RESEARCH ARTICLE

Saliva Supernatant miR-21: a Novel Potential Biomarker for Esophageal Cancer Detection

Zi-Jun Xie[1,2&], Gang Chen[3&], Xu-Chao Zhang[4], Dong-Feng Li[5], Jian Huang[6], Zi-Jun Li[1*]

Abstract

Objective: To identify whether saliva supernatant miR-21 can serve as a novel potential biomarker in patients with esophageal cancer (EC). Methods: 32 patients with EC and 16 healthy controls were recruited in this study. Total RNA was extracted from saliva supernatant samples for measurement of miR-21 levels using RT-qPCR and relationships between miR-21 levels and clinical characteristics of EC patients were analyzed. Results: miR-21 was significantly higher in the EC than control groups. The sensitivity and specificity were 84.4% and 62.5% respectively. Supernatant miR-21 levels showed no significant correlation with cancer stage, differentiation and nodal metastasis. Conclusions: Saliva supernatant miR-21 may be a novel biomarker for EC.

Keywords: Esophageal cancer - microRNA - diagnosis - biomarker

Asian Pacific J Cancer Prev, **13 (12)**, 6145-6149

Introduction

Several studies have demonstrated that aberrant expression of miRNAs is closely related to the pathogenesis and development of cancer, and miRNAs possess diagnostic value as cancer biomarkers (Alvarez-Garcia et al., 2005). Several studies have reported that miR-21 is aberrantly expressed in cancer tissue and plasma in patients with EC (Feber et al., 2008; Mishima et al., 2009; Komatsu et al., 2011). However, miRNA expression in the saliva of patients with EC has not yet been reported. Due to the extensive blood supply in salivary glands, saliva is considered to be a terminal product of blood circulation, and molecules that are present in plasma are also present in saliva (Lee et al., 2009). Hence, saliva is believed to mirror systemic health and reflect conditions such as cancers, infectious diseases, cardiovascular diseases, etc (Wong et al., 2006). Tissue, plasma, and saliva miRNAs share similar expression profiles (Taylor et al., 2008; Rabinowits et al., 2009; Park et al., 2009; Weber et al., 2010; Wiklund et al., 2010). Based on the our previous study on salivary miRNAs expression profile of EC, we made further efforts to detect the expression level of saliva supernatant miR-21 from patients with EC by RT-qPCR, and explored its potential diagnostic value in EC.

Materials and Methods

Sample size estimation

Preliminary experiment: We randomly selected 8 saliva supernatant samples from EC patients and 4 saliva supernatant samples from healthy controls, and detected the expression level of miR-21 in the saliva supernatant samples by RT-qPCR. The result showed that the expression level of miR-21 was significantly increased in these samples from EC patients .The diagnostic sensitivity of it for EC was 75%. The formula for sample size estimation was as follows:

$$n= u_{a/2}P(1-P)/\delta^2$$

In this formula, n is the number needed, $u_{\alpha/2}$ is the test level, α is the cutoff value of two-tailed normal distribution, P is the expected value of sensitivity, and δ is the permissible error.

According to the ability to attain the sample size for our study, we chose the values $\alpha = 0.05$ ($u_{\alpha/2} = 1.96$), $\delta = 0.15$, and the values were substituted into the formula. The result was $n = 1.962 \times 0.75 \times (1 - 0.75) / 0.152 = 32$. That is, in this study, the minimum number of cases needed for the EC group was 32, and 32 cases were chosen. According to the ability to attain the sample size for our study, we assumed the ratio of cases in the EC group to that in the healthy group to be 2:1 and 16 cases were chosen.

