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Original Article

Differential expression of miR-21 and miR-75 in esophageal carcinoma patients and its clinical implication

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Abstract: In Xinjiang, China, esophageal carcinoma has a high incidence in Kazak and Uighur populations. MicroRNA (miR)-21 and miR-375 are related to esophageal carcinoma. This study thus investigated their potencials in early diagnosis and prognosis in Kazak and Uighur populations, to provide evidences for serum markers of esophageal cancer. A total of 126 Kazak or Uighur esophageal cancer patients were enrolled as the disease group, along with 86 local Han patients as disease control cohort, and 80 healthy Kazak or Uighur individuals. MiRNA expression was detected by *in situ* hybridization in tissues and by qRT-PCR in serum. ROC approach was used to evaluate the diagnostic value of miRNA on esophageal carcinoma. Cox analysis was performed to screen factors governing prognosis. MiR-21 level was significantly elevated in both tissue and serum samples of esophageal cancer patients, while miR-375 was down-regulated. Such difference was more potent in disease group compared to disease control group. MiR expression was correlated with infiltration depth, TNM stage, vascular invasion, and lymph node metastasis. Elevated expression of miR-21 reduced the sensitivity of radio-therapy, and increased recurrence frequency. The diagnostic value of single assay for miR-21 or miR-375 was lower than the combined assay (AUC=0.812 or 0.739 vs. 0.858). They also affected patient prognosis (OR=1.53 or 0.652). MiR-21 and miR-375 presented abnormal expression in Kazak or Uighur esophageal carcinoma patients and were independent factors affecting prognosis. The combined assay of miR-21 and miR-375 may help to make early diagnosis of esophageal cancer.

Keywords: Esophageal carcinoma, microRNA-21, microRNA-375, early diagnosis, prognosis

Introduction

Esophageal cancer is one of common malignant tumors, and has the eighth highest incidence and sixth highest mortality among major cancers [1]. It is also frequent in China, where esophageal squamous cell carcinoma (ESCC) is the dominant pathological subtype, occupying around 90% of all cases [2]. The incidence of esophageal cancer showed geographic difference. Kazak or Uighur populations are major minority group in Xinjiang, China, and are also susceptible to esophageal carcinoma. In Kazak people, for example, the incidence of esophageal cancer is as high as 15.59 per 10^5 persons, which is significantly higher than average level (1.52 per 10^5 persons) of the state. Therefore, esophageal carcinoma is one major health concern in these regions [3]. Abnormal expression of miRNA (miR) in esophageal cancer tissues has been widely explored, as it is known to participate in the occurrence and progression of esophageal cancer [4-7]. Currently, no sensitive and specific markers have been established for esophageal cancer. Most patients were already at terminal stage at the time of primary diagnosis, causing its 5-year survival rate lower than 10% [8]. As two frequently studied miR molecules related with esophageal cancer, miR-21 and miR-375 play facilitating and inhibiting roles in cancer progression, respectively. Their expression levels largely reflect patient's survival and prognosis [9]. Tumor marker in peripheral blood has advantages in clinics, including minimal invasion, repeatable assay, and ease for multiple assays simultaneously, and has become promising method for tumor diagnosis and follow-up.

MiRNA has the potency for one complementary biomarker of tumors, as serum miRNA may provide new insights for tumor diagnosis [10, 11]. Previous study showed the stable existence of miR-21 and miR-375 in ESCC patient's serum and ease for assay. The monitor thus can reflect the progression and condition of disease [12]. This study thus investigated the correlation between serum miR and esophageal cancer in Kazak or Uighur populations in Xinjiang, China, in an attempt to provide new insights for early diagnosis, prognostic prediction and treatment of esophageal cancer.

Materials and methods

Clinical information

A total of 126 Kazak or Uighur esophageal cancer patients who received confirmed diagnosis by pathological examination in surgical resection in *A*ffiliated Tumor Hospital, Xinjiang Medical University from November 2011 to June 2015 were enrolled as the disease group. All tumor samples belong to ESCC subtype. There were 60 males and 66 females, with average age at 59.3 ± 9.8 years old. No patient received radio- or chemo-therapy before primary surgery. Clinical stage of tumors was judged according to TNM guideline stipulated by AJCC and UICC. 86 local Han patients with ESCC were assigned as disease control group (44 males, 42 females, average age=59.1 ± 10.1 years). Another cohort of 80 healthy individuals of Kazak or Uighur people (44 males, 36 females, average age=58.6 ± 8.1 years) were chose from physical examination as the healthy control group. Detailed analysis of clinical information and follow-ups were performed in all patients via telephone or mails. The duration of follow-ups ranged from 2 months to 66 months (average=19.9 ± 17.3 months). Peripheral venous blood samples were collected from both cancer and healthy people and were centrifuged at 2000 g for 10 min at room temperature to collect serum, which was further purified by 10000 g for 10 min at 4°C. Serum samples were stored at -80°C for further use. All tissue samples were extracted from surgery within 10 min. No hemorrhage or necrosis area was included in the central region. Tumor adjacent tissues were sampled as normal esophageal mucosa with distance larger than 5 cm beyond tumor boundary. All tissue samples were kept in liquid nitrogen and stored in -80°C fridge. This study has been pre-approved by the ethical committee of Affiliated Tumor Hospital, Xinjiang Medical University and has obtained written informed consents from all patients.

