MicroRNA Profiles to Predict Postoperative Prognosis in Patients with Small Cell Carcinoma of the Esophagus

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Abstract. *Background/Aim: Small cell carcinoma of the esophagus (SCCE) is a rare but very aggressive disease with poor prognosis. The aim of the present study was to identify a molecular signature to predict postoperative outcomes in patients with SCCE. Materials and Methods: Expression of microRNA was detected in surgically-removed SCCE tumors using microarrays. A SCCE cell line (TYUC-1) was established to investigate the biological role of differentially expressed microRNAs. Results: Hierarchical clustering of microRNA expression revealed two discrete clusters that were identical to the cases with rapid tumor relapse (n=3; median survival, 5.1 months) and the cases with long-term survival (n=3; median observation, 144.7 months), respectively. Eight microRNAs (miR-4323, miR-625, miR-3619-3p, miR-4419b, miR-1249, miR-4648, miR-4664-3p and miR-1203) showed significant correlation with tumor relapse ($p<0.01$). Migration of TYUC-1 was significantly inhibited by down-regulation of miR-625. Conclusion: The expression profiles of microRNAs in tumors may represent a novel predictor for postoperative outcomes in patients with SCCE.*

Primary small cell carcinoma of the esophagus (SCCE) is a rare disease accounting for 0.5-2.8% of all esophageal malignancies (1). SCCE is generally recognized as a very aggressive disease with a poor therapeutic response, leading to poor prognosis (2). Because a standard treatment has not yet been established, SCCE patients have been treated with various combined therapies, including surgical resection, chemotherapy and radiation therapy. However, the significance of surgery in the multimodality treatment of SCCE patients remains controversial (3). A number of previous studies recommended chemotherapy or chemo-radiotherapy, but not surgery, because SCCE is a systemic disease with a high risk of distant metastasis (4). On the other hand, several studies reported a long-term survival of SCCE patients after surgery with or without adjuvant chemo/chemo-radiotherapy (3, 5).

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs that play a key role in various biological processes through the post-transcriptional regulation of gene expression by targeting mRNA (6). Many miRNAs are known to be involved in the initiation and progression of malignancies and may also be potential biomarkers for therapeutic effects and prognosis in cancer patients (7, 8). However, the role of miRNAs in SCCE has not yet been examined.

In the present study, we first summarized the clinical characteristics of 6 SCCE patients who underwent surgery in our hospital to assess the role of surgery in the treatment of SCCE patients. We then investigated the expression profiles of miRNAs in surgically-removed SCCE specimens to assess the use of a miRNA signature as a postoperative prognostic predictor in this disease. Finally, the potential significance of miRNAs, which were differentially expressed between relapse and non-relapse cases, was assessed using a human esophageal small cell carcinoma cell line, *in vitro*.

Materials and Methods

Patients and surgical specimens. Written informed consent for this study was obtained from patients prior to surgery with approval by the Institutional Review Board in the University of Toyama Hospital (No. 20-75). Six cases underwent esophageal surgery for SCCE between 1983 and 2009 in our hospital. The clinical records of these patients were reviewed and formalin-fixed paraffin-embedded (FFPE) samples from the tumors and their normal counterparts were obtained from each case. Tumor of freshly removed specimen was obtained

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from a case (the Case 6) to establish a small cell cancer cell line. The observation period was calculated from the date of surgery until the patient succumbed to the disease or the last follow-up contact. All tumors were histologically diagnosed as SCCE by the Department of Clinical Pathology in the Hospital based on a neuroendocrine-like architectural pattern with positive immunostaining for neuroendocrine markers, such as synaptophysin and chromogranin A.

All cases were staged according to the International Union against Cancer TNM Classification 7th edition (9).

RNA extraction from FFPE specimens. Sections (10-μm) were prepared from each FFPE specimen. Paraffin was removed by a xylene treatment and tissues were then washed with ethanol twice to remove xylene. Tissues were treated with proteinase K at 37˚C overnight. Following centrifugation, the supernatant was processed with a silica-based spin column (Toray Industries, Tokyo, Japan) in order to obtain purified total RNA. The degrees of RNA cross-linking and RNA degradation were analyzed by electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

miRNA assays. miRNA profiling was examined using a Toray 3D-Gene® miRNA oligo chip (Toray Industries) on which 885 genes were mounted. The detailed procedure for this experiment has been described previously (10). In the present study, the expression level of each miRNA was normalized using the median of the signal strength for the entire gene in each chip.

Establishment of human esophageal small cell carcinoma cell line and cell culture. A human esophageal small cell carcinoma cell line (TYUC-1) was established in our laboratory from a surgically-removed tumor specimen of Case 6 in Table I. The tissue was mechanically minced with sharp scissors and the cells were initially cultured in Dulbecco's modified Eagles'medium (DMEM) /Ham's F12 medium (Wako, Osaka, Japan) with 5% fetal calf serum (FCS) containing antibiotics (GIBICO, Grand Island, NY, USA) at 4˚C. Then the cell culture was maintained in humidified incubators at 37˚C in an atmosphere of 5% CO_2 and 95% air. A human small cell lung cancer cell line (Lu-134-A-H) was purchased from Japanese Collection of Research Bioresources Cell Bank (http://cellbank.nibio.go.jp/english/) and cultured in RPMI1640 medium with 10% FCS. Human esophageal squamous carcinoma cell lines (KYSE520 and 790) were established by Shimada and colleagues and cultured in Ham's F12/RPMI-1640 with 2% FCS according to a previously reported method (11).

Immunocytochemical staining. Cell pellet of TYUC-1 was fixed in 10% formalin, embedded in paraffin and processed by standard methods and 4-μm sections were cut. Immunostaining was performed with Envision Plus kits/horseradish peroxidase/3,3'-diaminobenzidine (Dako Japan Inc, Kyoto, Japan) according to the manufacturer's instructions. Primary antibodies were incubated at room temperature for 1 h or at 4˚C overnight. Slides were counterstained with Mayer's hematoxylin. The primary antibodies used in this study were as follows: mouse monoclonal anti-CD56, clone 1 B6 (Novocastra, Newcastle, UK) dilution 1:100, mouse monoclonal anti-CD117, clone YR145 (Dako Japan) dilution 1:100, rabbit polyclonal anti- ChromograninA (Dako Japan) dilution1:800, mouse monoclonal anti- NSE, clone BBS/NC/VI-H14 (Dako Japan) dilution1:200.

