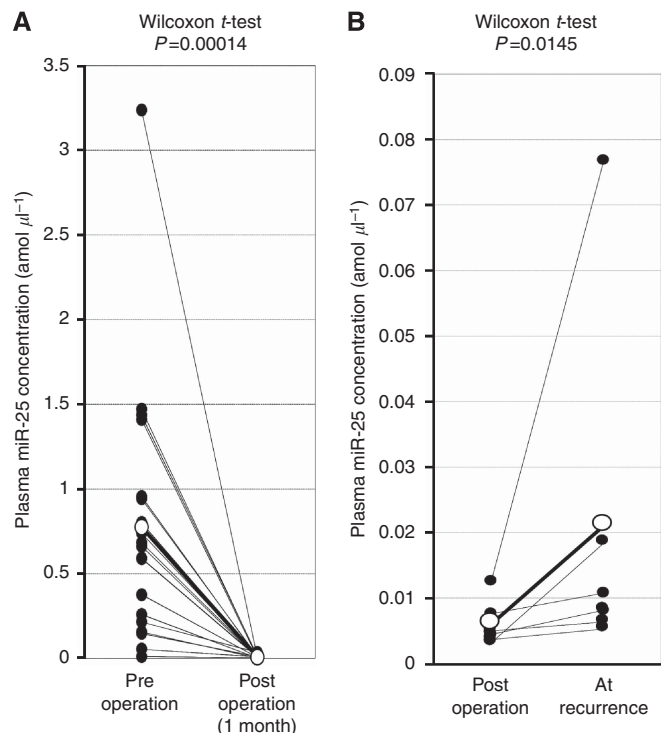
Figure 6. Plasma miR-25 levels in 20 consecutive superficial ESCC patients undergoing curative endoscopic resection and 50 healthy volunteers. (A) Plasma miR-25 levels were significantly higher in superficial ESCC patients than in healthy volunteers ( $P<0.0001$ ). (B) The area under the receiver operating characteristic curve was 0.8555.

ESCC patients that had been identified by the comprehensive array-based approach. Previous studies about miRNA profiles revealed that the majority of circulating miRNAs were co-fractionated with plasma protein complexes (Arroyo *et al*, 2011). Moreover, plasma might include more abundant protein levels, including coagulant-related proteins, than serum. Indeed, the detected candidate miRNAs were considerably different, because the profiles of cancer-associated miRNAs might be different between plasma and serum (Takeshita *et al*, 2013). These findings prompted us to further search for novel cancer-associated

miRNAs in plasma through a comprehensive miRNA microarray-based approach.

In this study, we finally identified a novel ESCC screening and monitoring plasma miRNA, miR-25, which is included in the miR-106b-25 cluster comprising miR-106b, miR-93, and miR-25 (Yu *et al*, 2006) and is located within the intron13 of MCM7 gene locus at 7q23.1. The region for these miRNAs was reported to be frequently overexpressed/amplified in ESCC tissue and cell lines (Shinomiya *et al*, 1999; Guo *et al*, 2008; Sakai *et al*, 2010; Zhu *et al*, 2011; Xu *et al*, 2012). This genomic aberration of the miR-106b-25





**Figure 7.** Evaluation of whether plasma miR-25 level could reflect tumor dynamics. **(A)** Comparison of plasma miR-25 concentrations between preoperative and postoperative samples from ESCC patients. Plasma miR-25 levels were significantly lower in postoperative samples than in preoperative samples ( $P=0.0001$ ). **(B)** Comparison of plasma miR-25 levels between postoperative and recurrence state samples from ESCC patients. In some patients from whom paired samples were collected after oesophagectomy and after recurrences, a significant re-elevation in plasma miR-25 levels was found at recurrence ( $P=0.0145$ ).