MicroRNA assay and in situ hypbridization

Tumors and adjacent normal tissues were used for *in situ* hybridization targeting miRNA. In brief, tissues were fixed in 4% paraformaldehyde, and were embedded in paraffin for sectioning. After de-wax in xylene and dehydration in gradient ethanol, tissue slides were treated with 200 μg/ml proteinase K at 37°C for 10 min, and were quenched in PBS containing 2% lysine. Pre-hybridization was performed at 56°C for 50 min followed by treatment in balance buffer for 30 min. Probes for miRNA (100 nM) with 5'-biotin labelled was added for overnight incubation at 56°C. Excess probes were washed, and hybridization buffer was detected by enzyme-labeled fluorescence (ELF) signal amplification kit following manual instruction: Slides were washed in 1X washing buffer for 5 min, followed by 100 μl blocking reagent for room temperature incubation (30~60 min). After removing blocking buffer, 100 μl streptavidin-alkaline phosphatase was added for incubation at room temperature for 30 min. Tissue slides were against washed in 1X washing buffer for 3 times (5 min each), followed by the addition of 100 μl ELF97 alkaline phosphatase substrate, which was incubated at room temperature for 40 min. After DAPI staining for 2 min, a fluorescent microscope was employed to observe tissue slides after mounting. Probe sequences were 5'-TGGCC CCTGC GCAAG GATG-3' and 5'-TCACG CGAGC CGAAC GAACA AA-3' for miR-21 and miR-375, respectively. Positive signals were deduced as green fluorescence inside cells. Results were interpreted as "-" (less than 10% positive cells), "+" (between 10% and 15% positive cells), "++" (from 15% to 50% of total cells) and "+++" (more than 50% positive cells).

Serum RNA was extracted by miRNeasy serum/plasma kit (Qiagen, US) following the manual instruction. Total RNA was used as the template to synthesize cDNA by specific stem-loop reverse transcription primers. Using cDNA as the template, PCR amplification was performed under TaqDNA polymerase using following primers: miR-21P_{RT}, 5'-GTCGT ATCCA GTGCA GGGTC CGAGG TATTC GCACT GGATA CGACT

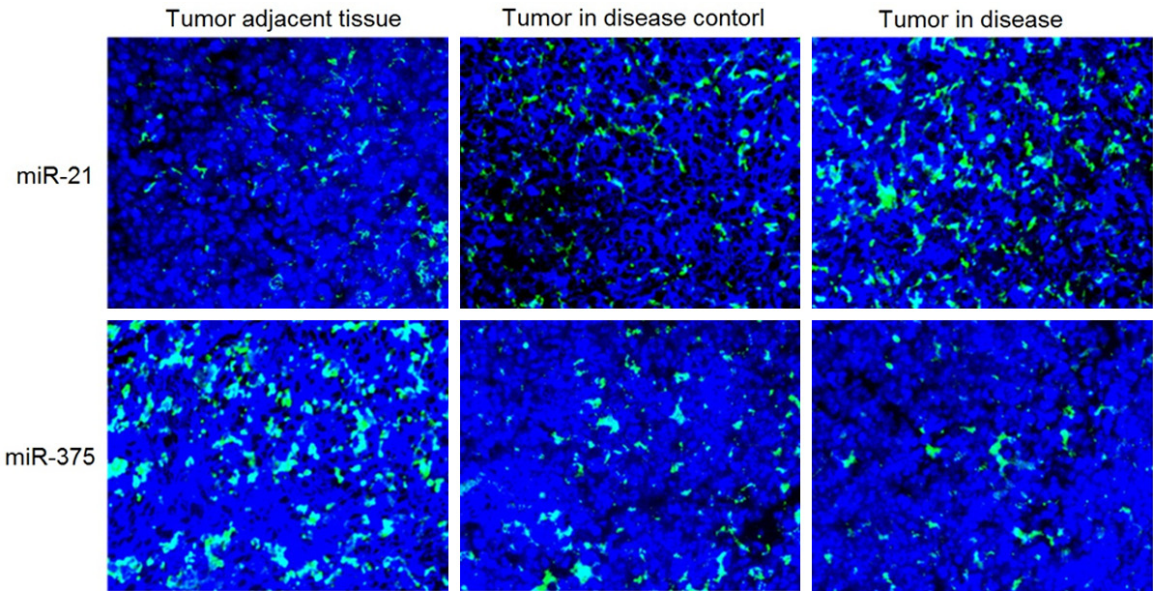

Figure 1. *In situ* hybridization of miR-21 and miR-375 (200×).

