Diagnostic and predictive significance of serum microRNA-7 in esophageal squamous cell carcinoma

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Received October 3, 2015; Accepted November 5, 2015

DOI: 10.3892/or.2015.4499

Abstract. MicroRNA-7 has been reported to participate in tumorigenesis and progression by several signaling pathways in various tumors. However, its potential as a serum diagnostic factor and predictive biomarker for esophageal squamous cell carcinoma (ESCC) has not been studied. Serum samples were collected from 105 pathologically proven ESCC patients and 30 age- and gender-matched healthy controls. All patients were treated with concurrent chemoradiotherapy (CRT). Real-time polymerase chain reaction was carried out to measure the serum miR-7 expression level. The data were compared among radio-sensitive and radio-resistant groups, and healthy volunteers to elucidate the diagnostic and predictive value of miR-7 expression. Finally, *in vitro* experiments are used to clarify the mechanisms of the miR-7. In the present study, we found that the serum miR-7 level of ESCC patients was 4.74-fold lower as compared with healthy subjects, indicating that serum miR-7 expression could be an excellent diagnostic factor. The serum miR-7 expression level for these responsive patients was 2.34-fold higher than that for non-responsive patients, indicating it as a valuable biomarker for predicting treatment response of ESCC patients to concurrent chemoradiation treatment. We also found that miR-7 levels are strongly correlated with tumor length and the status of lymph node metastasis ($P<0.05$). In contrast, the responsiveness of therapy is significantly correlated with CEA ($P<0.05$), Cyfra21-1 ($P<0.05$), serum miR-7 level ($P<0.05$) and myelosuppression ($P<0.01$). In addition, the experimental data also suggest that miR-7 can interfere with EGFR mRNA translation. In ESCC patients, serum miR-7 has the potential to serve as a noninvasive biomarker of diagnosis and predicting treatment responses to concurrent chemoradiation therapy. ESCC patients with lower Cyfra21-1 and CEA, higher miR-7 and severe myelosuppression were much more sensitive to CRT. In addition, miR-7 may function by interfering with EGFR mRNA translation, but not degradation.

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Key words: miR-7 expression, esophageal squamous cell carcinoma, chemoradiation therapy, radiosensitivity, EGFR

Introduction

Esophageal carcinoma is the sixth most common cause of cancer-related death worldwide. China is one of the high-incidence countries and esophageal cancer accounts for approximately 150,000 death each year, nearly a quarter of all cancer deaths (1) in the country. Esophageal cancer is usually diagnosed at an advanced stage, making curative surgical resection, which is initially recommend for early stage cases, feasible for only 30-40% of patients (2). The outcome of surgery for patients with such an aggressive tumor is still unsatisfactory, with a 5-year survival rate less than 20% (3). Accumulating evidence from randomized clinical trials supports the use of neoadjuvant chemoradiotherapy (CRT), which is shown to improve resectability and survival in patients with locally advanced esophageal cancer, although mixed results have been reported (4). The variations in clinical responses to CRT are most evident for esophageal squamous cell carcinoma (ESCC), and the survival rates between responders and non-responders are quite different even with the same clinical stage (5,6). Therefore, there is a compelling need to identify novel biomarkers that hold promise of precisely predicting tumor response to CRT to tailor treatments for different ESCC patients and enhance survival (7).

MicroRNAs (miRNAs) are endogenous, short, non-coding RNAs that can regulate the expression of target genes by binding to RNA-binding proteins to control the occurrence and development of the disease including cancer. It was first characterized in 1993, since then, more and more studies have focused on the role of miRNA-small molecules in the cellular processes and pathways including the differentiation, progression, apoptosis and proliferation of different disease. In 2002, Calin *et al* (8) first linked miRNAs with cancer progression. Since then, increased number of studies have reported the

