RESEARCH COMMUNICATION

Serum miR-21 Expression in Human Esophageal Squamous Cell Carcinomas

Er-Hui Cai[1&], Yong-Xin Gao[1&], Zhong-zhi Wei[1&], Wei-Ying Chen[2], Ping Yu[3], Ke Li[1*]

Abstract

To investigate the relationship between serum miRNA-21 (miR-21) expression in esophageal squamous cell carcinomas (ESCCs) and its clinicopathologic features, a 1:1 matched case-control study including 21 patients with ESCC and 21 age- and gender-matched healthy controls was carried out. Serum specimens were taken from all subjects. Total RNA was extracted and the stem-loop real time polymerase chain reaction was used to measure serum miR-21 in both groups. Clinical parameters were assessed to determine associations with serum miR-21 concentrations. Serum miR-21 expression in ESCC samples was significantly higher than in paired cancer-free samples ($P < 0.05$). Metastasis was associated with mir-21 expression in serum ($P < 0.05$), ESCC patients with metastasis having 8.4-fold higher serum miR-21 concentrations than healthy controls. There were no statistically significant associations between miR-21 expression and clinicopathologic parameters, such as gender ($P > 0.05$), age ($P > 0.05$), tumor location ($P > 0.05$), cell differentiation ($P > 0.05$), TNM staging ($P > 0.05$), whether chemo/radiotherapy had been administered ($P > 0.05$), or whether surgery had been performed ($P > 0.05$). These findings suggest that the detection of microRNA-21 in serum might serve as a new tumor biomarker in diagnosis and assessment of prognosis of ESCCs.

Keywords: miRNA-21-serum - esophageal carcinoma - case-control study - clinicopathologic features

Asian Pacific J Cancer Prev, **13**, 1563-1567

Introduction

Esophageal carcinoma is the seventh most common malignant tumor in the world, accounting for 410,000 new cases annually (WHO, 2003), the mortality of which occupies the sixth position among the malignant tumors worldwide (Kamangar et al., 2006). During the early stages (I, II) when the tumor is still small, cancer of the esophagus is usually asymptomatic. In the large majority of cases, dysphagia is the presenting symptom. Unfortunately, this occurs when significant obstruction is caused by a large tumor, and the prognosis is very likely to be poor. Although tumor markers greatly improve diagnosis, the invasive, unpleasant, and inconvenient nature of current diagnostic procedures limits their application. Hence, there is a great need for identification of novel non-invasive biomarkers for early tumor detection (Zhou and Wang, 2010).

miRNAs are a class of naturally occurring small non-coding RNAs that post-transcriptionally control gene expression by binding to specific sites at the 3'-UTR of target-mRNAs, resulting in mRNA cleavage and/or translation repression (Ambros, 2003). Mature miRNAs are the results of sequential processing of primary transcripts (pri-miRNAs) mediated by two RNase III enzymes, Drosha and Dicer (Cullen, 2004). Mature 18- to 24-nt-long miRNAs negatively regulate protein expression of specific target mRNA (Bartel, 2004). Early studies showed that miRNAs could regulate cellular differentiation, proliferation, and apoptosis, processes that are important in cancer aggravation. Recent bioinformatics data indicate that miRNAs are involved in almost all basic signaling pathways, including the expression of a series of important tumor-related genes (Alvarez-Garcia and Miska, 2005). Several studies reveal that altered miRNA expression has been reported in various cancers, and the profiles of tissue miRNAs exhibit great potential for an application in cancer definition (Esquela-Kerscher and Slack, 2006; Calin and Croce, 2006). Thus, these dysregulated miRNAs are often referred to as oncogenic microRNAs (oncomiRs).