CAACA-3'; miR-21P_F, 5'-GTGCA GGGTC CGAGG T-3'; miR-21P_R, 5'-GCCGC TAGCT TATCA GACTG ATGT-3'; miR-375P_{RT}, 5'-GTCGT ATCCA GTGCA GGGTC CGAGG TATTC GCACT GGATA CGACT CACGC-3'; miR-375P_F, 5'-AGCCG TTTGT TCGTT CGGCT-3'; miR-375P_R, 5'-GTGCA GGGTC CGAGG T-3'; U6P_{RT}, 5'-AACGC TTCAC GAATT TGCGT-3'; U6P_F, 5'-CTCGC TTCGG CAGCA CA-3'; U6P_R, 5'-AACGC TTCAC GAATT TGCGT-3'. Real-time fluorescent quantitative PCR was performed on ABI ViiA7 PCR cycler under the following conditions: 95°C denature for 5min, followed by 40 cycles each containing 95°C for 15 s and 60°C for 1 min. Collected data were standardized against U6 internal reference gene. Expression level of miR-21 and miR-375 was quantitatively analyzed by comparative Ct method ($2^{-\Delta Ct}$) method ($\Delta Ct=Ct^{microRNA}-Ct^{U6}$).

Evaluation of radio-therapy efficacy

Based on guideline for treatment efficacy of solid tumors by WHO [13], the complete remission was defined as the loss of tumor lesion. Partial remission was defined as the reduction of tumor by more than 50% without newly occurred lesion. Inefficacy was defined as existence of primary tumors.

Statistical analysis

SPSS18.0 software was used to process all data, of which measurement data were presented as mean ± standard deviation (SD) while enumeration data were presented as percentage. Chi-square test was used for compare enumeration data between groups. The comparison of serum miRNA was done by Mann-Whitney U rank-sum test. Speraman rank-correlation analysis was used to detect the relationship between serum miRNA level and tissue expression intensity. Kaplan-Meier approach was used to plot the survival curve of patients, which were compared by Log-rank test. Based on Cox regression model, multi-variate analysis was performed for related factors for prognosis. Receiver operating characteristic (ROC) curve was applied to evaluate the diagnostic value of miRNA expression level on esophageal carcinoma. MedCalcl 2.7.8 software was used to compare the difference of various test indexes on cancer diagnosis. A statistical significance was defined when $P<0.05$.

Results

Expressional profile of miR-21 and miR-375 in ESCC patients

In situ hybridization results revealed significantly elevated expression of miR-21 in tumor tissues compared to adjacent tissues, while miR-375 level was significantly depressed in tumors. In Kazak and Uighur patients, the extent of miR-21 up-regulation and miR-375 down-regulation was significantly higher than that in local Han

Table 1. Expression of miR-21 and miR-375 in ESCC tumor and adjacent tissues

microRNA	Group	Tissue expression intensity				P_a	P_b
		-	+	++	+++		
miR-21	Disease	12	20	16	78	0.027	<0.001
	Disease control	21	13	11	41		
	Adjacent tissue	149	38	13	12		
miR-375	Disease	73	14	20	19	0.004	<0.001
	Disease control	29	16	26	15		
	Adjacent tissue	14	21	15	162		

Note: P_a, disease group vs. disease control group; P_b, comparison among three groups.

patients (**Figure 1**). In cancer tissues, the positive percentage of miR-21 was significantly higher than adjacent tissues, while positive rate of miR-375 was lower than adjacent tissues (P<0.05 in both cases, **Table 1**). Comparison of positive rates for miR-21 and miR-375 between disease and disease control group also revealed statistical significance (P=0.027 and P=0.004), suggesting differential expressional patterns of miR-21 and miR-375 in Kazak and Uighur people as contrast to Han patients. These results provided the theoretical evidences and scientific implication of studying the correlation between miRNA expression and esophageal cancer in Kazak and Uighur people. qRT-PCR results showed significantly elevated serum miR-21 in disease group as compared to healthy control group (Mann-Whitney U value=38.00, P=0.023), while serum miR-375 level was significantly lower than control group (Mann-Whitney U value= 170.00, P=0.011, **Figure 2**). Spearman rank correlation analysis revealed significantly positive correlation between serum miR-21/miR-375 expression and their expression intensity in tumor tissues as shown by *in situ* hybridization (r=0.582 and 0.673, P=0.019 and 0.013, respectively).