role of miRNAs in carcinogenesis, indicating that miRNAs are closely related to the process of epithelial-mesenchymal transition (9,10), characteristic of cancer stem cells (10,11), the initiation of tumor invasion and metastasis (12,13), and the therapeutic response to chemotherapy or radiotherapy (14). There is growing evidence to indicate miRNAs as biomarkers, expression profile of miRNAs are different in cancer and normal tissues, even in different organs or tissues (15,16). Recent studies have reported that tissue-specific miRNAs are consistently detected in circulating samples, and cancer tissue-specific miRNAs also have been found in the circulation at different stages of the disease (17). In addition, more and more reports have demonstrated that tumor cells release miRNAs into the circulation and these circulating miRNAs are in a remarkably stable, cell-independent form which is protected from endogenous RNase activity in the bloodstream, suggesting potential opportunities for using circulating miRNAs as blood-based, non-invasive biomarkers for molecular diagnostics, physiological and pathological status, including cancer (18-21).

MicroRNA-7 (miR-7) is an intronic miRNA that resides in the first intron of the heterogeneous ribonuclear protein K gene on chromosome 9, and is evolutionarily conserved across all species. Previous findings suggested that miR-7 participates in tumorigenesis and progression by several signaling pathways in various types of tumors (22-27). Li and Carthew (28) have certified that 3'-untraslated regions of human EGFR contains miR-7 complementary sites, which enable it to act on EGFR expression. Furthermore, resent studies have reported that miRNA-7 can affect sensitivity to chemotherapy by MRP1 (29) and radiotherapy by EGFR (30), providing opportunities for the development of miRNA-based therapies and/or biomarkers in CRT of cancer patients.

To investigate the functional roles of miR-7 in radioresponse of ESCC and its underlying mechanism, we detected the serum miR-7 expression in ESCC and analyzed its association with clinical response of ESCC to CRT and evaluated the possibility of using serum miR-7 as a diagnostic and predicting factor for ESCC. Furthermore, we investigated the potential miR-7 role in transfected cellular model of ECA-109, identifying EGFR as a direct downstream target of miR-7.

Materials and methods

Ethics statement. This research involved human participants, thus serum-based specimen collection and studies were approved by the Institutional Review Boards of Shandong Cancer Hospital, the Shandong Academy of Medical Sciences. All participants provided written consent and indicated willingness to donate their blood samples for research.

Samples and treatment regimen. Serum samples were collected from the peripheral venous blood of 105 patients and 30 healthy volunteers at the Department of Radiation Oncology, Shandong Cancer Hospital. Clinical data of enrolled patients including gender, age, tumor locations, status of lymph node and distant metastases, the maximum diameter of tumor and tumor differentiation were recorded. Entry criteria and treatment regimen for the present study have been described in our published study (31). Immediately after collection, the serum samples were snap-frozen in liquid nitrogen and then stored at -80˚C for RNA extraction for quantitative RT-PCR (qRT-PCR). Response Evaluation Criteria In Solid Tumors (RECIST), the guidelines recommended by the World Health Organization (32) was applied in the present study to evaluate the response of ESCC patients to CRT (concurrent radio-chemotherapy): each patient's response was defined as complete remission (CR), partial remission (PR), stable disease (SD) or progressive disease (PD).

Cell line and cell culture. ECA-109 cells, a well differentiated human ESCC cell line, were provided by Tianjin Cancer Hospital. Cells were maintained in RPMI-1640 medium (HyClone, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, 100 U/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO_2. Cells were passaged every 2-3 days to maintain exponential growth.

miRNA transfection. The mimic of miR-7 was purchased from GenePharma (Shanghai, China). The ECA-109 cells were transfected with 100 nM of miR-7 mimic or their corresponding negative controls. Lipofectamine 2000 (Invitrogen, San Diego, CA, USA) was used for cell transfection. Transfection complexes were added into the culture plates and incubated for 4 h, and then replaced by fresh medium according to the manufacturer's instructions.

Screening and verification of circulating miRNAs by RT-PCR. Total RNA from human samples, including miRNAs, was extracted from 400 μl of serum using the mirVana PARIS RNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions with the cell-miR-39 spike-in (Sangon Biotech, Shanghai, China). A mirVana miRNA column (Ambion) was used to collect total RNA. The bound RNA was cleaned with the buffers provided by the manufacturer to remove impurities and eluted in a final volume of 50 μl. Total RNA from cells were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

To analyze the expression miR-7, quantitative real-time PCR was conducted using TaqMan fluorogenic probes. miR-7 was detected by qRT-PCR using the mirVana™ qRT-PCR primer set and the mirVana™ qRT-PCR miRNA detection kit (Applied Biosystems, San Diego, CA, USA) according to the manufacturer's instructions, which were previously described (31).

