MicroRNAs associated with postoperative outcomes in patients with limited stage neuroendocrine carcinoma of the esophagus

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Abstract. Esophageal neuroendocrine carcinoma (E-NEC) is an aggressive disease with a poor prognosis. The present study aimed to assess the role of surgery in the treatment of patients with resectable E-NEC, and identify a microRNA (miRNA/miR) signature in association with positive postoperative outcomes. Between February 2017 and August 2019, 36 patients with E-NEC who underwent curative surgery at the Japan Neuroendocrine Tumor Society partner hospitals were enrolled in the study. A total of 16 (44.4%) patients achieved disease-free survival (non-relapse group), whereas 20 (55.6%) patients developed tumor relapse (relapse group) during the median follow-up time of 36.5 months (range, 1-242) after surgery with a 5-year overall survival rate of 100 and 10.8%, respectively (P<0.01). No clinicopathological parameters, such as histological type or TNM staging, were associated with tumor relapse. Microarray analysis of 2,630 miRNAs in 11 patients with sufficient quality RNA revealed 12 miRNAs (miR-1260a, -1260b, -1246, -4284, -612, -1249-3p, -296-5p, -575, -6805-3p, -12136, -6822-5p and -4454) that were differentially expressed between the relapse (n=6) and non-relapse

(n=5) groups. Furthermore, the top three miRNAs (miR-1246, -1260a and -1260b) were associated with overall survival (P<0.01). These results demonstrated that surgery-based multidisciplinary treatment is effective in a distinct subpopulation of limited stage E-NEC. A specific miRNA gene set is suggested to be associated with treatment outcome.

Introduction

Neuroendocrine carcinoma (NEC) of the esophagus (E-NEC) is rare, accounting for ~1% of all esophageal malignancies (1,2). NEC is defined as a poorly differentiated carcinoma with neuroendocrine differentiation and a Ki-67 proliferation index >20%, consisting of small cell carcinoma and large cell carcinoma, as described in the World Health Organization (WHO) classification (3). It is an aggressive disease with high rates of lymph node and distant metastases at the time of diagnosis (4-6). In addition, even patients with surgically resectable E-NEC often experience postoperative tumor relapse, resulting in poor prognosis (6). Thus, a careful decision on the optimal surgery is required (7,8). However, postoperative prognostic predictors for E-NEC have yet to be established.

MicroRNAs (miRNAs/miRs) are small, non-coding RNAs of 18-22 nucleotides in length that bind to their target mRNAs and play a key role in cancer initiation and progression by regulating post-transcriptional gene expression (9). A number of miRNAs have been reported to be potential biomarkers for the detection, classification, therapeutic effects and prognosis of different types of cancer (10-12). In addition, miRNAs are stable and can be detected in formalin-fixed paraffin-embedded (FFPE) archive specimens (13,14), enabling the molecular features of rare diseases to be analyzed.

The aim of the present study was to assess the role of surgery in the multidisciplinary treatment of the patients with resectable E-NEC. Furthermore, the miRNAs that are associated with treatment outcome were investigated.

Materials and methods

Patients and surgical specimens. The present work was conducted as a multicenter retrospective study of the Japan Neuroendocrine Tumor Society (JNETS). The study protocol was approved by the ethics review board of the University of Toyama Hospital (approval no. R27-109; Toyoma, Japan), and by institutional review board of each participating JNETS partner hospital (Table SI). Written informed consent or opt-out consent was obtained from all participants.

A total of 36 patients with E-NEC who underwent curative surgery at JNETS partner hospitals and whose FFPE tumor sections were available were recruited. The mean age of the 36 enrolled patients was 62.6 years old (range, 52-75 years) with a male-to-female ratio of 2.6:1. The patients were enrolled and samples were collected between February 2017 and August 2019. Patients who met all of the following criteria were eligible: i) Patients who underwent curative surgery and were diagnosed with E-NEC based on histopathological findings of the resection specimen; ii) patients with a preserved FFPE block in both the cancerous and non-cancerous areas of the surgical specimen; iii) patients with available prognostic and clinicopathological data; and iv) patients between the ages of 20 and 80 years at the time of surgery. Patients with concurrent or iatrogenic multiple advanced cancers were excluded from this study.

All tumors were histologically diagnosed as poorly differentiated NEC, based on the WHO Classification of Endocrine Organs (2017) (15) and Digestive System (2019) histologic criteria (3). Pathological diagnosis was made by the bord certificated pathologist in each participating hospital. In available cases, the diagnosis was made with reference to the neuroendocrine marker expression, such as synaptophysin, chromogranin A, neural cell adhesion molecule 1 (NCAM) and neuron specific enolase (NSE). All cases were staged according to the 7th edition of the Union for International Cancer Control system (16). Response evaluation for preoperative treatment was performed according to the response evaluation criteria in solid tumors (RECIST) (17).

RNA extraction and miRNA expression profiling. The FFPE samples of the tumors (T) and their normal counterparts (N) were obtained for each case, sections (100 μm) were prepared, and total RNA was extracted. The detailed procedure of this experiment is previously described (14). The boundaries of the tumor margins in the tumor or the mucosal layer in the normal counterparts were marked with loupe images, and the RNA was extracted using a silica-based spin column (Toray Industries Inc.) after trimming the sections (Fig. S1), so that >70% of the target cells are harvested.

Samples in which electrophoresis was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) showed either the majority of RNAs at ≥4,000 nucleotides due to cross-linking, or the majority of RNAs at ≤1,000 nucleotides due to degradation, were deemed unsuitable for miRNA analysis.

Extracted RNA samples were labeled with the 3D-Gene miRNA labeling kit (Toray Industries, Inc.) and hybridized to a 3D-Gene Human miRNA Oligo chip (cat. no. IH201; Toray Industries Inc.) mounted with 2,632 genes. The fluorescent signals were scanned with the 3D-Gene Scanner (Toray Industries, Inc.). The miRNAs with low expression levels (less than threshold 100 of the global normalization value) were excluded from comparison between relapse and non-relapse groups. The tumor-to-normal ratio (T/N ratio) was calculated based on the miRNA expression levels in each tumor and the corresponding normal counterpart.