Correlation between serum miR-21/miR-357 and clinical features of patients

Satisfactory positive correlation was found between expression intensity and serum levels of miR-21/miR-375 in esophageal carcinoma. As serum miR has advantages of easy, rapid and low cost assays, it is preferable to apply serum assay in clinics. We thus analyzed the relationship between serum miRNA expressional level and clinical indexes of patients. Using the median value of serum miRNA expression level as the cut-off line, we divide all patients into high-expression and lowexpression sub-groups. The expression level of miR-21 was remarkably correlated with tumor infiltration depth, vascular invasion and lymph node metastasis (P<0.05) but not with age, sex, tumor size, differentiation grade and TNM stage (P>0.05). The expression level of miR-375 was also significantly different in patients with distinct TNM stage, vascular invasion and lymph node metastasis (P<0.05) but not with age, sex, tumor size, infiltration depth and differentiation grade (P>0.05, **Table 2**). Moreover, the expression level of miR-21 also affected the radio-therapy sensitivity and recurrence of patients. In brief, miR-21 high-expression individuals had lower radio-therapy sensitivity than those patients with low miR-21 expression, along with significantly higher probability of recurrence. No significant difference has been observed regarding radio-therapy sensitivity and recurrence between patients with different miR-375 expression levels.

Diagnostic value of serum miR-21 and miR-375 on esophageal carcinoma

By the help of ROC curve, we calculated diagnostic value of miR-21, miR-375 and miR-21/miR-375 ratio. Result showed certain diagnostic value of single assay of miR-21 or miR-375 in esophageal cancer (AUC=0.796 and 0.712), but was significantly lower than the value of miR-21/miR-375 ratio (AUC=0.832, P<0.05 in all cases). As previously described [14], we classified TNM stage I and stage II patients into early phase and TNM stage III and stage IV patients into late phase. The diagnostic value of serum miR-21, miR-375 and miR-21/miR-375 ratio was then evaluated in early and late phase, respectively. Results in early phase cancer showed certain diagnostic value of single assay for miR-21 or miR-375, both of which, however, were significantly lower than miR-21/miR-375 combined assay (P=0.021 and 0.014). In late phase, the diagnostic value of miR-375 was significantly lower than miR-21/miR-375 (P=0.033), which was indifferent with miR-21 (P=0.105). No significant difference can be found in all these three assays when diagnosing between early and late phase cancers (P>0.05, **Figure 3**).

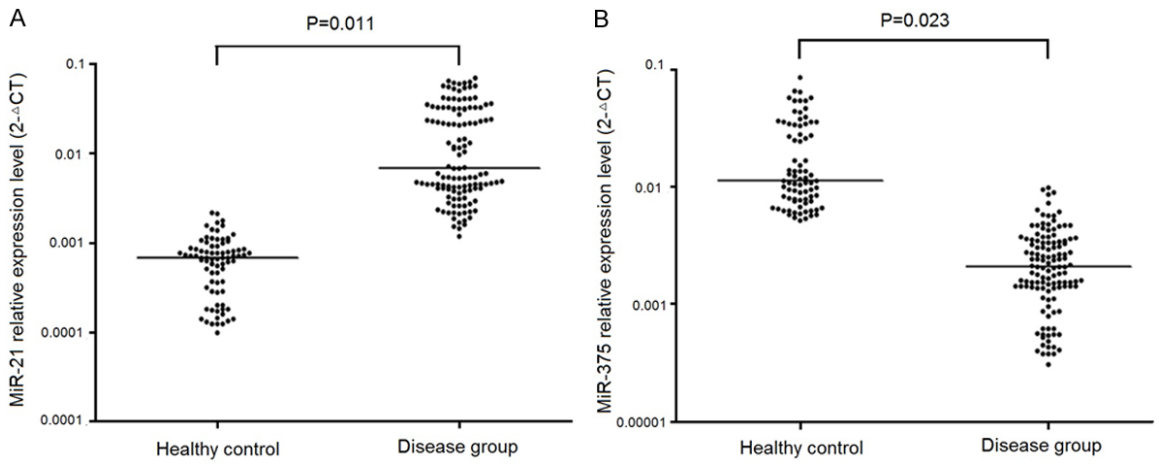

Figure 2. MiR-21 and miR-375 serum expression level in healthy control and disease group. A. Serum miR-21 level by qRT-PCR; B. Serum miR-375 level by qRT-PCR.

Table 2. Relationship between serum miRNA expression and clinical features

Clinical index		N	miR-21		χ^2	P	miR-375		χ^2	P
			High	Low			High	Low		
Age (year)	≤60	68	33	35	0.128	0.721	39	29	3.195	0.074
	>60	58	30	28			24	34		
Sex	Male	56	27	29	0.128	0.719	24	32	2.057	0.152
	Feamle	70	36	34			39	31		
Tumor size (cm)	≤5	46	20	26	1.233	0.267	22	24	0.137	0.711
	>5	80	43	37			41	39		
Infiltration depth	T1~T2	55	19	36	9.325	0.002	25	30	0.807	0.369
	T3~T4	71	44	27			28	33		
Differentiation grade	Good	36	20	16	3.311	0.191	19	17	0.178	0.915
	Moderate	60	25	35			29	31		
	Poor	30	18	12			15	15		
TNM stage	Stage I	11	6	5	2.927	0.403	8	3	13.649	0.003
	Stage II	51	24	27			33	18		
	Stage III	34	19	16			10	24		
	Stage IV	30	20	10			12	18		
Vascular invasion	Yes	46	36	10	23.146	<0.001	16	30	6.711	0.009
	No	80	27	53			47	33		
Lymph node metastasis	Yes	50	36	20	8.229	0.004	17	33	8.488	0.004
	No	76	27	43			46	30		
Radio-therapy sensitivity	Complete remission	26	7	19	11.539	0.003	10	16	1.762	0.414
	Partial remission	76	38	38			40	36		
	Inefficacy	24	18	6			13	11		
Recurrence	Yes	36	30	6	22.400	<0.001	16	20	0.622	0.430
	No	90	33	57			47	43		