Relative levels of EGFR mRNA in cells were examined by SYBR-Green real-time quantitative reverse transcription-PCR and normalized to β-actin mRNA. Reverse transcriptions using the PrimeScript® RT Master Mix (Perfect Real-Time), and qRT-PCR was performed using SYBR® Premix Ex Taq™ II (Perfect Real-Time) (both from Takara Bio, Inc., Japan) according to the manufacturer's instructions.

All qRT-PCRs were performed in duplicate, and the data are presented as mean ± standard error of the mean. Real-time polymerase chain reaction (RT-PCR) was carried out on the ABI 7900 Real-Time PCR System (Applied Biosystems). Relative microRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method (31,33), where $\Delta\Delta Ct$ = (Ct gene of interest - Ct normalized gene) of (CR+PR) - (Ct gene of interest - Ct normalized gene) of (SD+PD).

Western blot analysis. Cell lysates were prepared in lysis buffer [0.15 M NaCl, 50 mM Tris-Cl (pH 7.5), 2 mM EDTA, 0.5% Triton-100, 5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml apoptinin] following 48 h transfection. Protein concentrations of total cell lysates were measured by Bio-Rad protein assay, and 50 μg of total cell lysates/lane was separated by 10% SDS-PAGE. After electrophoresis, the protein was transferred to polyvinylidene defluoride (PVDF) membrane, which was blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) [50 mmol/l Tris-HCl (pH 7.6), 150 mmol/l NaCl, 0.1% Tween-20] for 1.5 h at room temperature. Immunoblotting was performed with rabbit anti-EGFR (1:500; CST), and mouse anti-β-actin (1:500; Santa Cruz) primary antibodies after washing the membrane 3 times in TBST buffer. Membranes were subsequently probed with horseradish peroxidase-conjugated secondary antibody (1:5,000; Zhongshan Biotechnology, China), developed by chemiluminescence and exposed to X-ray film. Densitometry was performed with gel imaging system (AlphaImager 2200; Pharmacia Biotech Co., USA). All experiments were performed in triplicate.

Statistical analysis. All clinicopathological variables and circulating miRNA levels were analyzed using PASW Statistics, Windows software version 17.0 (SPSS, Inc., Chicago, IL, USA). An unpaired t-test was performed to compare the differences in serum miRNA levels between groups. Chi-square test and logistic regression analysis were used to evaluate the association between serum miR-7 relative expression and clinical pathological variables. All tests were two-sided and P<0.05 was considered to indicate a statistically significant result.

Results

Relative levels of serum miR-7 expression. The result analyses revealed that the Ct of miR-7 expression was 15.15±1.80 [95% confidence interval (CI)] for the radiosensitive group and 16.38±1.44 (95% CI) for the radioresistant group, as shown in Fig. 1. Based on statistical analysis, the relative miR-7 serum level was significantly higher in the SD+PD group when compared with the CR+PR group (P<0.05). The mean miR-7 serum levels differ by 2.34-fold between these two groups of patients, indicating that miR-7 may serve as a biomarker for predicting the response of ESCC patient to CRT. Fig. 2 presents the relative expression of serum miR-7 between two different groups.

The serum miR-7 level of healthy volunteers was 13.41±1.56 (95% CI). As shown by the small range, compared with ESCC patients, the serum miR-7 level in healthy subjects is 3.35 times higher than that in the radiosensitive group (P<0.01) and 7.84 times higher than that in the radioresistant group (P<0.01). The comparison between the healthy subjects and ESCC patients illustrates the possibility that serum miR-7 may also hold promise as a valuable diagnostic marker. Furthermore, the ROC curve was plotted to identify a cut-off value that could distinguish ESCC from healthy control. ROC curve analysis showed that at the optimal cut-off, serum miR-7 had a 78.1% sensitivity and a 83.3% specificity in separating ESCC from normal healthy control with an AUC of 0.841 (Fig. 4).