Statistical analysis. Relationships between clinicopathological factors and postoperative tumor relapse were assessed using Fisher's exact test. Survival times were calculated either from the date of surgery to the death of the patient or the last clinical follow-up date, with survival distributions estimated using the Kaplan-Meier method and compared using the log-rank test. Relationships between miRNA expression and clinicopathological factors were assessed using unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Hierarchical clustering analysis was performed by Ward's method. Pearson's correlation coefficients of $0.6<r<1$ and $-1<r<-0.6$ ($|r|>0.6$) were considered as significant positive and negative relationships respectively.

For the importance analysis of miRNAs, machine-learning classifier was employed to distinguish between the relapse and non-relapse group. As a model-dependent method, a model

was trained to classify the presence of recurrence using Random Forest (18). The miRNAs that contributed to the classification in the model were evaluated using Gini importance. On the other hand, Permutation Importance (19) was used as a model-independent method to determine the importance of features associated with recurrence. A permutation importance $P<0.05$ and Gini importance (GI) ≥ 0.015 were considered to indicate a statistically significant difference.

Results

Clinical information and tumor characteristics of 36 enrolled patients. The mean age of the 36 enrolled patients was 62.6 years old (ranging from 52 to 75 years) with a male-to-female ratio of 2.6:1. A total of 34 (94.4%) patients had small cell type, whereas 2 (5.6%) patients had large cell type; 18 (50.0%) patients had pure NEC, whereas 18 (50.0%) patients had mixed neuroendocrine-non-neuroendocrine neoplasm (MiNEN), in which NEC was combined with squamous cell carcinoma or adenocarcinoma, or both. The neuroendocrine marker synaptophysin, chromogranin A, NCAM and NSE were positive in 27/28 (96.4%), 17/28 (60.7%), 16/21 (76.2%) and 5/11 (45.5%) of the examined tumors, respectively.

All 36 patients underwent surgery with no residual tumors (R0). Subsequently, 24 (85.7%) of 28 patients with stage II-IV disease received perioperative chemo- or chemoradio-therapy.

Among the 36 patients, 16 (44.4%) patients achieved disease-free survival within the median observation period of 144 (ranging from 46 to 242) months, whereas 17 (47.2%) patients died of tumor relapse and 3 (8.3%) patients survived with tumor relapse at a median observation period of 13 (1-106) months after surgery (P=0.004). These two groups were referred to as the non-relapse group (n=16) and relapse group (n=20).

As summarized in Table I, there was no statistical difference between the relapse and non-relapse group in terms of clinicopathological parameters, such as age, sex, tumor location, tumor depth, lymph node metastasis, distant metastasis (M1 lymph), TNM staging, histological type, neuroendocrine marker expression (synaptophysin, chromogranin A, NCAM, NSE), surgical procedures and perioperative chemo- or chemoradiotherapy. Within the 10 patients who received preoperative chemo- or chemoradiotherapy, no relationship was seen between treatment response (RECIST) and tumor relapse.

The median follow-up time of the 36 enrolled patients was 36.5 months (range, 1-242) and the 5-year overall survival (OS) rate was 51.7%. Kaplan-Meier curves of the OS stratified by TNM stage showed no significant difference among patients with stage I, stage II, stage III, and stage IV diseases (P=0.20; Fig. 1A). Patients with stage I-II diseases showed a trend to a longer survival time compared with those with stage III-IV diseases; however, there was no significant difference (P=0.08; Fig. 1B). Patients with negative lymph node metastasis trended towards a longer survival time compared with those with positive lymph node metastasis (P=0.06; Fig. 1C). The 5-year OS rate of the non-relapse and relapse group were 100% and 10.8%, respectively (P<0.01; Fig. 1D). There was no significant correlation between adjuvant therapies and postoperative survival (P=0.45; Fig. S2).

Clinical information and tumor characteristics of 11 patients whose samples underwent miRNA expression analysis. In the 36 enrolled patients, total RNA was extracted from 30 patients with sufficient FFPE blocks to cut sections. Then, high-quality RNA was obtained for paired T/N samples from 11 of the 30 patients and used in miRNA profiling (Fig. S3).

The mean age of the 11 patients was 62.7 years old (range, 52-75) with a male-to-female ratio of 1.8:1. All 11 patients had small cell type. Overall, 7 of 11 (63.6%) patients had pure NEC, whereas 4 (36.4%) patients had MiNEN. A total of 8 of 11 (72.7%) patients, and 6 of 7 (85.7%) patients with stage II-IV disease received perioperative chemotherapy (data not shown).

Among the 11 patients, 5 (45.5%) patients achieved disease-free survival with a median observation period of 51 (range, 46-82) months, whereas 5 (45.5%) patients died of tumor relapse and 1 (9.1%) patient survived with tumor relapse at a median observation period of 27.5 (range, 7-106) months after surgery (P=0.003) (Table I). These two groups were referred to as the non-relapse group (n=5) and relapse group (n=6). As summarized in Table I, there was no statistical difference between the relapse and non-relapse group in clinicopathological parameters.

The median follow-up time of the 11 patients was 46.0 (range, 7-106) months and the 5-year OS rate was 54.6% (data not shown). Kaplan-Meier curves stratified by TNM stage showed no significant difference among patients with stage I, stage II and stage III diseases (P=0.65; Fig. 1E).

Expression of miRNAs in FFPE samples of 11 cases detected by microarray. In the 2,632 miRNAs assessed using the miRNA oligo chip, two miRNAs were not detected in any of the samples, thus 2,630 miRNAs were analyzed. Comparison between the average miRNA expression levels in the 11 NEC tumors and 11 corresponding normal tissues revealed that the tumors expressed 20 miRNAs >2-fold higher, and two miRNAs were expressed >2-fold lower, compared with their normal counterparts (Fig. 2A). Hierarchical clustering based on 2,630 detected miRNAs did not show any relationship between clusters and postoperative tumor relapse (Fig. 2B).