One such oncomiR, miR-21, has been shown to be over-expressed in multiple malignancies including pancreatic cancer (Bloomston et al., 2007), esophageal cancer (Feber et al., 2008), and colon cancer (Schetter et al., 2008). This miRNA has been linked to tumor aggression and carcinogenesis, in part, by preventing apoptosis and, thus, functioning as an oncogene (Chan et al., 2005). Previous studies have primarily used real time polymerase chain reaction (RT-PCR) or miRNA microarray technology to evaluate miRNA expression in tissues (Jiang et al., 2005; Calin and Croce, 2006). Because serum and plasma are easily accessible, circulating biomarkers are one of the most promising means of diagnosis. Related studies have shown that human serum contains miRNAs and the

*[1]Department of Preventive Medicine, [2]Center for Teaching Laboratories, [3]Department of Computers, Shantou University Medical College, Shantou, China [&]Equal contributors *For correspondence: kli@stu.edu.cn*

expression pattern of these serum miRNAs can potentially be used to identify various types of cancer (Chen et al., 2008; Gilad et al., 2008; Mitchell et al., 2008; Ng et al., 2009; Resnick et al., 2009). Given that endogenous miRNAs exist in a form that is resistant to RNase activity (Patrick et al., 2008), identifying a unique serum miRNA expression in esophageal cancer can potentially assist tumor diagnosis and cancer treatment.

In this study, miR-21 was chosen to ascertain whether its serum expression can distinguish esophageal cancer from cancer-free controls. We conducted serum miR-21 expression by a stem-loop qRT-PCR assay. The relationship between miR-21 and ESCC was investigated by a case-control study.

Materials and Methods

Study designs and participants

A case-control study was carried out in the First Affiliated Hospital of Shantou University Medical College (FAHSTUMC) in Shantou of Guangdong during Oct 2009 to Jan 2011. The study included 21 patients aged 45-80 years who were diagnosed with ESCC and healthy controls. Control subjects were recruited from individuals who sought a routine health check-up at the Healthy Physical Examination Centre of the FAHSTUMC. Healthy controls were matched to cases by age (± 5 years) and gender. Ratio of cases to controls was 1:1.

Clinicopathologic information was extracted from clinical data of ESCC patients (Table 1). The histopathology was confirmed by two pathologists and tumor stage was determined based on PTNM staging and cellular differentiation. For patients who were unsuitable for surgical treatment, histopathology and tumor stage were confirmed by histobiopsy and imaging technology. Tumors were staged according to the tumor-node-metastasis staging system of the International Union against Cancer. Histological grade was assessed according to World Health Organization (WHO) criteria.

Blood samples from ESCC patients and healthy controls were obtained with informed consent and agreement, according to protocols approved by the Ethics Committee of Shantou University Medical College. All specimens were transported at 4 °C and stored at -80 °C until RNA extraction. Serum miR-21 concentrations were detected by using the stem-loop qRT-PCR.

Primers design

To investigate miR-21 expression between patients and controls by real-time RT-PCR, gene-specific primers for human miR-21 and U6 were selected from the Sanger Center miRNA Registry at http://www.sanger.ac.uk/Softwar/Rfam/mima.index.shtml.

miR-21 specific-primer: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA-3', PCR forward primer: 5'-CGGCGGTAGCTTATCAGACTGA-3', reverse primer: 5'-GTGCAGGGTCCGAGGT-3; U6 control gene primer: 5'-GCTTCGGCAGCACATATACTAAAAT-3', PCR forward primer: 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse primer: 5'- GTGCAGGGTCCGAGGT-3'. All primers were synthesized by Sangon Biotech (Shanghai, China).

RNA Extraction

Blood samples (~5 ml) were drawn and sera were centrifuged and prepared to collect the supernatant. Total RNA was isolated using a derivation of the single step Trizol method. The samples were mixed with an equal volume of Trizol reagent (Invitrogen, USA), then mixed thoroughly by inversion and incubated for 5 min at room temperature. Subsequently, 0.2 ml of chloroform was added, samples were mixed vigorously and centrifuged at 12,000 × g for 15 min at 4 °C. Then 500 µl RNA phase was precipitated from the aqueous phase by mixing with 0.5 ml of isopropanol. The samples were incubated at room temperature for 10 min and centrifuged at 12,000 × g at 4 °C. The supernate was removed and the RNA pellet was washed once with 75% ethanol. Then, the RNA pellet was air dried and dissolved in 10 μl DEPC-treated water, then incubated for 10 to 15 min at 55 °C to 60 °C. Finally, RNA concentrations and purify were quantified by a spectrophotometer (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA, USA). RNA integrity was analyzed by 2% agarose gel electrophoresis.