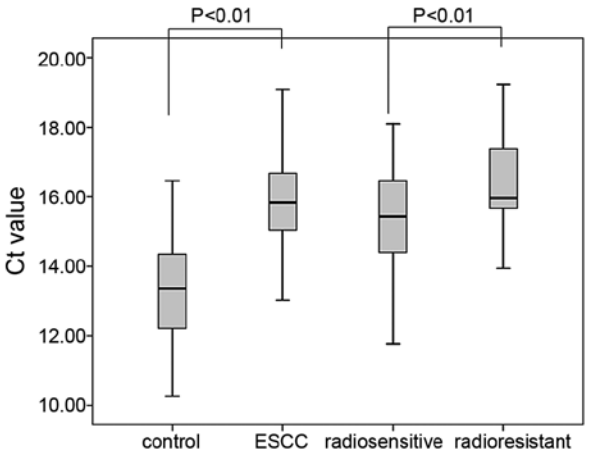

Figure 1. Ct value of serum microRNA-7 detected by real-time PCR.

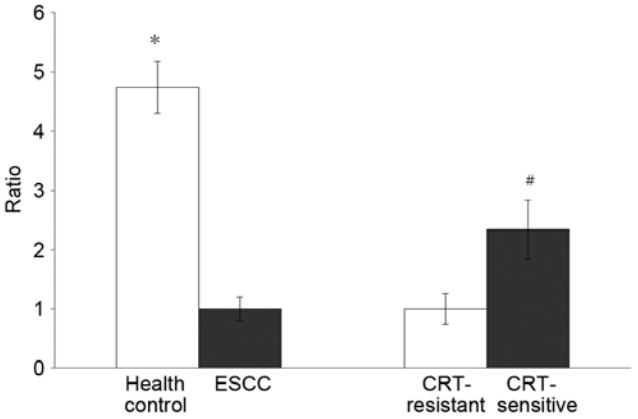

Figure 2. Relative serum microRNA-7 expression: left, the serum miR-7 level in healthy subjects is 4.74 times higher than that in the ESCC patients; right, the serum miR-7 level in CRT-sensitive is 2.34 times higher than that in the CRT-resistant group (*P<0.01; $^{\#}$P<0.05).

Relationship between serum miRNA-7 expression and clinicopathological features. In addition to examining the expression of miRNAs in serum, the relationship between miR-7 expression and clinicopathological features of enrolled ESCC patients was examined. By the median value, we demarcate high and low miR-7 levels. As shown in Table I, there is no correlation between age and gender and relative miR-7 serum levels. The relative miR-7 serum level is significantly correlated with tumor length and the status of lymph node metastasis. The relative miR-7 serum level is significantly lower in patients with longer tumor compared with patients with shorter ones (P<0.05) and in patients with lymph node involvement compared with patients without lymph node involvement (P<0.05). Furthermore, the relationship between the Ct value of miR-7 and tumor length was examined by calculating Pearson's correlation coefficient. Our results showed a correlation (r=0.489; P<0.01) between the serum miR-7 levels and the tumor length in ESCC patients (Fig. 3).

Moreover, Table I also presents the relationships between effectiveness of CRT and clinicopathological factors. As is shown in the table, the responsiveness of therapy is significantly correlated with carcinoembryonic antigen (CEA) (P<0.05),

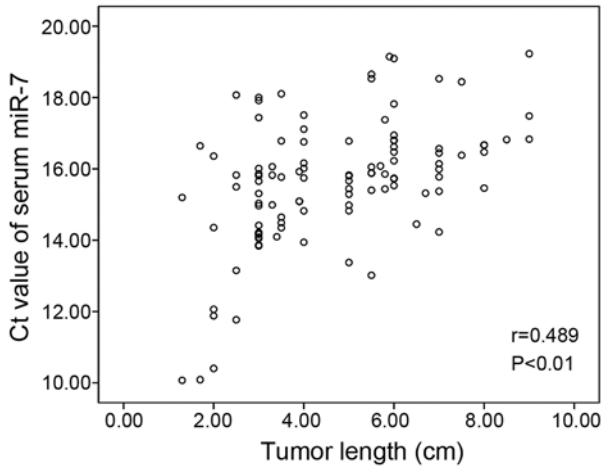

Figure 3. Relationship between levels of serum miR-7 and tumor length.