Serum miR-21 and miR-375 level and patient prognosis

Based on median level of serum miR-21 and miR-375 expression level in esophageal cancer patients, we divided them into high-expression and low-expression groups. MiR-21 high-expression patients had steeper survival curve than low-expression patients. In contrast, miR-375 low-expression patients showed steeper

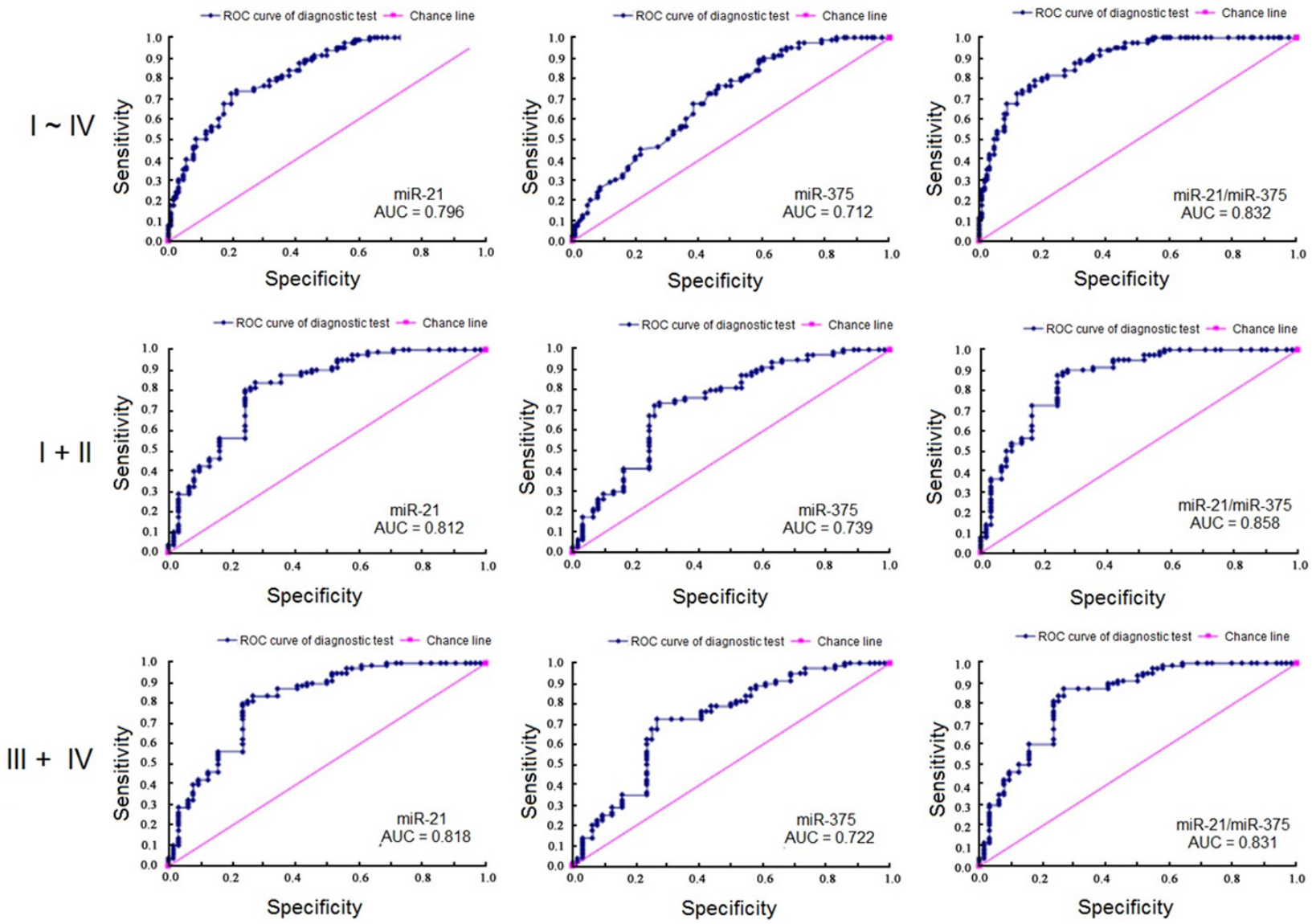

Figure 3. ROC analysis of serum miR-21 and miR-375 on esophageal cancer diagnosis.