Quantitative real-time polymerase chain reaction (RT-PCR) analysis for miRNAs. Total RNA was extracted from cells using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to a standard protocol. cDNA was prepared from total RNA samples using the Taq Man microRNA reverse transcription kit on the ABI Prism 7000 real-time PCR system, according to the manufacturer's instructions (Applied Biosystems). Predesigned Taq Man microRNA assays for hsa-miR-625 (Assay ID 002432), hsa-miR-4323 (Assay ID 242550), hsa-miR-3619-3p (Assay ID 464743) and RNU6B (Assay ID 001093) were purchased from Applied Biosystems. qRT-PCR was performed using a Taq Man universal PCR master mix, according to the manufacturer's protocol (Applied Biosystems). miRNA quantities were analyzed in duplicate and normalized against U6B as an internal control. Results were expressed as relative gene expression using the ΔΔCt method.

Transfection with miR-625 inhibitor. TYUC-1 (200,000 cells per well) were plated onto 24-well culture dishes and transfected with mirVana miRNA inhibitor (miR-625-3p; Applied Biosystems) using the Lipofectamine RNAiMAX Transfection Reagent according to the manufacturer's instructions (Invitrogen). A nonspecific control miRNA (mirVana miRNA Inhibitor Negative Control; Applied Biosystems) was used as a negative control. The cells were harvested at 24 hours after transfection and processed for further analysis.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded into 96-well plates at 20,000 cells/well, allowed to grow overnight and then treated with various concentrations of cisplatin (CDDP; Wako, Osaka, Japan). Following 48 hours of treatment, 20 μl of 5 mg/ml MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 4 hours. The viability of the treated cells was calculated from the average OD 490 values compared with that of the untreated cells. Each treatment was carried out in triplicate.

Cell migration assay. Cell migration ability was examined using a 24-well transwell plate with 8 mm pore polycarbonate membrane inserts according to the manufacturer's protocol (Corning, Corning, NY, USA). Five thousands cells were seeded on the upper chamber with 200 μl of serum-free medium at 24 h after transfection with miRNA inhibitor or negative control. Medium with 20% serum was added to the lower chamber as a chemoattractant. After 48 h incubation, the membranes were fixed and stained with Difquick according to the manufacturer's protocol (Sysmex, Kobe, Japan). Five visual fields (×200) were randomly selected from each membrane and the cell numbers were counted under a light microscope.

Statistical analysis. All analyses were carried-out with the JMP 9.0 software (SAS Institute Inc., Cary, NC, USA). The tumor-to-normal ratio (T/N ratio) was calculated based on the miRNA expression levels in the each tumor and the corresponding normal counterpart. Differences in miRNA expression levels between two variables were analyzed by the Student's *t*-test. Hierarchical clustering analysis was performed with the Ward's method. $p<0.05$ was used for significance.

Results

Patients' characteristics. Six cases (Case 1 - Case 6 in Table I) underwent esophageal surgery for SCCE between 1983 and 2009 in the University of Toyama Hospital. The clinical

Table I. *Patients' information and characteristics of primary small cell carcinoma of the esophagus.*

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Year of Surgery	1983	1985	1990	1993	2008	2009
Age (years)	80	58	63	56	73	54
Gender	M	M	M	M	M	F
Tumor Location	Lower	Middle	Middle	Middle	Middle	Middle
Tumor size (cm)	3.0	6.0	5.0	2.8	1.5	5.5
Tumor type	Ulcerative	Ulcerative	Ulcerative	Ulcerative	Ulcerative	Medullary
sT stage	1	4	1	3	3	4
N stage	0	3	4	0	2	1
M stage	0	0	0	0	0	0
TNM stage	1	4	4	2	3	3
Surgery	Radical	Radical	Radical	Radical	Radical	Radical
Postoperative chemotherapy	PEP	CDDP	CDDP 5FU	CDDP 5FU VP16	CDDP VP16	VP16 CPT-11
Observation period (months)	56.1	1.7	144.7	173.0	5.1	8.5
Outcome	Dead	Dead	Alive	Alive	Dead	Dead
Relapse	Free (Heart Disease)	Local recurrence	Free	Free	Liver Mediastinal Lymph node	Mediastinal/ Abdominal Lymph node
miRNA analysis	Yes	Yes	No	Yes	Yes	Yes

information and tumor characteristics of these patients are shown in Table I. The median age of the patients was 60.5 years (range=54-80 years). The TNM classification was stage 1-2 in Cases 1 and 4, and stage 3-4 in Cases 2, 4, 5 and 6. None of the patients received preoperative adjuvant therapy. All patients underwent radical surgery with no residual tumors (R0). All patients received postoperative chemotherapy with regimens including cisplatin (CDDP), peplomycin (PEP), 5-fluorouracil (5FU), etoposide (VP16) and irinotecan (CPT-11) according to the standard therapy for small cell lung cancer at that time. Two patients (Cases 3 and 4) received postoperative radiotherapy that targeted the mediastinal and cervical areas. Two patients (Cases 3 and 4) achieved long-term disease-free survival with an observation period of 144.7 and 173.6 months, respectively. Case 1 died of heart disease without tumor relapse at 56.1 months after surgery. Three patients (Cases 2, 5 and 6) died of tumor relapse at 1.7, 5.1 and 8.5 months after surgery. When these six cases were divided into a relapse group (cases 1, 3 and 4) and non-relapse group (cases 2, 5 and 6), the median observation period was 144.7 months (range=56.1-173.0) and 5.1 months (range=1.7-8.5), respectively. No correlation was observed between postoperative tumor relapse and clinicopathological characteristics.

The expression of miRNAs in primary small cell carcinoma of the esophagus. We extracted total RNA from tumors and corresponding normal esophageal epithelia using the archival FFPE samples of all six patients; high-quality RNA was obtained from five patients. In the 885 miRNAs assessed using the miRNA oligo chip, the signals of 410 miRNAs were detected in each examined sample. Comparisons between the average expression levels of miRNAs in the five SCCE tumors and those in the five corresponding normal tissues revealed that the expression of 22 miRNAs, including miR-17, miR-21 and miR-25, was more than 2-fold higher, while that of the 3 miRNAs miR-145, miR-4328 and miR-203, was more than 2-fold lower in the tumors than in their normal counterparts (Figure 1A). Hierarchical clustering based on all 410 detected miRNAs showed two discrete clusters: Cluster 1 represented patients with long-term postoperative survival (Cases 1 and 4), whereas cluster 2 represented patients with rapid postoperative tumor relapse (Cases 2, 5 and 6) indicating the predictive significance of miRNA expression profiles in patients with SCCE (Figure 1B).