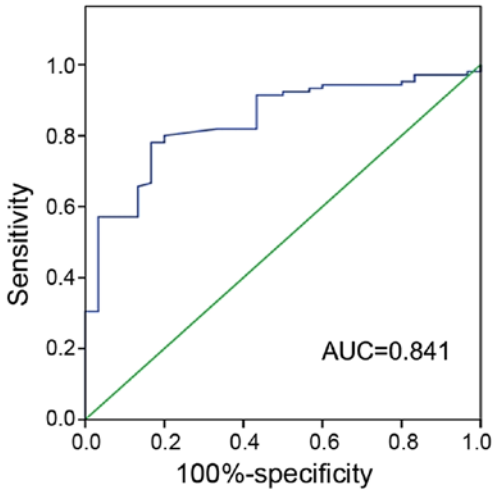

Figure 4. The ROC curve for miR-7. ROC analysis was performed to determine the sensitivity and specificity with the value of AUC.

Cyfra21-1 (P<0.05), serum miR-7 level (P<0.05) and the myelosuppression (P<0.01). Furthermore, by logistic regression analysis, the CR+PR rates of CRT were significantly associated with the levels of Cyfra21-1 [P=0.038; overall remission (OR)=0.296; 95% CI for OR=0.094-0.934], CEA (P=0.022; OR=0.276; 95% CI for OR=0.091-0.833), miR-7 (P=0.019; OR=0.265; 95% CI for OR=0.087-0.800), and myelosuppression (P=0.000, OR=3.347, 95% CI for OR=1.737-6.449) before treatments shown in Table II. Thus, ESCC patients with lower Cyfra21-1 and CEA, higher miR-7 and severe myelosuppression were much more sensitive to CRT.

Downregulation of EGFR by miR-7. In previous studies, EGFR has been identified as an important downstream factor of miR-7 (22,28,30), therefore, we focused on EGFR for further functional analyses. Compared with control subjects without any treatment, transfection of miR-7 into ECA-109 cells suppressed EGFR protein expression (Fig. 5A). However, no significant change of EGFR mRNA level was found after transfection of miR-7 mimic (Fig. 5B), suggesting that miR-7 can interfere with EGFR mRNA translation, but not degradation.

Discussion

Chemoradiotherapy (CRT) play a very important role in the treatment of esophageal cancer, however, it remains unclear on the prediction of treatment responsiveness to CRT. It is important to identify robust factors that will predict response to CRT, and will also facilitate appropriate patient selection and avoid unnecessary delays in patients at high risk of locoregional recurrence upon chemoradiation (4). Wieder *et al* (34) and Suzuki *et al* (35) have given some clues from uptake value and changes in metabolic activity in PET/CT that indicating tumor response and patient survival.

Recently, numerous studies focused on the factors that affect tumor responses to CRT, such as the presence of tumor hypoxia (36), tumor microenvironment (37), DNA damage repair (38), cell cycle checkpoint, apoptosis (39) and radio-related signal transduction pathways (40). Understanding the regulatory mechanisms of miRNA in tumor radiosensitivity from these diverse aspects has also become an intense area of interest. Although there has been great progress in the diagnosis, prediction of response to treatment and prognosis of ESCC in the past decades, identifying new biomarkers may greatly benefit the early detection screening methods.