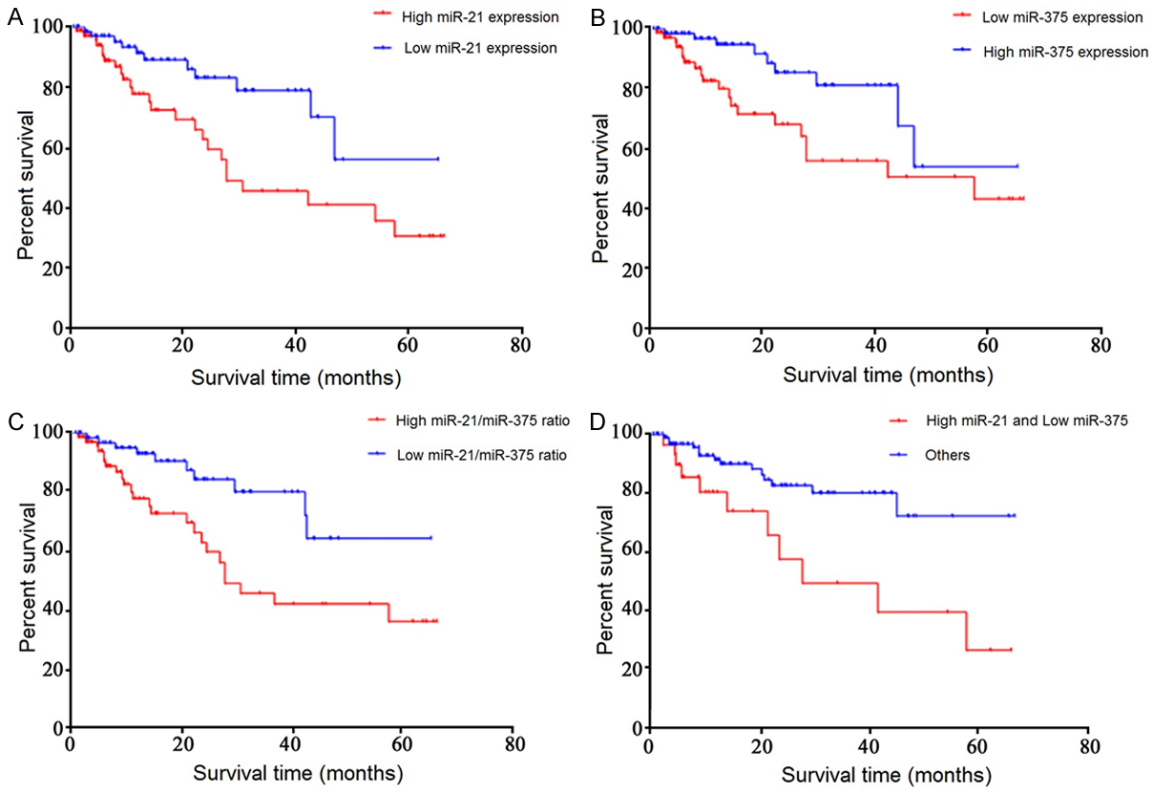

Figure 4. Serum miR-21 and miR-375 and patient prognosis. A. Survival curve of serum miR-21 high/low expression patients; B. Survival curve of serum miR-375 high/low expression patients; C. Survival curves of patients with different miR-21/miR-375 ratios; D. Survival curve with high miR-21 and low miR-375 and other patients.

survival curve than that of miR-375 high-expression (**Figure 4A** and **4B**). Log-rank test showed significantly lower survival rate in miR-121 high-expression patients (χ^2=7.210, P=0.008) and in miR-375 low-expression patients (χ^2=5.475, P=0.019). Using the median value of serum miR-21/miR-375 ratio, we also observed worse prognosis in high miR-21/miR-375 ratio patients than those with low ratio (χ^2=7.995, P=0.005, **Figure 4C**). Those patients with both high miR-21 and low miR-375 expression had significantly worse prognosis than other patients (χ^2=8.018, P=0.004, **Figure 4D**).

Independent factors affecting patient survival

Cox multi-variate analysis was used to check the effect of age, sex, tumor size, infiltration depth, differentiation grade, TNM stage, vascular invasion, lymph node metastasis, radiotherapy sensitivity, miR-21 expression level, miR-375 expression level and miR-21/miR-375 ratio. The maximum likelihood estimation was employed as the parameter in Cox regression function in this study. Result revealed several independent risk factors affecting survival of esophageal patients, including TNM stage, vascular invasion, lymph node metastasis, radiotherapy sensitivity, miR-21 expression level, miR-375 expression level, and miR-21/miR-375 ratio (**Table 3**). The risk of death of miR-21 high-expression patients was 1.853-fold of those patients with low miR-21 expression (P=0.012). Those patients with high miR-21/miR-375 ratio had 2.014-fold death risk than those with low ratios (P=0.009). High-expression of miR-375 was a protective factor for patient survival, as it can decrease death risk by 34.8% (P=0.041). Those patients with vascular invasion or lymph node metastasis had 2.074-fold and 1.914-fold higher death risk than those without such factors (P=0.023 and 0.030, respectively). Late TNM stage also was correlated with unfavorable prognosis (OR=1.936, P=0.018).