Differentially expressed miRNAs between patients with and without postoperative tumor relapse. When the expression (T/N ratio) of miRNAs was compared between relapse and non-relapse cases, the higher expression of 8 miRNAs (miR-4323, miR-625, miR-3619-3p, miR-4419b, miR-1249, miR-4648, miR-4664-3p and miR-1203) correlated with postoperative tumor relapse ($p<0.01$). The up-regulation of the other 29 miRNAs and down-regulation of 2 miRNAs (miR-1260b and miR-4474-3p) correlated with tumor relapse ($p<0.05$, Table II). Hierarchical clustering using the 39 miRNAs listed in Table II more clearly differentiated between the relapse and non-relapse groups than that using all 410 detected miRNAs (Figure 1B, Figure 2).

Table II. *Expression of miRNAs associated with postoperative tumor relapse.*

	Non-relapse		Relapse		*t*-test
	Average	SD	Average	SD	*p*-Value
hsa-miR-4323	0.747	0.019	1.326	0.045	0.0005
hsa-miR-625	0.763	0.055	1.420	0.049	0.0008
hsa-miR-3619-3p	0.769	0.192	2.153	0.051	0.0010
hsa-miR-4419b	0.879	0.114	1.554	0.060	0.0029
hsa-miR-1249	0.798	0.115	1.404	0.067	0.0045
hsa-miR-4648	1.191	0.096	3.467	0.397	0.0048
hsa-miR-4664-3p	0.778	0.022	1.142	0.063	0.0049
hsa-miR-1203	0.777	0.041	1.854	0.237	0.0090
hsa-miR-574-3p	0.743	0.152	1.245	0.068	0.0130
hsa-miR-1260b	1.142	0.061	0.442	0.174	0.0135
hsa-miR-4440	0.865	0.068	1.184	0.075	0.0170
hsa-miR-1307	0.811	0.147	1.224	0.060	0.0192
hsa-miR-486-3p	0.859	0.201	1.579	0.158	0.0200
hsa-miR-16	0.719	0.270	5.949	1.635	0.0237
hsa-miR-371-5p	0.773	0.111	1.335	0.163	0.0251
hsa-miR-2392	0.752	0.029	1.209	0.146	0.0253
hsa-miR-4433	0.853	0.076	1.339	0.149	0.0259
hsa-miR-3131	0.719	0.162	1.845	0.353	0.0268
hsa-miR-92a	1.099	0.411	3.448	0.768	0.0312
hsa-miR-296-5p	0.694	0.072	1.765	0.374	0.0319
hsa-miR-4726-5p	0.748	0.014	1.915	0.417	0.0330
hsa-miR-4728-3p	0.841	0.158	1.280	0.112	0.0338
hsa-miR-4723-5p	0.782	0.348	2.176	0.448	0.0352
hsa-miR-3656	0.860	0.037	1.077	0.076	0.0362
hsa-miR-557	0.683	0.171	1.485	0.278	0.0382
hsa-miR-4474-3p	0.871	0.114	0.592	0.068	0.0384
hsa-miR-4640-5p	0.892	0.002	1.334	0.169	0.0393
hsa-miR-4758-5p	0.849	0.126	1.173	0.086	0.0394
hsa-miR-4689	0.764	0.157	2.023	0.472	0.0401
hsa-miR-4673	0.616	0.126	0.860	0.032	0.0408
hsa-miR-4665-3p	0.871	0.144	1.315	0.144	0.0432
hsa-miR-940	0.814	0.076	1.710	0.356	0.0446
hsa-miR-1290	0.935	0.364	7.531	2.654	0.0451
hsa-miR-4706	0.802	0.211	1.435	0.211	0.0463
hsa-miR-4776-5p	0.773	0.147	1.176	0.129	0.0467
hsa-miR-3151	0.832	0.166	1.873	0.412	0.0470
hsa-miR-3187-5p	0.702	0.035	1.256	0.229	0.0481
hsa-miR-4274	0.757	0.068	1.747	0.409	0.0484
hsa-miR-1231	0.804	0.176	1.306	0.171	0.0499

Biological significance of the miRNAs that were differentially expressed between relapse and non-relapse cases. We established a human esophageal small cell carcinoma cell line (TYUC-1) from surgically-removed tumor specimen of Case 6. TYUC-1 represented semi-attached round cells and actively grew with sphere formation. We also comfirmed the expression of neuroendocrine markers, such as CD56, CD117, Chromogranin A and NSE by immunochemistry (Figure 3A). Among miR-625, miR-3619-3p and miR-4323, which were the three most significantly correlated molecules with postoperative tumor relapse (Table II), miR-625 was significantly up-regulated both in TYUC-1 and a human small cell lung cancer cell line (Lu-134-A-H), compared to human esophageal squamous carcinoma cell lines (KYSE520 and 790), while the expression of miR-3619-3p and miR-4323 were unditectable in all 4 examined cell lines (Figure 3B). To investigate the role of miR-625 in malignant potential of SCCE, the expression of miR-625 was found down-regulated in TYUC-1 to assess its effect on cell proliferation, chemosensitivity and cell migration. The expression of miR-625 was significantly reduced in the cells transiently transfected with miR-625 inhibitor compared to the cells transfected with negative control at 24 h and 72 h after transfection (Figure 4A). Cell proliferation was not affected by the transfection of miR-625 inhibitor (Figure 4B). MTT assay under incubation of TYUC-1 with various concentration of CDDP showed no significant effect of miR-625 in chemosensitivity (Figure 4C). On the other hand, the migration of TYUC-1, which was assessed by a transwell assay, significantly decreased after transfection with the miR-625 inhibitor (Figure 4D).

Discussion

SCCE is known to be a very aggressive malignancy with poor prognosis (2). A standard therapy has not yet been established and the role of surgery in its multimodality treatment remains controversial (1-5). In the six patients treated with R0 surgery in combination with chemotherapy in our Hospital, two patients with limited disease and a patient with extensive disease achieved long-term disease-free survival, while three patients with limited disease died of tumor relapse very rapidly after surgery. These results suggested that combination therapy, including surgery, improved the survival rates of a distinct sub-population of the SCCE patients. Furthermore, the poor outcome observed in the counter population was not associated with small cell histology itself or disease stage, which indicated the need for novel biomarkers to predict postoperative outcomes. Previous studies have demonstrated the significance of biomarkers, such as serum levels of neuron-specific enolase (12) and miR-92a (13), for small cell lung cancer; however, predictive biomarkers have not yet been identified for SCCE.