Reverse Transcription

The cDNA was reverse transcribed from total RNA using specific miRNA primer and M-MLV First-Strand Synthesis System for qRT-PCR reagents (Invitrogen, USA). Briefly, 3 µl of total RNA template in 5 µl of sterile distilled water plus 1 µl of primer, and 1 µl of dNTP mix were incubated in a PTC-100 TM Programmable Thermal Controller (MJ Reserch, Inc, USA) at 65°C for 5 min to denature the RNA. After placing on ice for at least 1 min, 4 µl of 5 × first-strand buffer was added, plus 2 µl of 0.1M DTT, 1 µl Rnase inhibitor, 1 µl M-MLV reverse transcriptase, and 2 µl DEPC-treated water were added to form a 10 µl cDNA synthesis mix Reaction mixtures were incubated at 16 °C for 30 min, at 42 °C for 30 min, at 85 °C for 5 min and then held at 4 °C. The diluted cDNA templates were either stored at -20 °C or used to proceed with quantitative polymerase chain reactions.

Real-time RT-PCR using SYBR Green

The Two-Step M-MLV Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, USA) was used for real time PCR. The 20 µl reaction mixtures containing 10 µl SYBR-Green qPCR SuperMix-UDG mixed with 0.4 µl Rox, 0.4 µl 1 µM forward and reverse primers, respectively, 2 µl cDNA and 6.8 µl DEPC-water. The SYBR Green PCR was performed on an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems, USA) under the following PCR conditions: an initial cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. In PCR strip tubes, each reaction was performed in triplicate along with the endogenous U6 control gene. At the end of the real-time PCR cycles, a dissociation curve was performed to check for the presence of non-specific dsDNA SYBR Green hybrids, such as primer-dimers. Data analysis was performed using ABI Prism 7300 SDS

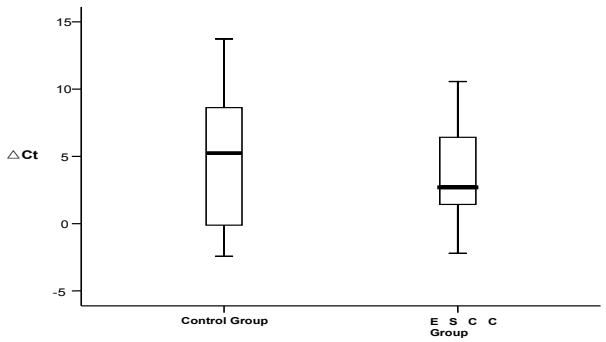

Figure 1. The ΔCt Values of miR-21 in Human Esophageal Squamous Cell Carcinoma Serum and Healthy Control Serum

Software V1.3.1 (Applied Biosystems, USA). The relative expression level was calculated using the ΔCt method and the equation $2^{-\Delta Ct}$, where ΔCt = Ct (miRNA) - Ct (U6). A lower ΔCt value indicates a higher miRNA expression in ESCC patient serum compared to healthy control serum.

Statistical Analysis

Statistical analyses were performed with SPSS 13.0 software (SPSS, Inc., Chicago, IL). Since the distributions were skewed, data obtained from qRT-PCR were expressed as median ± standard deviation (SD). The Wilcoxon signed rank test was used to compare the serum miR-21 levels in both case and control groups. The Mann-Whitney test and Kruskal-Wallis test were used to compare the differences among clinicopathologic parameters. Two-tailed P-value of <0.05 was considered statistically significant.