Discussion

Esophageal carcinoma is one common malignant tumor in Kazak and Uighur people in

Table 3. Analysis of risk factors affecting survival of esophageal cancer patients

Parameters	Regression coefficient (B)	Standard error (S.E)	Wald	P	OR	95% *CI*
Age	1.251	0.439	1.205	0.122	1.473	0.656-3.211
Sex	-0.974	0.263	1.366	0.308	0.739	0.542-2.736
Tumor size	1.352	0.764	1.973	0.097	1.502	0.883-2.122
Infiltration detph	1.356	0.352	3.038	0.087	1.264	0.746-3.263
Cell differentiation grade	1.523	1.067	2.851	0.168	1.312	0.456-3.021
TNM grade	1.644	0.112	4.837	0.018	1.936	1.235-2.876
Vascular invasion	1.863	0.103	5.874	0.023	2.074	1.342-4.156
Lymph node metastasis	1.789	0.097	4.652	0.030	1.914	1.358-3.428
Radio-therapy sensitivity	1.155	0.681	2.511	0.236	1.629	0.677-3.194
miR-21	1.876	0.079	5.271	0.012	1.853	1.475-6.242
miR-375	-1.480	0.256	2.176	0.041	0.652	0.271-0.861
miR-21/miR-375	1.987	0.103	5.943	0.009	2.014	1.362-5.187

Xinjiang. Most patients showed insidious onset even until terminal stage, leading to relatively low diagnostic rate. Current treatment strategy consisting of surgery has reached a plateau regarding its efficacy. Due to complicated mechanism, routine treatment had unfavorable outcomes. Patients frequently had unfavorable prognosis and high mortality, with 5-year survival rate at 3%~10% [8]. However, surgery intervention for stage I cancer can elevate 5-year survival rate to more than 90%. Therefore the early diagnosis and timely treatment are of critical importance [15].

Non-coding microRNA is an important component in epigenetics regulation. It participates in multiple biological processes including body growth/development, cell proliferation/apoptosis, cell metabolism and differentiation [16]. MicroRNA has stable property and wide arrays of functions in tumor tissues. It has significant value in tumor diagnosis, treatment efficacy and prognosis evaluation. The abnormal expression of microRNA in esophageal cancer has been widely reported, as it is closely correlated with tumor occurrence and progression [4-7]. MiR-21 is one widely studies microRNA molecule. Similar to oncogene, it regulates various biological properties of tumor cells including proliferation, transformation and metastasis. Its abnormally high expression is correlated with the occurrence of pulmonary carcinoma, breast cancer and pancreatic tumors [17]. The role of miR-21 in ESCC has also drawn research interests, as lots of studies have confirmed its abundant expression in esophageal tumor tissues [18] and patient's serum [19], suggesting its facilitating role in pathogenesis of esophageal cancer as one oncogene.

MiR-375 can depress the expression of multiple tumor genes such as AEG-1, AP1, IGF1R and PDK1, which exert tumor suppression roles in pathogenesis [20]. The down-regulation of miR-375 caused by abnormal transcriptional factors or methylation of promoter region can facilitate tumor occurrence, and affect patient's survival and prognosis by its expression level [20]. In esophageal cancer patients, miR-375 has been found to have depressed expression [21, 22], suggesting it inhibitory role in cancer pathogenesis as tumor suppressor gene. Few study has been reported regarding the correlation of microRNA and esophageal cancer in minority groups such as Kazak and Uighur people in Xinjiang. We thus investigated the expression of miR-21 and miR-375 in esophageal cancer patients in Kazak or Uighur populations in Xinjiang, China, along with their correlation with clinical features, early diagnosis and prognosis, in an attempt to reveal their potency as early diagnosis and prognostic prediction markers for esophageal cancer in those people. Results showed remarkably higher positive rate and expression intensity of miR-21 in tumor tissues in esophageal cancer patients, as consistent with Hiyoshi *et al*, who found more miR-21 expression in ESCC tissues than adjacent tissues by *in situ* hybridization [23]. In contrast with miR-21, miR-375 expression intensity and positive rate was significantly lower in cancer tissues compared to adjacent tissues, as consistent with Li et al [21]. Tanaka et al found higher serum miR-21 level in ESCC patients