Our microarray analysis identified 25 miRNAs that were differentially expressed between paired SCCE tumors and their corresponding normal samples (22 miRNAs were up-regulated and 3 miRNAs were down-regulated in the tumors). In the 22 miRNAs that were up-regulated in tumors, 16 miRNAs were associated with the progression and therapeutic resistance of various types of tumors. Ten molecules, including miR-17 (14), miR-21 (15), miR-25 (16), miR-93 (17), miR-1246 (18, 19), miR-106b (16), miR-103a (8), miR-107 (8) and miR-223 (20), have been associated with the malignant potential of esophageal cancer.

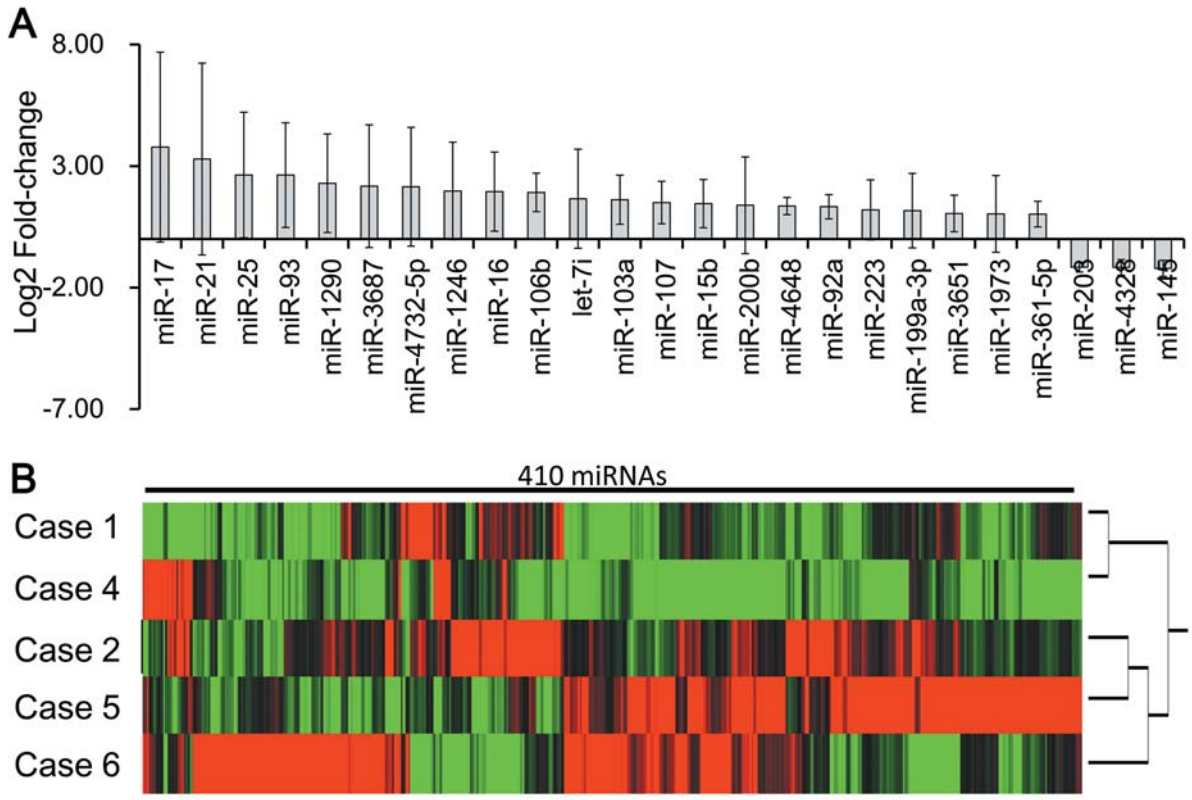

Figure 1. *Expression of miRNAs in primary small cell carcinoma of the esophagus. A. The average expression level (tumor-to-normal (T/N) ratio) of each miRNA in 5 SCCE cases was calculated and log2-transformed. Twenty-five miRNAs showed more than a 1 log2-fold change. B. Cluster analysis. The diagram shows the results of the hierarchical clustering of miRNAs and samples. Columns: 410 miRNAs that were detected in all 5 cases. Rows: samples. Red color represents a higher expression level; Green color represents a lower expression level.*

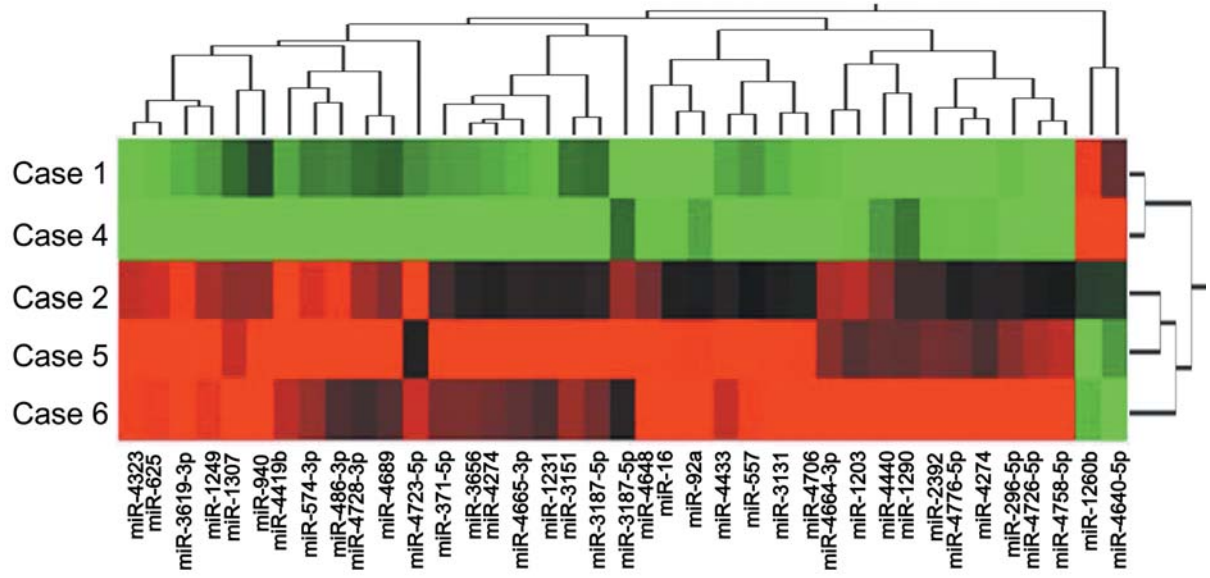

Figure 2. *Cluster analysis based on the 39 differentially expressed miRNAs. The diagram shows the results of the hierarchical clustering of miRNAs and samples. Columns: 39 miRNAs that were differentially expressed between patients with and without postoperative tumor relapse. Rows: samples. Red color represents a higher expression level; Green color represents a lower expression level.*