Results

Expression of miRNAs and correlation with clinicopathologic features in esophageal cancer

Twenty one ESCC patients and age, sex-matched controls were included in the study. The median age of the patients was 53 years, and the range was between 45 and 80 years. Of the twenty-one patients, four were female, the others were male. The details regarding clinicopathologic features only included patients (Six of the patients came for follow-up treatment. Pathological features could not be obtained in some of the cases). Most patients had a tumor in the middle third of the esophagus (14/21, 66.7%). A well differentiated tumor was seen in one patient, moderate and poor differentiated tumors comprised 42.8% (n = 9) and 28.6% (n = 6) of the ESCC patient population, respectively. Of the patients who had TNM staging information (n = 12), two (2/12, 12.5%) had stage I disease, five (5/12, 41.7%) had stage II, and five (5/12, 41.7%) had stage III. Eight out of twenty one had metastasis presence. Fifteen patients had received chemo/radiotherapy, and twelve patients had an esophagus dissection within a month.

We initially compared miR-21 concentrations in serum from ESCC patients and healthy volunteers. The Ct values of miR-21 in ESCC and control group ranged from 26.2 to 36.8 and from 26.0 to 37.2 respectively. The Ct values of U6 in ESCC and control group spanned from 20.4 to 33.8 and from 20.5 to 36.3, respectively. Mean Ct values in case and control group were 31.6 ± 3.1 and 33.1 ± 3.7, respectively. Figure 1 showed the ΔCt values obtained from both groups. miR-21 concentrations in serum were elevated in ESCC group compared to control group (P < 0.05). Serum miR-21 expression of ESCC patients was 2.9 times higher than that of control group (Fold=$2^{-\text{Average }\Delta Ct\text{ (ESCC)}}/2^{-\text{Average }\Delta Ct\text{ (control)}}$).

Table 1. Summary of Clinicopathologic Features of the ESCC Patients

Variables		"miRNA-21expression Median ± SD"	P Value
Gender			0.885
Male	17	2.83±3.91	
Female	4	0.97±4.11	
Age			0.107
≥55	13	2.70±3.51	
<55	8	2.50±6.97	
Tumor location			0.941
Middle	14	1.12±4.70	
Lower	7	1.99±2.85	
Differentiation			0.484
Well	1	6.76	
Moderate	9	2.17±3.14	
Poor	6	2.35±4.34	
Missing	5	7.66±6.08	
TNM stage			0.958
I	2	3.62±3.22	
II	5	2.83±4.24	
III	5	2.17±5.00	
Missing	9	2.70±5.31	
Metastasis			0.041[a]
Presence	8	1.71±0.61	
Absence	13	5.79±4.57	
Chemo/Radiotherapy			0.095
After	15	1.99±4.02	
Before	6	5.81±5.26	
Surgery			0.218
After	13	2.17±3.70	
Before	8	2.70±4.92	

[a]P<0.05

For clinicopathologic factors, the expression did not seem to differ substantially between younger and older groups (>55y vs. ≤55y), male and female, and different tumor locations, extents of differentiation, as well as pathologic stages, whether the patient received chemo/radiotherapy, or whether surgery was performed within a month. However, a higher miR-21 expression was associated with metastasis (P <0.05). Patients with metastasis had 8.4 times higher expressions than those who did not have metastasis. Table 1 below shows the median levels of miR-21 expression in various groups of patients classified by different clinical and pathological features.

Discussion

Seeking non-invasive tumor markers for diagnosis is currently one of the most rapidly developing areas in cancer research (Duffy, 2007). In the best currently available blood tests, carcinoembryonic antigen exhibits low sensitivity and specificity, particularly in the context of early disease. Comprehensive proteomic analysis has introduced a group of differentially expressed proteins as

disease indicators and significantly increased the accuracy of diagnosis (Leman et al., 2007), but this protein assay procedure is not easy to apply in clinical diagnosis. Surprisingly, high concentrations of miRNAs exist in highly stable, cell-free form in peripheral blood (Mitchell et al., 2008).

The presence of miRNAs suggests that although serum contains ribonuclease and there should be no intact RNA, they are considerably resistant to RNase digestion. Also, serum treated under harsh conditions, such as boiling, low/high pH, extented storage, freeze-thaw cycles, yielded no significant differences compared to non-treated serum, thereby greatly facilitating clinical use of such tests (Chen et al., 2008).