Differentially expressed miRNAs between patients with and without postoperative tumor relapse. To identify miRNAs that accurately differentiate patients with and without postoperative tumor relapse, miRNAs with very low expression (less than threshold 100 of the global normalization value) were excluded, yielding 337 miRNAs for further analysis. When the expression (T/N ratio) of miRNAs was compared between relapse (n=6) and non-relapse (n=5) cases, based on the difference between the averages of the two groups using the t-test, the lower expression of five miRNAs (miR-1246, miR-1249-3p, miR-296-5p, miR-6805-3p and miR-12136) and higher expression of nine miRNAs (miR-1260a, miR-1260b, miR-4284, miR-612, miR-575, miR-6822-5p, miR-5088-5p, miR-7977 and miR-4454) correlated with postoperative tumor relapse (P<0.05; data not shown).

Taking into account correlation coefficients, the lower expression of five miRNAs and higher expression of 12 miRNAs correlated with postoperative tumor relapse ($|r|>0.6$). Using random forest, the lower expression of four miRNAs

Table I. Clinicopathological characteristics of the patients.

	All patients (n=36)			Patients with miRNA analysis (n=11)		
Characteristic	Non-relapse	Relapse	P-value	Non-relapse	Relapse	P-value
Case number	16	20		5	6	
Age (years), mean ± SD	62±6	63±6	0.933	65±6	61±9	0.486
Sex						
Male	11	15	0.723	3	4	>0.999
Female	5	5		2	2	
Tumor location						
Upper/Middle thoracic	9	13	0.734	4	3	0.546
Lower thoracic	7	7		1	3	
Pathological tumor depth						
T1-2	9	14	0.493	4	6	0.455
T3-4	7	6		1	0	
Lymph node metastasis						
N0	7	3	0.073	3	2	0.567
N1-2	9	17		2	4	
Distant metastasis						
M0	15	18	0.840	5	6	-
M1 lymph	1	2		0	0	
TNM stage						
1-2	11	8	0.107	5	5	>0.999
3-4	5	12		0	1	
Histology						
Small cell	16	18	0.492	5	6	-
Large cell	0	2		0	0	
Pure NEC	8	10	>0.999	4	3	0.546
MiNEN	8	10		1	3	
SCC	7	7		1	2	
Adeno	1	2		0	0	
SCC + Adeno	0	1		0	1	
Lymphatic vessel invasion						
Negative	5	8	0.533	1	3	0.546
Positive	10	11		4	3	
Unknown	1	1		0	0	
Venous invasion						
Negative	5	7	0.861	2	5	0.242
Positive	9	13		3	1	
Unknown	2	0		0	0	
Synaptophysin						
Negative	1	0	0.472	1	0	0.456
Positive	11	16		4	6	
Unknown	4	4		0	0	
Chromogranin A						
Negative	5	6	0.901	1	1	0.355
Positive	8	9		4	3	
Unknown	3	5		0	2	
NCAM						
Negative	2	3	0.964	1	0	0.452
Positive	7	9		2	4	
Unknown	7	8		2	2	

Table I. Continued.

Characteristic	All patients (n=36)			Patients with miRNA analysis (n=11)		
	Non-relapse	Relapse	P-value	Non-relapse	Relapse	P-value
NSE						
Negative	5	1	0.057	1	1	0.491
Positive	3	2		1	0	
Unknown	8	17		3	5	
Operation						
Subtotal esophagectomy	15	20	0.444	5	6	-
Lower esophagectomy	1	0		0	0	
Lymph node dissection						
Two-field	7	6	0.493	1	2	>0.999
Three-field	9	14		4	4	
D1	1	0	0.459	0	0	0.567
D2	11	13		2	4	
D3	4	7		3	2	
Curability						
R0	16	20	-	5	6	-
R1	0	0		0	0	
Preoperative chemotherapy	2	7	0.133	0	2	-
DCF	1	2		0	1	
FP	1	1		0	0	
CDDP/CPT11	0	3		0	1	
CDDP/ETP	0	1		0	0	
Preoperative CRT (FP + radiation)	0	1	>0.999	0	0	-
Response to preoperative treatment (RECIST)						
CR	0	0	0.856	0	0	-
PR	1	4		0	1	
SD	1	3		0	1	
PD	0	0		0	0	
Unknown	0	1		0	0	
Postoperative chemotherapy	10	10	-	4	3	0.546
CDDP/5FU	4	4		0	0	
CDDP/ETP	2	0		2	0	
CDDP/CPT11	2	5		2	3	
NDP/DOC	1	0		0	0	
TS1	1	1		0	0	
Postoperative CRT	2	0	0.191	0	0	-
Observation period (median, range)	144 (46-242)	13 (1-106)	0.004	51 (46-82)	27.5 (7-106)	0.003
Duration between surgery and tumor relapse	-	6 (1-25)	-	-	8 (3-25)	-
Type of tumor relapse						
H		7		1	-	
Ly		5		2		
L		0		0		
Pl		0		0		
P		0		0		
H + Ly		3		3		

Table I. Continued.