Great advances concerning miRNA-based therapeutics have been made in various human diseases, including cancer. Recent studies have verified that dysregulation of miRNA expression in human diseases may act as diagnostic, and prognostic factors as well as predicting response to chemotherapy or/and radiotherapy biomarkers. miR-7 has previously been characterized as a tumor-suppressor miRNA in several human cancers by targeting a number of key signaling molecules (22,25,41). The present study showed that serum miR-7 levels were downregulated (4.74-fold-change) in patients with ESCC compared with healthy controls, indicating that it may be a useful biomarker for early diagnosis. However, the downregulation is not unique to ESCC. For example, the level of serum miR-7 is decreased in glioblastoma, and increased miR-7 inhibits glioma cell proliferation by inhibiting the EGFR and AKT pathways (24). Downregulation of miR-7 in breast cancer has also been proven in the study by Kong *et al* (42), who demonstrated the inhibition of epithelial-to-mesenchymal transition and metastasis of breast cancer cells via targeting FAK expression. Previously studies have demonstrated that miR-7 is also downregulated in advanced tongue squamous cell carcinoma cell lines (43,44). Therefore, our current results are consistent with previous findings.

The present study shows that the presence of ESCC is associated with a suppressed level of serum miR-7, and the degree of suppression is correlated with tumor length and lymph node status. An interesting feature of our study is the existence of a statistically significant correlation between lower serum miR-7 expression in ESCC and longer tumor, as well as positive lymph node metastasis, which are the main prognostic factors for ESCC. ROC curve analysis showed that at the optimal cut-off, serum miR-7 had a 78.1% sensitivity and a 83.3% specificity in separating ESCC from normal healthy control with an AUC of 0.841 (Fig. 3). Such findings imply that miR-7 may be involved in the initiation and progression of cancer.

Table I. Relationships between effectiveness of chemoradiotherapy (CRT) and clinicopathological factors as well as serum levels of tumor markers.

	MicroRNA-7				Effectiveness			
Elements	High expression	Low expression	χ^2	P-value	CR+PR	SD+PD	χ^2	P-value
Gender			0.116	0.733			0.012	0.914
Male	35	34			41	28		
Female	17	19			21	15		
Age (years)			0.97	0.325			1.155	0.282
≥60	38	34			40	32		
<60	14	19			22	11		
Tumor location			0.457	0.796			3.545	0.17
Upper third	16	14			22	8		
Middle third	28	32			32	28		
Lower third	8	7			8	7		
Length			7.505	0.023			1.662	0.436
≤4.0	33	20			34	19		
4.1-6.0	13	19			16	16		
>6.0	6	14			12	8		
Tumor differentiation			1.157	0.561			1.557	0.459
High	13	11			13	11		
Moderate	28	26			35	19		
Poor	11	16			14	13		
Lymph node metastasis			4.197	0.04			0.264	0.607
Positive	21	32			30	23		
Negative	31	21			32	20		
Distant metastasis			0.03	0.861			0.012	0.912
Positive	12	13			15	10		
Negative	40	40			47	33		
Smoking history			0.234	0.628			0.367	0.545
Positive	26	24			28	22		
Negative	26	29			34	21		
Drinking history			0.106	0.745			0.024	0.876
Positive	19	21			24	16		
Negative	33	32			38	27		
Family history			0.003	0.958			0.007	0.935
Positive	12	12			14	10		
Negative	40	41			48	33		
CEA			0.497	0.481			5.237	0.021
≤3.3	35	39			49	25		
>3.3	17	14			13	18		
Cyfra21-1			0.293	0.589			5.901	0.015
≤3.4	35	33			46	22		
>3.4	17	20			16	21		
Myelosuppression			4.024	0.403			25.076	0.000
0	17	13			7	23		
I	20	20			26	14		
II	10	15			21	4		
III	3	5			6	2		
IV	2	0			2	0		
miR-7 level							4.418	0.036
Higher					36	16		
Lower					26	27		

CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease; CEA, carcinoembryonic antigen.

Table II. Multivariate analysis of the clinicopathological factors related to responsiveness of therapy.