In our study, miR-21 expression in the sera of ESCC patients was up-regulated remarkably. Zhang has confirmed that miR-21 was significantly increased in ESCC patients compared with healthy controls (Zhang et al., 2010). As one of the most prominent biomarkers, miR-21 was implicated in genesis and progression of human cancer. Our experiment also verified that patients with metastasis had higher miR-21 levels in serum, which is consistent with existing evidences. But the statistics showed that miR-21 concentration in serum had no relationships with gender, age, tumor locations and other clinicopathologic factors, which is consistent with existing reports (Dong et al., 2011). These indicate that further work is needed to clarify these relationships, because even in different individuals of the same race, gene expressions may vary widely (Matsuo et al., 2000).

To date, several direct targets of miR-21 have been identitied, with all of them being tumor suppressors: the PTEN phosphatease, the actinbinding protein tropomyosin I, gene ANP32A and SMARCA4. Knock down of miR-21 was shown to cause increased apoptosis and reduced invasiveness in glioblastoma, reduced cell proliferation in breast and cervix cancer, as well as decreased cellular invasion and metastasis in colorectal cancer. Conversely, miR-21 overexpression resulted in increased tumor cell proliferation, migration and invasion in hepatocellular carcinoma. These indicate that miR-21 acts as key oncomiR by favouring neoplastic transformation on multiple levels and the network of miR-21-induced gene expression changes needs to be explored (Schramedei et al., 2011).

Circulating miRNAs are packed in complexes, called exosomes or microvesicles, and are released by normal and tumor cells through unknown mechanisms. Emerging evidence has indicated that such external miRNAs are also involved in cell-to-cell signal transduction and genetic information exchange (Kosaka et al., 2010). Aberrantly expressed miRNAs vary among different tumor types and some of them are secreted into blood (Mitchell et al., 2008).

miR-21 is one of the most prominent miRNAs implicated in the genesis and progression of human cancer. Current methods for detection and qualification of miRNAs are largely based on cloning, northern blotting, primer extension, or micro arrays. However, they are relatively limited in terms of sensitivity and specificity. Low sensitivity is an issue for miRNA quantification due to the difficulty in amplifying these short RNA targets. On the other hand, low specificity may lead to false positive signals from closely related miRNAs, precursors and genomic sequences. Consequently, real-time PCR, which has been designed as a conventional PCR assay from miRNAs averaging ~22 nt in length, turns out to be a better choice (Chen et al., 2005).

Blood samples were used to conduct the experiments. At present, gastrointestinal endoscopy remains the primary screening tool by which the suspected lesions can be biopsied for histopathological analysis. This invasive test, even though it has been proven to increase early detection of tumors and therefore can prolong the survival of the patient, is generally considered to be inconvenient and painful. Because of its limitations, research to facilitate discovery of novel predictive markers for ESCC is needed (Schramedei et al., 2011). The discovery of miRNAs, has shown their great potential regulating roles in many aspects of biological and pathological processes. miR-21 has been shown to be over expressed in many different solid tumors, including breast, colon, lung, pancreas, prostate, and stomach, as well as in cholangio carcinoma cell lines. Thus, it is highly likely that miR-21 plays a fundamental role in tumor cell behavior and malignant transformation (Zhou and Wang, 2010) and our findings may potentially have relevance to other tumors in which miR-21 is over-expressed.

In conclusion, stable and detectable miRNAs in serum of patients with esophageal cancer have been reported in this study. miR-21 could be a useful biomarker for the progression of esophageal carcinoma. If validated in a large-scale case-control study, this test may be useful in future as a non-invasive screening test for esophageal cancer.

Acknowledgements

This study was supported by the Science and Technology Project Foundation of Guangdong Province, the Guangdong Innovative Experimental Project of Higher education Students, and Outstanding Students Research Foundation of Shantou University Medical College 2010.