[24], further confirming our results. Differential expressional patterns of miR-21 and miR-375 occurred in ESCC patients of Kazak or Uighur populations in Xinjiang, as contrast to local Han patients, consisting one possible reason for higher mortality of ESCC in those minority groups. Serum level of miR-375 in ESCC patients was significantly lower than control group, as consistent with Komatsu *et al* [12]. This study also revealed positive correlation between serum level and tissue positive rate regarding miR-21 and miR-375, indicating that serum microRNA assay might work as one alternative way for tissue pathological assay. This study also analyzed the correlation between serum miR-21/miR-375 expression level and clinical features and found significant correlation between miR-21 level and tumor infiltration depth, vascular invasion, or lymph node metastasis. For example, those patients with infiltration depth T3~T4 had higher serum miR-21 level than T1~T2 individuals, agreeing with Mori et al, who reported higher serum miR-21 in T3~T4 patients [25]. Vascular invasion and lymph node metastasis of tumors all depend on cell invasion and migration potency. Liu et al found that miR-21 could facilitate cell growth and migration via targeted inhibition of programmed cell death 4 (PDCD4) in ESCC patients of Kazak group [3]. This study found higher miR-21 level in patients with vascular invasion and lymph node metastasis compared to those with no such phenomena, as proved by Cai *et al* [19]. Kong et al found higher percentage of low miR-375 expression in patients with late TNM stage [14]. Moreover, patients with vascular metastasis had 85.71% with down-regulation of miR-375. It was significantly lower in those without vascular metastasis (only 13.21%). These results were consistent with our observation, in which difference existed in miR-375 expression level across patients with differential TNM stages, vascular invasion and lymph node metastasis. This can be explained by the over-expression of insulin-like growth factor 1 receptor (IGF1R) as the consequence of miR375 down-regulation [14]. Winther et al found lower sensitivity for radio-therapy in those patients with miR-21 high-expression compared to lower level of miR-21 [9]. This is probably with the inhibition of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is one homolog of tumor suppressor gene, by elevated miR-21 expression [26]. This study also found differential expression levels of miR-21 across patients with different sensitivities of radio-therapy but not for miR-375. Moreover, the recurrence probability of patients with miR-21 over-expression was also significantly higher than those with low miR-21 expression, as agreed with Komatsu *et al* [12]. They also found remarkably decreased serum miR-21 level after surgical treatment, and re-surge levels at the time of tumor recurrence [12]. Such amplitude and sensitivity of miR-21 expression was even higher than carcinoembryonic antigen (CEA), suggesting the potency of miR-21 as one sensitive marker for monitoring esophageal cancer recurrence.

Serum microRNA assay had advantages such as easy sampling, rapid and lower cost, making it one preferable approach in clinical diagnosis of tumors. This study found certain diagnostic value of single assay for miR-21 or miR-375 in esophageal cancer, but still lower than that of miR-21/miR-375 ratio. Komatsu *et al* indicated that the diagnostic value of this ratio as 0.816 [12], which was close to 0.832 in our study, indicating higher sensitivity of combined assay of miR-21 and miR-375 than single assay. As most esophageal cancer patients were already at late or terminal stage when having significant symptoms, early diagnosis is of critical importance. Current diagnostic technique, including endoscopy and pathology examination, have lower sensitivity, and are thus hard to indicate delicate alternation of esophageal epithelial cells. Serum microRNA as tumor markers thus provides new insights for early diagnosis of esophageal cancer. Ye *et al* compared the diagnostic values of miR-21 across different stages of esophageal tumors and found no significant difference [15]. This study found certain diagnostic values of miR-21, miR-375 and their ratio for both early and late stage of esophageal cancers, but without significant difference, as consistent with Ye *et al* [15]. Analysis of survival curves found unfavorable prognosis in those patients with miR-21 up-regulation compared to down-regulation ones, and better prognosis in miR-375 high-expression patients, suggesting the tumor facilitating or inhibiting roles by miR-21 or miR-375, respectively. These interpretations were consistent with Liu *et al* [3] and Kong *et al* [14]. Those patients with both miR-21 high-expression and miR-375 down-regulation had even worse prognosis, as proved by Komatsu *et al* [10]. Cox regression analysis found the eleva-

tion of death risk in high serum miR-21 patients by 85.3% compared to miR-21 low-expression ones. OR value in miR-375 high-expression patients was 0.652. Combined assay of miR-21 and miR-375 had more potent values for predicting prognosis, as its OR value as high as 2.014. Komatsu *et al* found higher mortality in patients with both miR-21 over-expression and miR-375 down-regulation than other patients [10], with OR value as high as 3.789. These results suggested that those esophageal cancer patients with miR-21 high-expression and miR-375 low-expression should draw more focus in future treatment to improve the prognosis.

This study observed Kazak and Uighur people in Xinjiang, both of which had the highest incidence of esophageal cancer in China. Due to the relative isolation of research population, both disease and healthy control samples came from the same cohort of people with similar genetic background. This should benefit the minimization of genetic mutation caused by differential background, thus helping us to explore major genetic factors related with disease.

In summary, this study demonstrated abnormal expression of miR-21 and miR-375 in Kazak and Uighur people in Xinjiang. Such differential expression was independent factor affecting patient prognosis. The combined assay of miR-21 and miR-375 is of higher diagnostic value for early stage of esophageal cancer. With further investigation for the role of miR-21 and miR-375 in pathogenesis mechanism of esophageal cancer, they may work as novel serum markers for early diagnosis and prognosis prediction for esophageal cancer, as well as novel drug targets and candidates.

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Disclosure of conflict of interest

None.

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