cluster was also accumulated in various other cancers, including gastric cancer (Petrocca *et al*, 2008), hepatocellular carcinoma (Li *et al*, 2009), prostate cancer (Poliseno *et al*, 2010), multiple myeloma (Pichiorri *et al*, 2008), ovarian cancer (Zhang *et al*, 2012), and breast cancer (Wu *et al*, 2012). Furthermore, several recent studies have demonstrated that the miR-106b-25 cluster and its individual miRNAs have pro-oncogenic functions, including mediating pro-proliferative and antiapoptotic phenotypes (Ren *et al*, 2006; Petrocca *et al*, 2008; Kan *et al*, 2009; Li *et al*, 2009; Poliseno *et al*, 2010; Martens-Uzunova *et al*, 2011). Some of the identified crucial functions of miR-106b-25 are the inhibition of tumour-suppressor genes such as *PTEN* (Poliseno *et al*, 2010), *p21/WAF1* and *Bim* (Petrocca *et al*, 2008; Kan *et al*, 2009; Li *et al*, 2009), *TP53* (Kumar *et al*, 2011), *CDH1* (Xu *et al*, 2012), and *DR4* (Razumilava *et al*, 2012) in several cancers and the activation of *E2F1*, which leads to impaired *TGF- $\beta$*  signalling and to proliferation, dysregulated cell cycling, and increased invasiveness in HNSCC (Hui *et al*, 2010). Furthermore, these oncogenic miRNAs in the miR-106b-25 cluster have been shown to be upregulated in cancer stromal tissues compared with normal stroma (Nishida *et al*, 2012). These findings strongly suggested that miR-25 may have a pivotal role in carcinogenesis and the development of tumours and that upregulation of plasma miR-25 might be associated with the molecular behaviours of ESCC. Indeed, a high plasma miR-25 level was proved to be significantly associated with early tumour stage in ESCC ( $P=0.0115$ ), and in superficial ESCC patients undergoing endoscopic resection, the value for the AUC, which was used to assess the feasibility of using plasma miRNA levels as a diagnostic tool for the early detection of ESCC, was great (0.8555). This suggests that miR-25 has an important role in the

early phase of cancer development. Thus plasma miR-25 levels may be a useful biomarker for the early detection of ESCC.

To further evaluate the clinical possibility of miR-25 for monitoring tumour dynamics, we investigated whether plasma miR-25 levels could reflect tumour dynamics in ESCC by three different analyses. The first analysis was the confirmation of comparative higher miR-25 expression in primary ESCC tissues and ESCC cell lines than in normal oesophageal tissues and fibroblast cells. The second analysis was to confirm that the miR-25 levels were significantly reduced after curative oesophagectomy and endoscopic resection in patients with high preoperative plasma miR-25 levels (Figure 7A). The third analysis was to determine that the significant re-elevation in the plasma miR-25 levels was found at recurrences after curative oesophagectomy or endoscopic resection (Figure 7B). These findings were similar to those of our previous reports (Tsujiura *et al*, 2010; Komatsu *et al*, 2011; Morimura *et al*, 2011; Konishi *et al*, 2012; Hirajima *et al*, 2013; Kawaguchi *et al*, 2013) and clearly demonstrated that plasma miR-25 levels reflected tumour dynamics and may be available as a new plasma biomarker for monitoring the tumour status of ESCC patients.

Recently, Pritchard and his colleagues reported a caution in a cancer biomarker study of circulating miRNAs because the circulating miRNAs might have been derived from peripheral blood cells (Pritchard *et al*, 2012; Cheng *et al*, 2013). The specific miRNAs associated with haemolysis and the secretion of miRNAs from blood cells might affect their plasma levels in clinical application. Therefore we evaluated the correlation between both miR-25 and miR-19 levels in plasma and peripheral blood cells in both 20 ESCC patients as a test study and in 105 consecutive ESCC patients as a validation cohort study. A significant association was observed between the plasma miR-19 levels and concentrations of red blood cells and haemoglobin in the peripheral blood (Figure 4A). However, as for miR-25, no association was found between them in both the 20 ESCC patients as a test study and the 105 consecutive ESCC patients as a validation cohort study (Figure 4B, Supplementary Figure S3). These results might exclude the possibility that the secretion of miRNAs from blood cells or haemolysis affected the miR-25 level in clinical settings. However, because secretion, kinetics, and metabolism in plasma miRNAs have not been clearly elucidated, this issue is currently under evaluation.

Taken together, through the integration of two comprehensive miRNA array-based approaches, we clearly demonstrated that plasma miR-25 levels may potentially be useful for screening cancer and monitoring tumour dynamics in ESCC patients. Nevertheless, many issues must be addressed before these findings can be translated into a clinically useful, non-invasive screening strategy for ESCC patients. Therefore we will prospectively confirm the usefulness of plasma miR-25 in a large number of patients. Furthermore, we believe that more sensitive plasma miRNA biomarkers could be identified by different methods such as a recent next-generation sequence or digital PCR-based approach and could translate circulating miRNAs into the clinical setting. These strategies are currently under evaluation, and we will report in the near future.

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