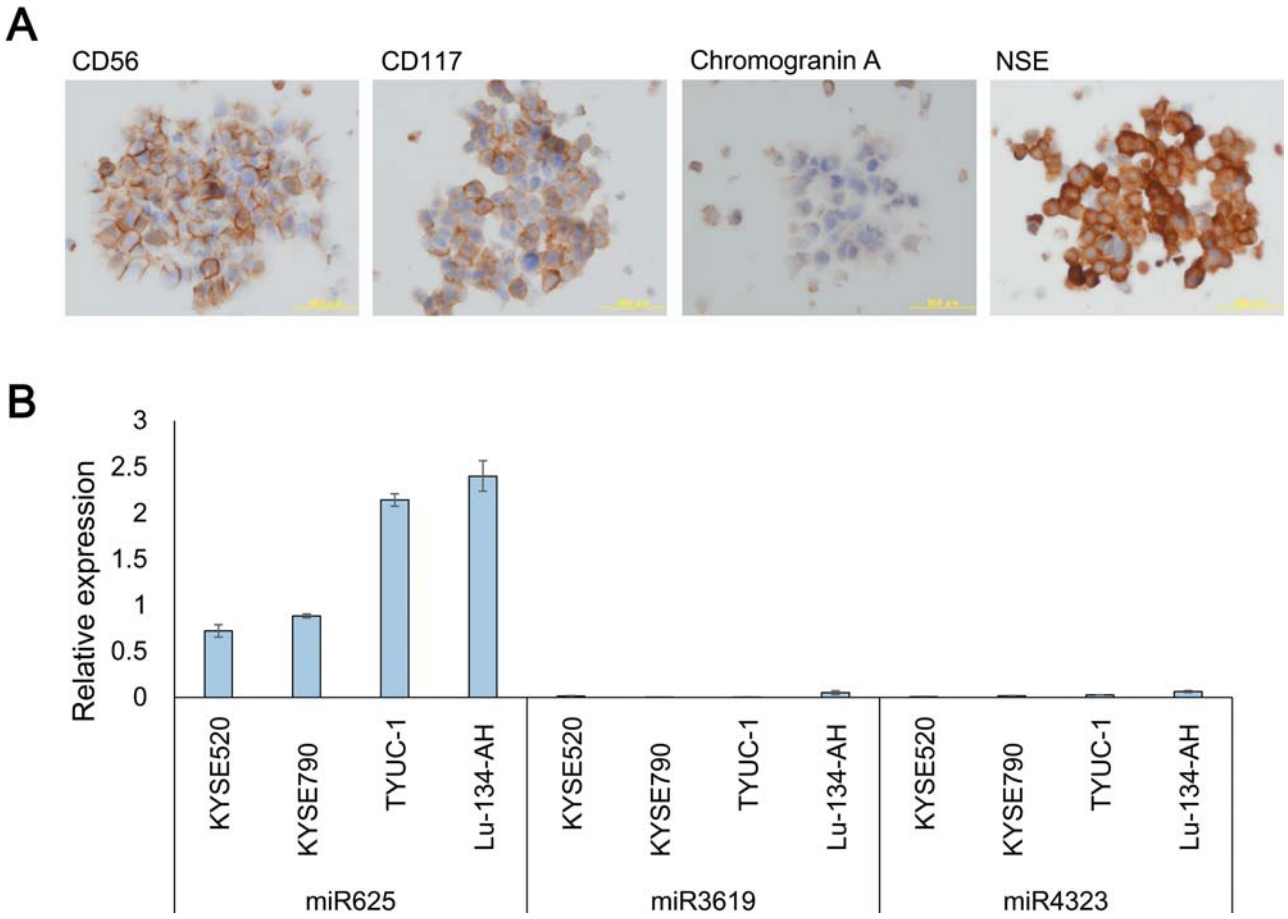

Figure 3. *Expression of the differentially expressed miRNAs in human esophageal small cell carcinoma cell line (TYUC1). A. A human esophageal small cell carcinoma cell line (TYUC1) was established from surgically removed tumor specimen of Case 6 and the expression of neuroendocrine markers, such as CD56, CD117, Chromogranin A and NSE, were detected by immunocytochemistry. Original magnification, ×200. B. Relative expression of the most up-regulated miRNAs in the cases with postoperative tumor relapse were detected in TYUC1, a human small cell lung cancer cell line (Lu-134-A-H)- and human esophageal squamous carcinoma cell lines (KYSE520 and 790) by quantitative RT-PCR (mean±SD, *p<0.05, **p<0.01).*

In the 3 miRNAs that were down-regulated in SCCE tumors, miR-203 (21) and miR-145 (22) were shown to have a tumor suppressor effect in esophageal cancer. These results indicate that the archival FFPE samples were successfully used to identify differentially expressed miRNAs in this study and suggested the crucial roles of these miRNAs in the development and progression of SCCE. Hierarchical clustering of miRNA expression levels revealed two distinct sub-groups that were identical to the patients with long-term postoperative survival and to those with rapid tumor relapse, respectively. These results suggested the existence of a specific miRNA signature that could be used to predict postoperative outcomes. In the 39 miRNAs that were significantly expressed differentially between the two groups ($p<0.05$), 15 miRNAs have been associated with the malignant features, such as metastasis, chemo-resistance and/or radio-resistance, of various tumors (Table III) (23-27), and thus shown to be prognostic predictors (28-32).