	All patients (n=36)			Patients with miRNA analysis (n=11)		
Characteristic	Non-relapse	Relapse	P-value	Non-relapse	Relapse	P-value
H + Ly + L		1		0		
H + Ly + L + Pl		1		0		
H + Ly + L + P		1		0		
Ly + L		1		0		
Ly + P		1		0		
Chemotherapy after relapse		18		-	5	-
CDDP/5FU		3			1	
CDDP/ETP		6			3	
CDDP/CPT11		6			1	
Others		3			0	
RT after relapse		7			0	
Prognosis						
Disease-free survival	16	0		5	0	
Survival with relapse	0	3		0	1	
Cancer death						
Cause-specific death	0	17		0	5	
Death of other diseases	0	0		0	0	

NEC, neuroendocrine carcinoma; MiNEN, mixed neuroendocrine-non-neuroendocrine neoplasm; SCC, squamous cell carcinomas; Adeno, adenocarcinoma; NCAM, neural cell adhesion molecule 1; NSE, neuron specific enolase; DCF, docetaxel, cisplatin plus 5-FU; FP, 5-FU plus cisplatin; CDDP, cisplatin; CPT11, irinotecan; ETP, etoposide; CRT, chemoradiotherapy; CR, complete response; PR, partial response; SD, stable disease; PD, progress disease; DOC, docetaxel; H, hematological; Ly, lymph node; L, local; Pl, pleural; P, peritoneal.

and higher expression of 10 miRNAs correlated with post-operative tumor relapse (GI≥0.015). Using permutation importance, the lower expression of five miRNAs and higher expression of 13 miRNAs correlated with postoperative tumor relapse (P<0.05). A merged table of miRNA lists identified 22 miRNAs extracted by these analyses (Table II).

Hierarchical clustering based on the 22 miRNAs did not show relationships between clusters and postoperative tumor relapse (Fig. S4); however, a maximum 12 miRNAs (miR-1260a, miR-1260b, miR-1246, miR-4284, miR-612, miR-1249-3p, miR-296-5p, miR-575, miR-6805-3p, miR-12136, miR-6822-5p and miR-4454) and a minimum of three miRNAs (miR-1260a, miR-1260b and miR-1246) within the 22 miRNAs differentiated between relapse and non-relapse groups (Fig. 3).

Survival analysis and prognosis. Based on a ROC curve analysis to differentiate patients with relapse from those without, the cut-off value, the largest AUC value, sensitivity and specificity for the expression of the 12 miRNAs in Fig. 3A are presented in Table III.

The Box-and-Whisker plots for the top three miRNAs (miR-1260a, miR-1260b and miR-1246) demonstrated that the plots of the relapse and non-relapse groups were clearly separated near the cutoff value (Fig. 4A) and thus the miRNAs differentiated postoperative tumor relapse with both sensitivity and specificity of 1. The overall survival rates of patients with a high expression of miR-1260a and miR-1260b, as well as a low expression of miR-1246, were significantly higher, with a 5-year survival rate of 100% (Fig. 4B-D; P<0.01).

Discussion

E-NEC is an aggressive disease with a lower survival rate compared with squamous cell carcinomas (SCC) and adeno-carcinomas of the esophagus (6), with the role of surgery controversial even in the resectable disease stage (20). Earlier researchers regarded E-NEC as a systemic disease and recommended systemic treatments (6,20). Whereas, recent meta-analysis of large population-based cohorts suggested that radical esophagectomy is an effective primary treatment for certain patients with limited disease stage E-NEC (5,20). Several potential postoperative favorable prognostic indicators are reported, such as absence of lymph node metastasis (4), earlier TNM stage (5,21) and adjuvant therapy (5,22,23).

In the present study, the postoperative outcome of the 36 enrolled patients who underwent radical surgery for the limited disease stage of E-NEC achieved a 5-year survival rate of 51.7%, almost the same postoperative prognosis as patients with esoph-ageal SCC in Japan (24), suggesting that radical esophagectomy was effective in the present cohort. The Kaplan-Meier curves of the overall survival stratified by lymph node metastasis (N0 vs. N1) and TNM stage (stage I-II vs. III-IV) showed a trend with prognosis; however, even patients with stage III-IV disease and patients with positive lymph node metastasis achieved 5-year survival rate of 38.5 and 40.5% respectively, suggesting the