References

Alvarez-Garcia I, Miska EA (2005). MicroRNA functions in animal development and human disease. *Cell*, **132**, 4653-62.

Ambros V (2003). MicroRNA pathways in flies and worms: Growth, death, fat, stress and timing. *Cell*, **113**, 673-6.

Bartel DP (2004). MicroRNAs: Genomics, biogenesis, mechanism and function. *Cell*, **116**, 281-97.

Bloomston M, Frankel WL, Petrocca F, et al (2007). MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA*, **297**, 1901-8.

Calin GA, Croce CM (2006). MicroRNA signatures in human cancers. *Nat Rev Cancer*, **6**, 857-66.

Calin GA, Croce CM (2006). MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res*, **66**, 7390-4.

Chan JA, Krichevsky AM, Kosik KS (2005). MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res*, **65**, 6029-33.

Chen C, Ridzon DA, Broomer AJ, et al (2005). Real-time quantification of microRNAs by stem–loop RT–PCR. *Nucleic Acids Res*, **33**, e179.

Chen X, Ba Y, Ma L, et al (2008). Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res,* **18**, 997-1006.

Cullen BR (2004). Transcription and processing of human microRNA procursors. *Mol Cell*, **16**, 861-5.

Dong Y, Wu WK, Wu CW, et al (2011). MicroRNA dysregulation in colorectal cancer: a clinical perspective. *Br J Cancer*, **104**, 893-8.

Duffy MJ (2007). Role of tumor markers in patients with solid cancers: a critical review. *Eur J Intern Med*, **18**, 175-84.

Esquela-Kerscher A, Slack FJ (2006). Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer*, **6**, 259-69.

Feber A, Xi L, Luketich JD, et al (2008). MicroRNA expression profiles of esophageal cancer. *J Thorac Cardiovasc Surg*, **135**, 255-60.

Gilad S, Meiri E, Yogev Y, et al (2008). Serum microRNAs are promising novel biomarkers. *PLoS One*, **3**, e3148.

Jiang J, Lee EJ, Gusev Y, et al (2005). Real-time expression profiling of microRNA precursors in human cancer cell lines. *Nucleic Acids Res*, **33**, 5394-403.

Kamangar F, Dores GM, Anderson WF (2006). Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *J Clin Oncol*, **24**, 2137-50.

Kosaka N, Iguchi H, Ochiya T (2010). Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci*, **101**, 2087-92.

Leman ES, Schoen RE, Weissfeld JL, et al (2007). Initial analyses of colon cancer-specific antigen (CCSA)-3 and CCSA-4 as colorectal cancer-associated serum markers. *Cancer Res*, **67**, 5600-5.

Matsuo K, Lin A, Procter JL, et al (2000). Variations in the expression of Granulocyte antigen NB1. *Transfusion*, **40**, 654-62.

Mitchell PS, Parkin RK, Kroh EM, et al (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*, **105**, 10513-8.

Ng EK, Chong WW, Jin H, et al (2009). Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut*, **58**, 1375-81.

Patrick S. Mitchell, Rachael K. et al (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*, **105**, 10513-8.

Resnick KE, Alder H, Hagan JP, et al (2009). The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. *Gynecol Oncol*, **112**, 55-9.

Schetter A, Leung SY, Sohn JJ, et al (2008). MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA*, **299**, 425-36.

Schramedei K, Morbt N, Pfeiferl G, et al (2011). MicroRNA-21 targets tumor suppressor genes ANP32A and SMARCA4. *Oncogene*, **30**, 2975-85.

WHO (2003). Global cancer rates could increase by 50% to 15 million by 2020. http://www.who.int/mediacentre/news/releases/2003/pr27/en/.

Zhang C, Wang C, Chen X, et al (2010). Expression Profile of MicroRNAs in Serum: A Fingerprint for Esophageal Squamous Cell Carcinoma. *Clin Chem*, **56**, 1871-9.

Zhou SL, Wang LD (2010). Circulating microRNAs: Novel biomarkers for esophageal cancer. *World J Gastroenterol*, **16**, 2348-54