Our investigation, using TYUC-1, revealed that down-regulation of miR-625, which is one of the most up-regulated miRNAs in cases with postoperative tumor relapse, resulted in inhibition of cell migration *in vitro*. These results further suggested that the altered expression of the miRNAs identified in this study plays key roles in the widespread metastasis and/or therapeutic resistance associated with SCCE, thus, highlight the relevance of miRNA signatures to predict clinical outcomes following curative surgery in SCCE patients.

In summary, the clinicopathological characteristics of six SCCE patients who underwent curative surgery in our hospital showed that multimodality treatment including

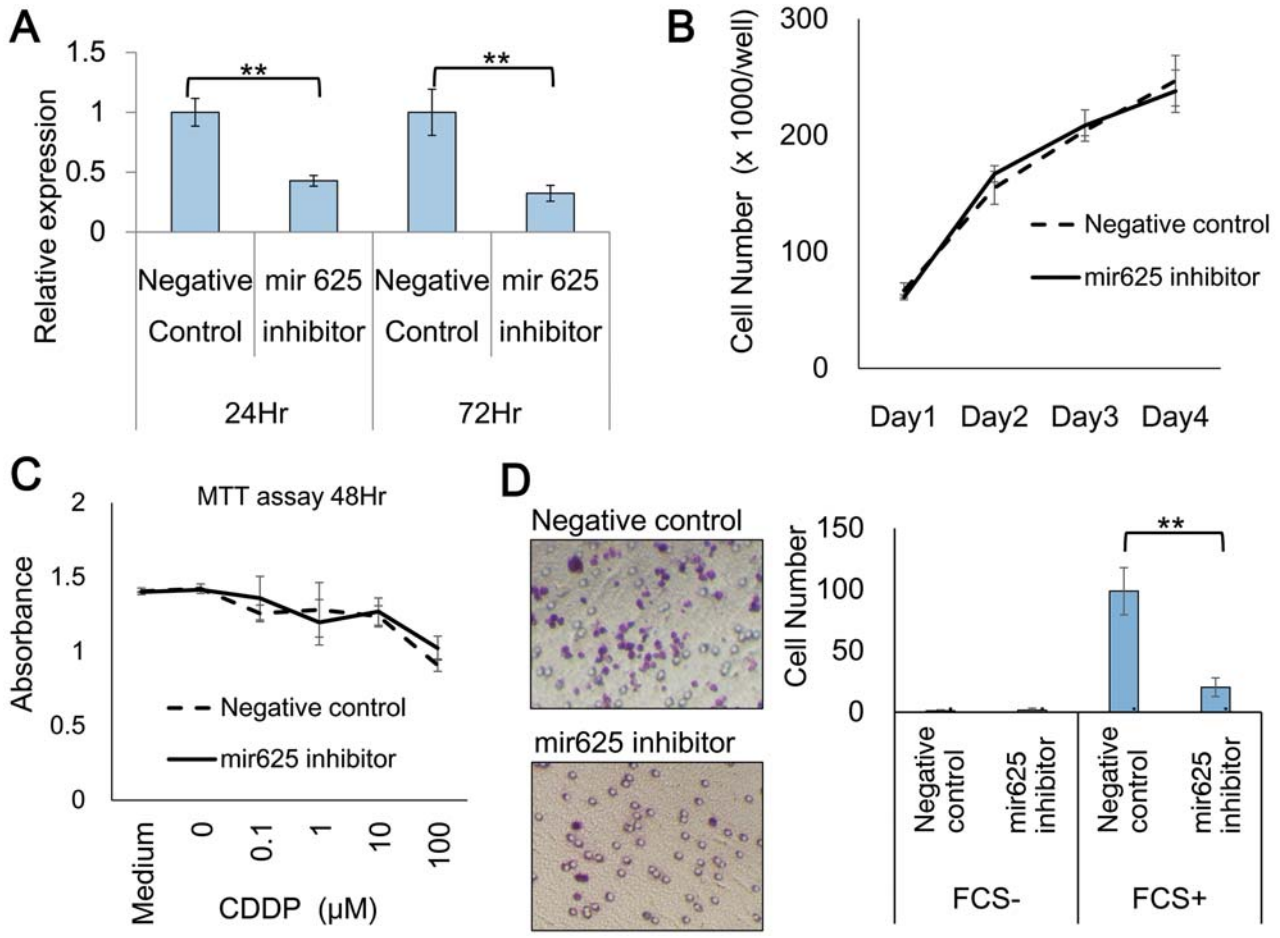

Figure 4. *The role of miR-625 in malignant potential of TYUC1. A. Relative expression of miR-625 in TYUC-1 at 24 and 72 h after transfection with miR-625 inhibitor or negative control detected by quantitative RT-PCR (mean±SD, **p<0.01). B. Growth curves of TYUC-1 after transfection with miR-625 inhibitor or negative control (n=3, mean±SD). C. The effect of miR-625 on chemo-sensitivity was assessed by MTT assay under 48 h incubation of the cells with various concentration of cisplatin (CDDP) (n=3, mean±SD). D. The effect of miR-625 on cell migration was assessed by a transwell assay in which the number of the cells under the membrane were stained and counted (n=3, mean±SD, *p<0.05, **p<0.01).*

surgery improved the prognosis of a distinct sub-population of SCCE patients. Hierarchical clustering of miRNA expression in surgically-removed SCCE tumors revealed two discrete clusters that were identical to the cases with long-term postoperative disease-free survival and to the cases with rapid tumor relapse, respectively. In the 39 miRNAs that were significantly differentially expressed between the two groups, 15 miRNAs have been associated with the malignant features. Down-regulation of miR-625, which is one of the most significantly up-regulated miRNAs in cases with postoperative tumor relapse, resulted in inhibition of TYUC-1 cell migration. These results suggested the use of miRNA expression profiles as a predictor for postoperative outcomes in patients with SCCE.

Although we only examined a small number of the cases, to the best of our knowledge, this is the first study to examine miRNA expression profiles in SCCE patients. Further investigations based on the large-scale collection of samples may confine the most useful gene set and then assess its clinical use.

Disclosure Statement

The Authors declare that they have no competing interest related to their research.