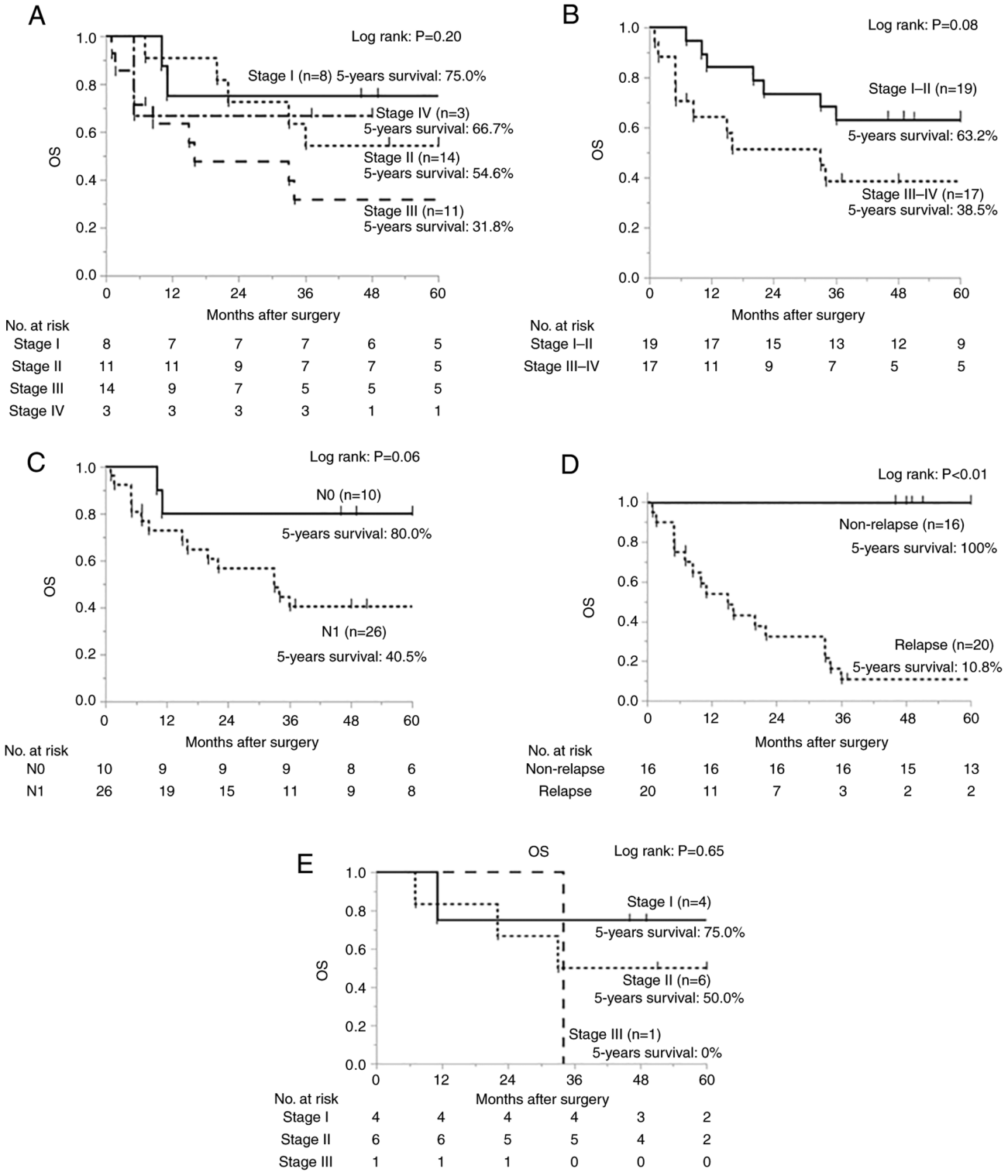

Figure 1. Patient postoperative outcome. (A) Kaplan-Meier curves of the OS for the patients stratified using TNM stage. (B) Kaplan-Meier curves of the OS for the patients stratified using stage I-II and stage II-IV. (C) Kaplan-Meier curves of the OS for the patients stratified using the status of lymph node metastasis. (D) Kaplan-Meier curves of the OS for the patients stratified by recurrence status. (E) Kaplan-Meier curves of the OS for the 11 patients stratified by TNM stage. TNM, tumor node metastasis; OS, overall survival.

existence of subgroups independent of TNM staging and the need for novel indicators for patient response to surgery based multidisciplinary treatment.

In the present study, postoperative prognosis was improved compared with reports from other countries. For example, the 5-year postoperative survival of 25 patients with localized lymph node-negative (TanyN0M0) small cell NEC in the US population has been reported as 50% (4), whereas in the present study, for 10 patients with TanyN0M0, it was 80%. Similarly, another report involving 72 patients with limited stage (TNM stage I-III) E-NEC in the Chinese population found a postoperative 5-year survival rate of 28.4% (5),

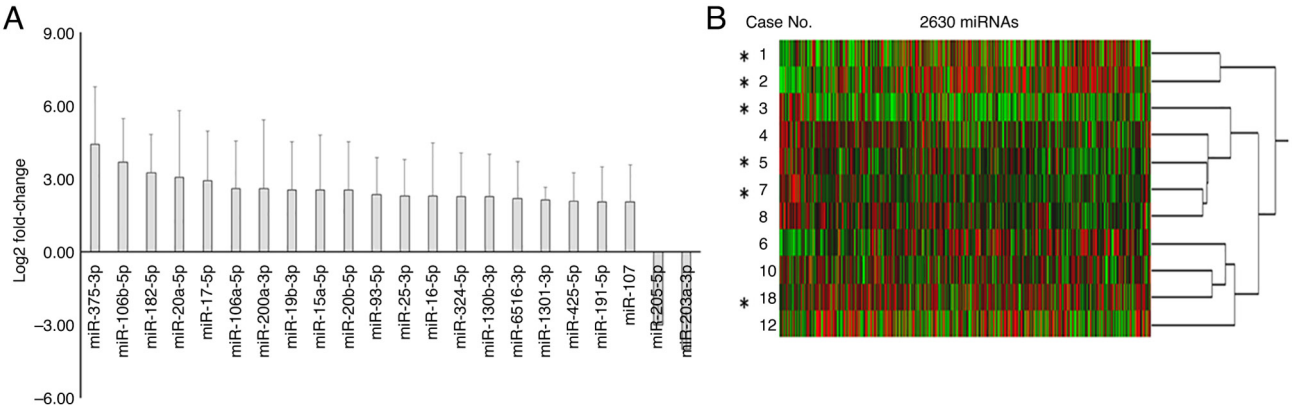

Figure 2. Expression of miRNAs in E-NEC. (A) A total of 22 miRNAs were expressed in E-NEC tumors with a more than two-fold difference compared with corresponding normal counter parts. (B) Hierarchical clustering of 2,630 detected miRNAs and 11 samples. Red represents a higher expression level; green represents a lower expression level. *Cases with postoperative tumor relapse. miRNA/miR, microRNA; E-NEC, esophageal neuroendocrine carcinoma.