Variables	B	SE	Wals	P-value	OR	95% CI for OR	
						Lower	Upper
Gender	-0.105	0.743	0.020	0.888	0.901	0.210	3.862
Age (years)	0.596	0.602	0.981	0.322	1.815	0.558	5.909
Lymph node metastasis	-0.098	0.571	0.029	0.864	0.907	0.296	2.778
Distant metastasis	0.534	0.685	0.608	0.435	1.705	0.446	6.525
Tumor location	-0.304	0.419	0.526	0.468	0.738	0.324	1.679
Length	-0.186	0.350	0.283	0.595	0.830	0.418	1.648
Tumor differentiation	0.284	0.370	0.591	0.442	1.329	0.644	2.744
Smoking history	0.390	0.748	0.272	0.602	1.477	0.341	6.397
Drinking history	-0.245	0.686	0.127	0.721	0.783	0.204	3.003
Family history	-0.114	0.612	0.035	0.852	0.892	0.269	2.961
Myelosuppression	1.208	0.335	13.037	0.000	3.347	1.737	6.449
miR-7 level	-1.330	0.565	5.541	0.019	0.265	0.087	0.800
CEA	-1.287	0.564	5.214	0.022	0.276	0.091	0.833
Cyfra21-1	-1.216	0.585	4.314	0.038	0.296	0.094	0.934
Constant	4.092	2.421	2.858	0.091	59.877		

CEA, carcinoembryonic antigen; CI, confidence interval; OR, overall remission.

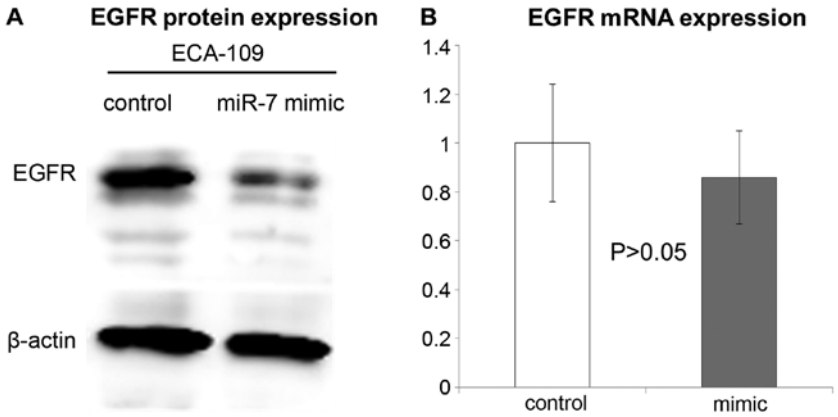

Figure 5. EGFR is a target of miR-7. miR-7 mimic transfection alters EGFR protein (A) expression but not mRNA levels (B) in ECA-109 cells.

Furthermore, the expression level analysis revealed that serum miR-7 is a valuable biomarker for differentiating the responsiveness to CRT of ESCC patients. As shown in Table II, the responsiveness to CRT is significantly associated with the levels of Cyfra21-1, CEA, miR-7 and myelosuppression before treatments. *i.e*. ESCC patients with lower Cyfra21-1 and CEA, higher miR-7 and severe myelosuppression were much more sensitive to CRT, which is similar to previous studies (45-47). However, this conclusion should be confirmed in a study with larger and more homogeneous samples.

The higher expression of miR-7 in ESCC may be more sensitive to CRT, which can be explained with activated EGFR and AKT associated pathways (24,30). Recent studies have reported that EGFR can modulate DNA repair (48-50), consequently influence the responsiveness to CRT. While the present study showed that miR-7 has the ability to down-regulate the expression of EGFR, which is similar to other studies (24,30,51). These results suggested that serum miR-7 may serve as a biomarker for the noninvasive diagnostic and predictive marker for ESCC.

In conclusion, the present study showed that serum miR-7 levels were significantly suppressed in patients with ESCC compared with control subjects, and the degree of suppression is correlated with longer tumor and positive lymph node metastasis. ESCC patients with lower Cyfra21-1 and CEA, higher miR-7, and severe myelosuppression were much more sensitive to CRT. These findings indicate that serum miR-7 may serve as a novel diagnostic and response predictive marker for ESCC patients, and it can also play a potential role in selecting CRT. Furthermore, we demonstrated that miR-7 mediated its function by repressing EGFR, which could be a novel mechanism of the ESCC patients responsiveness to CRT, suggesting that

miR-7 possibly could be employed as an effective therapeutic target for ESCC.

Acknowledgements

We are grateful to Professor Cedric X. Yu (Department of Radiation Oncology, University of Maryland School of Medicine, Baltimore, MD, USA) for reviewing the manuscript.

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