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Table III. *Reported oncological function of miRNAs that were differentially expressed between patients with and without postoperative tumor relapse.*

miRNA	Functions	Type of tumor	Literature
miR-625	Chemo-resistance and metastasis	Colorectal/gastric cancer	(23, 24)
miR-1249	Tumor pathogenesis	Hepatocellular carcinoma (HCC)	(33)
miR-574-3p	Tumor suppressor	Head and neck/esophageal cancer	(34)
miR-1260b	Oncogene	Prostate cancer	(35)
miR-486-3p	Postoperative prognostic predictor	HCC	(28)
miR-16	Prognostic marker	Esophageal/lung cancer	(29, 30)
miR-371-5p	Tumor growth promotion	HCC	(36)
miR-2392	Predictor of lymph node metastasis	Cervical cancer	(19)
miR-92a	Promotes lymph node metastasis	Esophageal cancer	(25)
	Predictor of chemoresistance	Small cell lung cancer	(13)
miR-296-5p	Promote tumor growth	Gastric cancer	(37)
miR-557	Tumor pathogenesis	HCC	(33)
miR-4758	Associated with tumor relapse	Gastric cancer	(31)
miR-1290	Chemo-resistance	Bladder cancer	(26)
	Radio-resistance	Cervical cancer	(27)
miR-3151	Associated with shorter survival	Myeloidleukemia	(32)
miR-1231	Susceptibility of the tumor	HCC	(38)

References

1 Kukar M, Groman A, Malhotra U, Warren GW, Bogner P, Nwogu CE, Demmy TL and Yendamuri S: Small cell carcinoma of the esophagus: a SEER database analysis. Ann Surg Oncol *20*: 4239-4244, 2013.

2 Al Mansoor S, Ziske C and Schmidt-Wolf IG: Primary small cell carcinoma of the esophagus: patient data meta analysis and review of the literature. Ger Med Sci 11, 2013.

3 Chen SB, Yang JS, Yang WP, Weng HR, Li H, Liu DT and Chen YP: Treatment and prognosis of limited disease primary small cell carcinoma of esophagus. Dis Esophagus *24*: 114-119, 2011.

4 Gao R, Zhang Y, Wen XP, Fu J and Zhang GJ: Chemotherapy with cisplatin or carboplatin in combination with etoposide for small-cell esophageal cancer: a systemic analysis of case series. Dis Esophagus 2013. doi: 10.1111/dote.12149. Epub ahead of print

5 Zhu Y, Qiu B, Liu H, Li Q, Xiao W, Hu Y and Liu M: Primary small cell carcinoma of the esophagus: review of 64 cases from a single institution. Dis Esophagus *27*: 152-158, 2014.

6 Baek D, Villén J, Shin C, Camargo FD, Gygi SP and Bartel DP: The impact of microRNAs on protein output. Nature *455*: 64-71, 2008.

7 Lin RJ, Xiao DW, Liao LD, Chen T, Xie ZF, Huang WZ, Wang WS, Jiang TF, Wu BL, Li EM and Xu LY: MiR-142-3p as a potential prognostic biomarker for esophageal squamous cell carcinoma. J Surg Oncol *105*: 175-182, 2012.

8 Guo Y, Chen Z, Zhang L, Zhou F, Shi S, Feng X, Li B, Meng X, Ma X, Luo M, Shao K, Li N, Qiu B, Mitchelson K, Cheng J and He J: Distinctive microRNA profiles relating to patient survival in esophageal squamous cell carcinoma. Cancer Res *68*: 26-33, 2008.

9 Sobin LH, Gospodarowicz M and Wittekind C, (editors): TNM Classification of Malignant Tumours, 7th Edition, UICC International Union Against Cancer 2010. Hoboken, NJ: Wiley-Blackwell; 2010.

10 Osawa S, Shimada Y, Sekine S, Okumura T, Nagata T, Fukuoka J and Tsukada K: MicroRNA profiling of gastric cancer patients from formalin-fixed paraffin-embedded samples. Oncol Lett *2*: 613-619, 2011.

11 Shimada Y, Imamura M, Wagata T, Yamaguchi N and Tobe T: Characterization of 21 newly established esophageal cancer cell lines. Cancer *69*: 277-284, 1992.

12 Zhao WX and Luo JF: Serum neuron-specific enolase levels were associated with the prognosis of small cell lung cancer: a meta-analysis. Tumour Biol *34*: 3245-3248, 2013.

13 Ranade AR, Cherba D, Sridhar S, Richardson P, Webb C, Paripati A, Bowles B and Weiss GJ: MicroRNA 92a-2*: a biomarker predictive for chemoresistance and prognostic for survival in patients with small cell lung cancer. J Thorac Oncol *5*: 1273-1278, 2010.

14 Xu XL, Jiang YH, Feng JG, Su D, Chen PC and Mao WM: MicroRNA-17, MicroRNA-18a, and MicroRNA-19a Are Prognostic Indicators in Esophageal Squamous Cell Carcinoma. Ann Thorac Surg *97*: 1037-1045, 2013.

15 Nouraee N, Van Roosbroeck K, Vasei M, Semnani S, Samaei NM, Naghshvar F, Omidi AA, Calin GA and Mowla SJ: Expression, tissue distribution and function of miR-21 in esophageal squamous cell carcinoma. PLoS One 8: e73009, 2013. doi: 10.1371/journal.pone.0073009.

16 Li X, Yang C, Wang X, Zhang J, Zhang R and Liu R: Expression, tissue distribution and function of miR-21 in esophageal squamous cell carcinoma. PLoS One 8: e73009, 2013. doi: 10.1371/journal.pone.0073009.

17 Gu J, Wang Y and Wu X: MicroRNA in the pathogenesis and prognosis of esophageal cancer. Curr Pharm Des *19*: 1292-1300, 2013.

18 Takeshita N, Hoshino I, Mori M, Akutsu Y, Hanari N, Yoneyama Y, Ikeda N, Isozaki Y, Maruyama T, Akanuma N, Komatsu A, Jitsukawa M and Matsubara H: Serum microRNA expression profile: miR-1246 as a novel diagnostic and prognostic biomarker for oesophageal squamous cell carcinoma. Br J Cancer *108*: 644-652, 2013.

19 Chen J, Yao D, Li Y, Chen H, He C, Ding N, Lu Y, Ou T, Zhao S, Li L and Long F: Serum microRNA expression levels can predict lymph node metastasis in patients with early-stage cervical squamous cell carcinoma. Int J Mol Med *32*: 557-567, 2013.

20 Mathé EA, Nguyen GH, Bowman ED, Zhao Y, Budhu A, Schetter AJ, Braun R, Reimers M, Kumamoto K, Hughes D, Altorki NK, Casson AG, Liu CG, Wang XW, Yanaihara N, Hagiwara N, Dannenberg AJ, Miyashita M, Croce CM and Harris CC: MicroRNA expression in squamous cell carcinoma and adenocarcinoma of the esophagus: associations with survival. Clin Cancer Res *15*: 6192-6200, 2009.