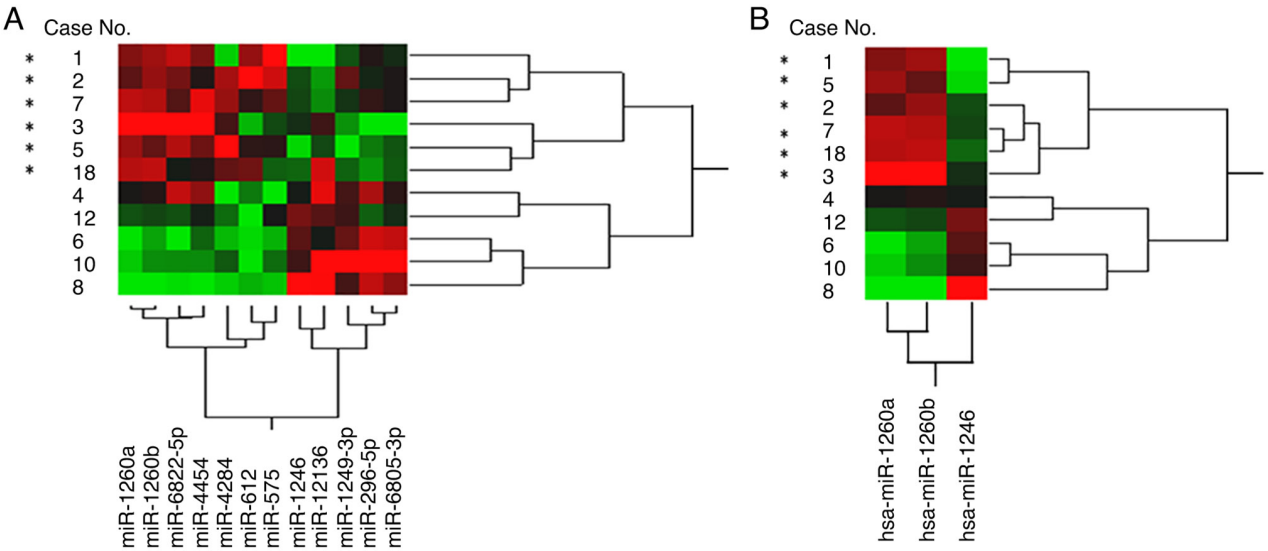

Figure 3. Differentially expressed miRNAs between patients with and without postoperative tumor relapse. (A) Hierarchical clustering of the 12-candidate miRNA set and 11 samples. Red represents a higher expression level; green represents a lower expression level. (B) Hierarchical clustering of top 3 candidate miRNA set and 11 samples. Red represents a higher expression level; green represents a lower expression level. *Cases with postoperative tumor relapse. miR/miRNA, microRNA.

whereas the 33-patient cohort of the present study showed a 5-year survival rate of 50.3%. One explanation for this are differences in adjuvant therapies. In the current study, 75.0% of all patients and 85.7% of the stage II-IV patients received cisplatin-based adjuvant treatment, which was higher compared with in previous reports in which only 48.6-66.0% of the patients received adjuvant therapies (5,21). Although the impact of adjuvant therapy in the present study was not significant, possibly due to limited cases, it is suggested that surgery should be performed as part of multidisciplinary treatment with adjuvant therapies.

In the current study, as all patients received surgery, the benefit of surgery compared with definitive chemo- or chemoradiotherapy cannot be assessed. A Japanese report found a 5-year survival rate of 45.4% in chemoradiotherapy treatment for patients with locally advanced (stage II-IV) E-NEC (25), indicating that if poor postoperative-outcome subgroups are identified, then chemoradiotherapy may be recommended as a primary treatment.

Therefore, as for other types of GI-NEC (26), novel postoperative prognostic indicators are urgently needed to define a subgroup of limited disease stage E-NEC patients who may benefit from surgery, to individualize future treatment.

The current microarray analysis using the miRNA oligo chip with 2,630 miRNAs identified 22 miRNAs that were differentially expressed in tumors compared with their normal counterparts. These miRNAs are well-known to be cancer related, and six of these, miR-375-3p (27), miR-17-5p (28), miR-182-5p (29), miR-25-3p (30), miR-107 (31) and miR-191-5p (32), are involved with neuroendocrine tumors. The present study suggests these miRNAs function in the development of E-NEC and shows successful use of hospital archival FFPE samples to detect differentially expressed miRNAs.

Based on these results, we focused on 12 miRNAs that were differentially expressed between relapse and non-relapse cases. Furthermore, the top three miRNAs, miR-1246,

Table II. miRNAs identified by either of the statistical methods.

miRNA	Non-relapse[a]	Relapse[a]	Up- or downregulation[b]	P-value[c]	R-value[d]	Random forest			Permutation importance (P-value)	Hierarchical cluster analysis	
						Giniimportance	95% CI			≤12 differentiated[e]	≥3 differentiated[e]
							Upper	Lower			
miR-1260a	-1.10±0.40	-0.12±0.17	0.99	0.003	0.878[f]	0.037[f]	0.03734	0.03742	0.002[f]	Yes	Yes
miR-1260b	-1.22±0.48	-0.17±0.17	1.05	0.006	0.860[f]	0.037[f]	0.03734	0.03741	0.002[f]	Yes	Yes
miR-1246	1.54±0.33	0.97±0.21	-0.57	0.013	-0.761[f]	0.037[f]	0.03733	0.03740	0.002[f]	Yes	Yes
miR-4284	-0.36±0.22	0.52±0.60	0.88	0.013	0.721[f]	0.018[f]	0.01756	0.01761	0.035[f]	Yes	
miR-612	-0.51±0.04	-0.22±0.20	0.29	0.016	0.722[f]	0.018[f]	0.01805	0.01809	0.013[f]	Yes	
miR-1249-3p	0.00±0.10	-0.18±0.11	-0.19	0.017	-0.692[f]	0.017[f]	0.01736	0.01741	0.016[f]	Yes	
miR-296-5p	0.17±0.12	-0.04±0.12	-0.21	0.020	-0.690[f]	0.016[f]	0.01577	0.01581	0.023[f]	Yes	
miR-575	-0.62±0.13	-0.25±0.27	0.37	0.020	0.677[f]	0.018[f]	0.01749	0.01754	0.010[f]	Yes	
miR-6805-3p	0.14±0.09	-0.02±0.10	-0.16	0.023	-0.672[f]	0.016[f]	0.01579	0.01583	0.022[f]	Yes	
miR-12136	0.62±0.15	0.26±0.28	-0.35	0.029	-0.645[f]	0.012	0.01172	0.01176	0.027[f]	Yes	
miR-6822-5p	-0.86±0.47	-0.25±0.20	0.61	0.040	0.696[f]	0.015[f]	0.01501	0.01505	0.029[f]	Yes	
miR-5088-5p	-0.14±0.11	0.08±0.19	0.22	0.042	0.607[f]	0.005	0.00530	0.00532	0.289		
miR-7977	-0.86±0.60	-0.13±0.26	0.74	0.049	0.674[f]	0.007	0.00690	0.00693	0.082		
miR-4454	-1.13±0.70	-0.27±0.34	0.85	0.049	0.663[f]	0.015[f]	0.01531	0.01535	0.020[f]	Yes	
miR-375-5p	-0.79±0.58	-0.13±0.32	0.66	0.063	0.626[f]	0.007	0.00666	0.00669	0.125		
miR-7975	-1.51±0.97	-0.45±0.28	1.05	0.072	0.648[f]	0.008	0.00845	0.00848	0.105		
miR-4286	-1.39±1.06	-0.34±0.26	1.05	0.091	0.619[f]	0.015[f]	0.01500	0.01504	0.027[f]		
miR-4746-3p	-0.28±0.09	-0.15±0.13	0.13	0.091	0.521	0.015[f]	0.01535	0.01540	0.013[f]		
miR-4443	-0.95±0.69	-0.32±0.19	0.63	0.114	0.581	0.015[f]	0.01468	0.01473	0.039[f]		
miR-8069	-0.15±0.29	-0.07±0.15	0.08	0.611	0.186	0.006	0.00569	0.00572	0.041[f]		
miR-4690-5p	-0.45±0.17	-0.22±0.19	0.23	0.067	0.566	0.009	0.00889	0.00892	0.043[f]		
miR-3917	-0.34±0.20	-0.18±0.20	0.17	0.207	0.416	0.009	0.00874	0.00877	0.048[f]		

[a]Mean ± SD. [b]Mean ± SD of relapse group-mean ± SD of non-relapse group, where positive numbers indicate upregulation of expression in the relapse group. [c]unpaired t-test. [d]Correlation coefficient. [e]Between relapse and non-relapse groups. [f]Statistical significance found. miR or miRNA, microRNA.

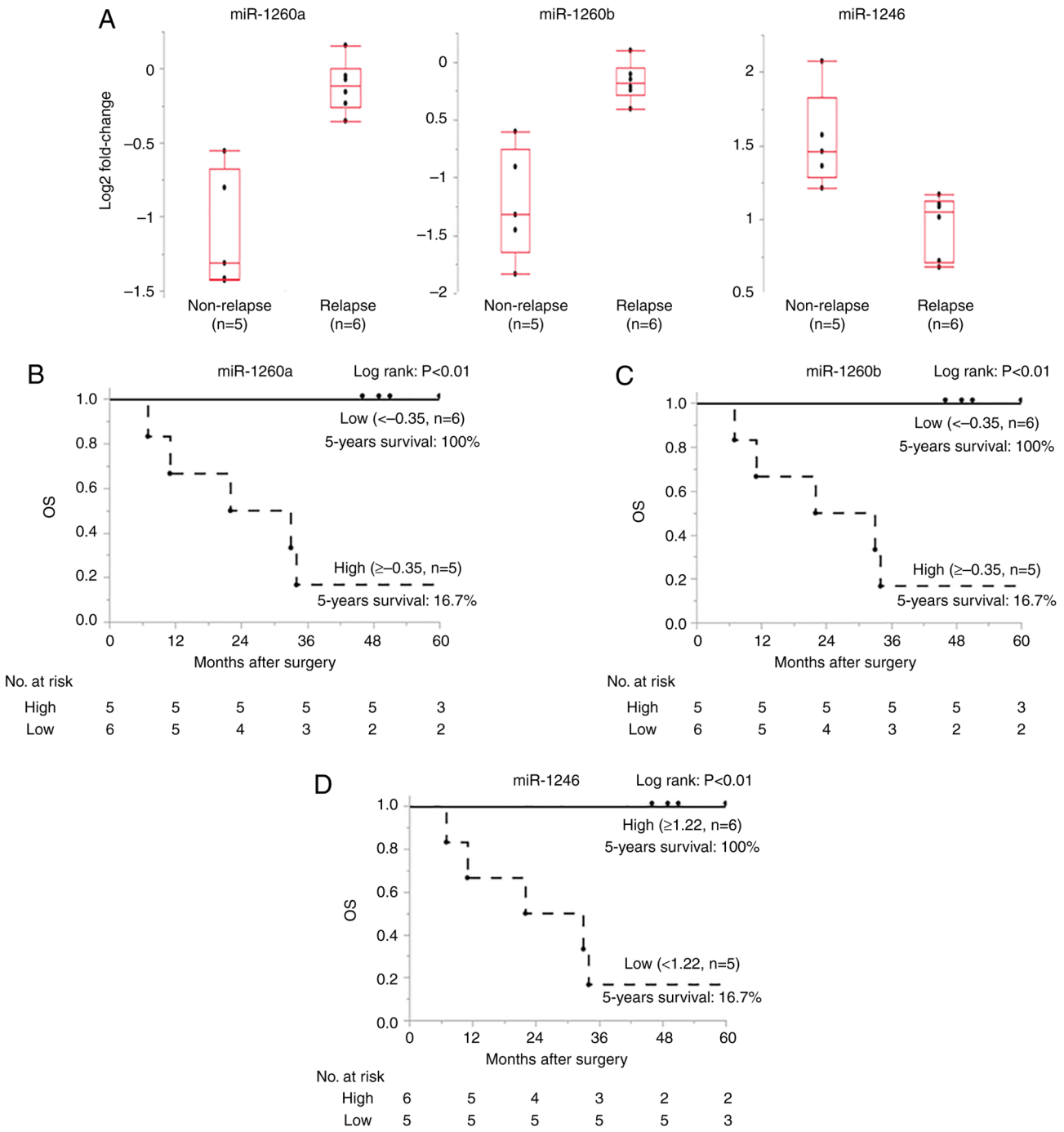

Figure 4. Relationship between the expression of miRNAs (miR-1260a, miR-1260b, miR-1246) and patient outcome. (A) Box-and-Whisker plots for the top three miRNAs in non-relapse and relapse cases. Kaplan-Meier curves of the OS for the 11 patients stratified by (B) miR-1260a, (C) miR-1260b and (D) miR-1246 expression levels. miRNA/miR, microRNA.

miR-1260a and miR-1260b, completely differentiated patients who had postoperative tumor relapse with both sensitivity and specificity of 1. Because radical surgery is a local treatment, response to surgery based multidisciplinary treatment is dependent on the presence of distant micrometastases and sensitivity to chemotherapy. Therefore, it possible that the aberrant expression of miR-1246, miR-1260a and miR-1260b are linked to metastasis and chemosensitivity in E-NEC, as reported in colorectal (33) and breast (34,35) cancer.