21 Okumura T, Shimada Y, Moriyama M, Takei Y, Omura T, Sekine S, Nagata T, Shimizu K and Tsukada K: MicroRNA-203 inhibits the progression of esophageal squamous cell carcinoma with restored epithelial tissue architecture *in vivo*. Int J Oncol *44*: 1923-1932, 2014.

22 Kano M, Seki N, Kikkawa N, Fujimura L, Hoshino I, Akutsu Y, Chiyomaru T, Enokida H, Nakagawa M and Matsubara H: miR-145, miR-133a and miR-133b: Tumor-suppressive miRNAs target FSCN1 in esophageal squamous cell carcinoma. Int J Cancer *127*: 2804-2814, 2010.

23 Rasmussen MH, Jensen NF, Tarpgaard LS, Qvortrup C, Rømer MU, Stenvang J, Hansen TP, Christensen LL, Lindebjerg J, Hansen F, Jensen BV, Hansen TF, Pfeiffer P, Brünner N, Ørntoft TF and Andersen CL: High expression of microRNA-625-3p is associated with poor response to first-line oxaliplatin based treatment of metastatic colorectal cancer. Mol Oncol *7*: 637-646, 2013.

24 Kirschner MB1, Cheng YY, Badrian B, Kao SC, Creaney J, Edelman JJ, Armstrong NJ, Vallely MP, Musk AW, Robinson BW, McCaughan BC, Klebe S, Mutsaers SE, van Zandwijk N, Reid G: Increased circulating miR-625-3p: a potential biomarker for patients with malignant pleural mesothelioma. J Thorac Oncol *7*: 1184-1191, 2012.

25 Chen ZL, Zhao XH, Wang JW, Li BZ, Wang Z, Sun J, Tan FW, Ding DP, Xu XH, Zhou F, Tan XG, Hang J, Shi SS, Feng XL and He J: microRNA-92a promotes lymph node metastasis of human esophageal squamous cell carcinoma via E-cadherin. J Biol Chem *286*: 10725-10734, 2011.

26 Kozinn SI, Harty NJ, Delong JM, Deliyiannis C, Logvinenko T, Summerhayes IC, Libertino JA, Holway AH and Rieger-Christ KM: MicroRNA Profile to Predict Gemcitabine Resistance in Bladder Carcinoma Cell Lines. Genes Cancer *4*: 61-69, 2013.

27 Zhang B, Chen J, Ren Z, Chen Y, Li J, Miao X, Song Y, Zhao T, Li Y, Shi Y, Ren D and Liu J: A specific miRNA signature promotes radioresistance of human cervical cancer cells. Cancer Cell Int *13*: 118, 2013.

28 Huang YH, Lin KH, Chen HC, Chang ML, Hsu CW, Lai MW, Chen TC, Lee WC, Tseng YH, Yeh CT: Identification of postoperative prognostic microRNA predictors in hepatocellular carcinoma. PLoS One *7*: e37188, 2012. doi: 10.1371

29 Hu Y, Correa AM, Hoque A, Guan B, Ye F, Huang J, Swisher SG, Wu TT, Ajani JA and Xu XC: Prognostic significance of differentially expressed miRNAs in esophageal cancer. Int J Cancer *128*: 132-143, 2011.

30 Navarro A, Diaz T, Gallardo E, Viñolas N, Marrades RM, Gel B, Campayo M, Quera A, Bandres E, Garcia-Foncillas J, Ramirez J and Monzo M: Prognostic implications of miR-16 expression levels in resected non-small-cell lung cancer. J Surg Oncol *103*: 411-415, 2011.

31 Omura T, Shimada Y, Nagata T, Okumura T, Fukuoka J, Yamagishi F, Tajika S, Nakajima S, Kawabe A and Tsukada K: Relapse-associated microRNA in gastric cancer patients after S-1 adjuvant chemotherapy. Oncol Rep *31*: 613-618, 2014.

32 Mrózek K, Whitman SP, Metzeler KH, Mendler JH, Wu YZ, Liyanarachchi S, Patel R, Baer MR, Powell BL, Carter TH, Moore JO, Kolitz JE, Wetzler M, Caligiuri MA, Larson RA, Tanner SM, de la Chapelle A and Bloomfield CD: miR-3151 interplays with its host gene BAALC and independently affects outcome of patients with cytogenetically normal acute myeloid leukemia. Blood *120*: 249-258, 2012.

33 Katayama Y, Maeda M, Miyaguchi K, Nemoto S, Yasen M, Tanaka S, Mizushima H, Fukuoka Y, Arii S and Tanaka H: Identification of pathogenesis-related microRNAs in hepatocellular carcinoma by expression profiling. Oncol Lett *4*: 817-823, 2012.

34 Liu SG, Qin XG, Zhao BS, Qi B, Yao WJ, Wang TY, Li HC and Wu XN: Differential expression of miRNAs in esophageal cancer tissue. Oncol Lett *5*: 1639-1642, 2013.

35 Hirata H, Hinoda Y, Shahryari V, Deng G, Tanaka Y, Tabatabai ZL and Dahiya R: Genistein downregulates onco-miR-1260b and upregulates sFRP1 and Smad4 via demethylation and histone modification in prostate cancer cells. Br J Cancer *110*: 1645-1654, 2014.

36 Liu RY, Diao CF, Zhang Y, Wu N, Wan HY, Nong XY, Liu M and Tang H: miR-371-5p down-regulates pre mRNA processing factor 4 homolog B (PRPF4B) and facilitates the G1/S transition in human hepatocellular carcinoma cells. Cancer Lett *335*: 351-360, 2013.

37 Li T, Lu YY, Zhao XD, Guo HQ, Liu CH, Li H, Zhou L, Han YN, Wu KC, Nie YZ, Shi YQ and Fan DM: MicroRNA-296-5p increases proliferation in gastric cancer through repression of Caudal-related homeobox 1. Oncogene *33*: 783-793, 2014.

38 Zhou C, Yu Q, Chen L, Wang J, Zheng S and Zhang J: A miR-1231 binding site polymorphism in the 3'UTR of IFNAR1 is associated with hepatocellular carcinoma susceptibility. Gene *507*: 95-98, 2012.

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