As for biological pathways of these miRNAs, miR-1246 has been reported to play a suppressive role in the regulation of the EMT by targeting dual-specificity tyro-sine-(Y)-phosphorylation-regulated kinase 1A (DYRK1A) and progranulin (PGRN) in a breast cancer cell line (36). DYRK1A is linked to a number of cellular processes, including self-renewal, DNA damage, apoptosis, and cancer stem cell maintenance (37). PGRN has been reported to promote lymphangiogenesis and is an independent risk factor in esophageal cancers (38). An oncogene nuclear factor I/B, which is overexpressed in neuroendocrine carcinoma (39,40), has also been identified as a direct target of miR-1246 (41). An onco-miR-1260b has been reported to

Table III. Cut-off values for predicting relapse analyzed using receiver operating characteristic curves.

Name	Cut-off	AUC	Sensitivity	Specificity
miR-1246	1.172	1.00	1.00	1.00
miR-1260a	-0.352	1.00	1.00	1.00
miR-1260b	-0.404	1.00	1.00	1.00
miR-1249-3p	-0.131	0.90	0.83	1.00
miR-4284	0.338	0.90	0.83	1.00
miR-612	-0.310	0.93	0.83	1.00
miR-296-5p	0.076	0.90	1.00	0.80
miR-575	-0.530	0.93	1.00	0.80
miR-6805-3p	0.056	0.90	1.00	0.80
miR-12136	0.304	0.90	0.67	1.00
miR-6822-5p	-0.547	0.83	1.00	0.80
miR-4454	-0.622	0.87	1.00	0.80

miR, microRNA; AUC, area under the curve.

regulate secreted frizzled-related protein 1 and Smad4 (42), those were reported to upregulated in neuroendocrine tumors (43,44).

In the present study, neither the expression of the candidate target molecules, nor the interaction with the three miRNAs were investigated, however, these reported findings suggested possible roles of miR-1246, miR-1260a, and miR-1260b in regulating malignant potential in E-NEC. Investigation of the molecular mechanisms of these miRNAs may help us to further understand the importance of them as biological markers, and aid in developing novel therapeutic strategies, such as miRNA replacement therapy based on the development of tumor suppressor miRNA delivery systems (45). After accounting for low-quality RNA samples, the small remaining number of cases prevented validation group creation to fully assess the candidate gene set in postoperative outcome prediction. In comparison with a previous report, a microarray analysis using gene chips with 885 miRNAs in five cases of esophageal small cell NEC presented 39 miRNAs to predict postoperative tumor relapse (46). Despite the differences in the number of genes carried on the chip, methods of RNA evaluation and statistical analysis, miR-1260b was also included in the candidate gene set. Nevertheless, larger multi-institutional studies are required to validate the use of our miRNA gene set to predict postoperative outcomes.

Recently, liquid biopsy entered use as a less invasive sample collection for various types of cancer. Disrupted levels of molecules listed in the present study, including miR-1246 (11), miR-1260a (47) and miR-1260b (48), are reported to be diagnostic and prognostic biomarkers in peripheral blood for several cancer types, suggesting the use of these molecules in liquid biopsy.

There are several limitations in the present study. First, this was a retrospective observational study with limited number of cases. Second, as perioperative chemotherapies with different regimens have been administered in many cases, the significance of surgery and chemotherapy in the treatment outcome and the link to candidate microRNA function are yet to be elucidated. Third, the sample size for the microarray analysis (11 cases) was too small to determine the use of the miRNAs as predictive markers for recurrence. Fourth, validation of the microarray results by RT-qPCR using the same RNA samples was not performed due to either insufficient amount or denaturation of the remaining RNA. Fifth, a validation cohort was not used due to the exclusion of cases after RNA quality check. Therefore, further validation studies based on larger multi-institutional prospective studies, preferably dealing with frozen tissue samples or blood, are needed to assess the use of our miRNA gene set to predict treatment outcomes of the surgery based multidisciplinary treatment.

In conclusion, the present results demonstrated that radical esophagectomy was effective as part of multidisciplinary treatment basically in combination with adjuvant therapies, for a distinct subpopulation of limited stage E-NEC. The expression levels of miRNAs such as miR-1246, miR-1260a and miR-1260b, in tumors were strongly associated with this postoperative outcome, suggesting possible involvement of these miRNAs in metastasis and chemoresistance in E-NEC. Further investigation is needed to assess possible use of this miRNA gene set as an indicator of surgery based multidisciplinary treatment in patients with E-NEC.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the GEO repository (accession no. GSE221075; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi).

Authors' contributions

TO was involved in the conception and design, experiments, data analysis, data interpretation and manuscript writing. KTe was involved in the data analysis using machine learning classifiers, data interpretation and manuscript writing. TO and KTe confirm the authenticity of all the raw data. TKo, STak, TKa, KTo, SH, TM, MY, STan, YaS, MF, YH, IH, YN and HY were involved in case enrollment and sample collection. TF, SU, YuS, HMat, SO, HMak and MI were involved in the conception and design, supervised the conduct of this study. All authors critically revised the report, commented on drafts of the manuscript, and have read and approved the final manuscript.

Ethics approval and consent to participate

The protocol of the present study was approved by the ethics review board of University of Toyama Hospital (approval no. R27-109). Then the study was approved by each institutional review board of participating JNETS partner sites.

Written informed consent or opt-out consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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