Subject selection

32 saliva supernatant samples from 32 patients with EC and 16 saliva supernatant samples from 16 age-, gender-, and ethnically-matched healthy individuals were obtained from Guangdong General Hospital between July, 2011 and January, 2012. Patient histopathology results were confirmed by endoscopic biopsy, and the EC patients had

*[1]Department of Gastroenterology, [3]Department of Thoracic Surgery, [4]Guangdong Lung Cancer Institute, [5]Research Center of Medical Sciences, [6]Physical Examination Center of East Ward, Guangdong Academy of Medical Sciences, Guangdong General Hospital, [2]Graduate School, Southern Medical University, Guangzhou, China [&]Equal contributors *For correspondence: zijunli2005@yahoo.com.cn*

no concomitant organic, systemic, or oral diseases, such as hepatitis, diabetes mellitus, etc, that could influence EC marker expression levels. Cancer staging was based on the UICC/TMN staging system. Stages I, II, and III were based on the histopathology results after surgical resection. Stage IV was based on histopathology results of puncture biopsy of metastatic nodes or PET-CT results. "N/A (not available)" was assigned when patients refused further tests or treatments. Healthy subjects were collected as controls based on negative health examination results including blood tests, chest X-rays, oral examinations, abdominal ultrasound examinations, fecal occult-blood testing, and digital rectal examinations. None of these controls had been diagnosed with any type of malignancy either previous to the study or at the time of sample collection. This study was approved by the Institutional Review Board and Ethics Committee at Guangdong General Hospital. All participants were provided written consent for their information to be stored in the hospital database and used for research.

Saliva collection

Subjects were asked to refrain from eating, drinking, smoking, and oral hygiene procedures for at least 2 hours before the collection. To stimulate glandular salivary flow, subjects received a 2% citric acid solution for application to the bilateral posterior lateral surfaces of the tongue with a cotton swab for 5 s every 30 s. The citric acid stimulation continued at 30-second intervals during the entire collection procedure. Up to 3 mL of saliva from each subject was collected in a 50-mL centrifuge tube. The 3 mL of saliva samples was centrifuged at 3,000 × g for 15 min at 4°C to spin down exfoliated cells, and the supernatant was transferred into microcentrifuge tubes followed by a second centrifugation at 12,000 × g for 10 min at 4°C to completely remove cellular components. Samples were stored at -80°C until use. The procedure mentioned above must be finished within 2 h (Michael et al., 2010).

The detection of saliva supernatant miR-21

The expression level of saliva supernatant miR-21 was detected by RT-qPCR using saliva supernatant samples from 32 patients with EC and 16 healthy controls. The mirVana PARIS Kit (Ambion) was used to isolate total RNA from 1 mL of saliva supernatant, according to the manufacturer's protocol. Finally, RNA was eluted in 30 μL of preheated nuclease-free water (95°C) and stored at -80°C until use. The reverse transcription reaction was first carried out with 11 μL of mixture containing 2 μL of RNA extract, 2 μL of RT primer (Ribo, China), and 7 μL of nuclease-free water. The 11-μL mixture was incubated at 70°C for 10 min and in ice for 2 min. Next, 5 μL of RT buffer, 2 μL of dNTP (2.5 mM), 0.5 μL of RNase inhibitor (40 U/μl), 0.5 μL of reverse transcriptase (200 U/μL), and 6 μL of nuclease-free water were added to the 11-μL mixture. The reverse transcription reaction continued at 42°C for 60 min, 70°C for 10 min, and 4°C for ∞. cDNA solution (3 mL) was amplified using 9 mL of SYBR Premix Ex Taq (TaKaRa, China), 2 μL of miRNA forward primer, 2 μL of miRNA backward primer, and 4 μL of nuclease-free water in a final volume of 20 mL. Quantitative PCR was run on a Biorad CFX96 2.1 (Biorad Biosystems), and the reaction mixtures were incubated at 95°C for 2 min, followed by 50 cycles of 95°C for 5 s and 60°C for 10 s. At the end of the PCR cycles, melting curve analysis was performed to validate generation of the expected PCR product. The setting of melting curve was 65.0 to 95.0°C at increments of 0.5°C for 0.05 min + plate read. Each sample was analyzed in triplicate. All Ct values were <36. The expression level of miR-21 was normalized to that of miR-16 (McDonald et al., 2011). All expression levels were calculated utilizing the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001). Briefly: $Sample^{i}\Delta Ct_{miR\text{-}21} = sample^{i}Ct_{miR\text{-}21} - sample^{i}Ct_{miR\text{-}16}$; $sample^{i}\Delta\Delta Ct_{miR\text{-}21} = Sample^{i}\Delta Ct_{miR\text{-}21}$ - the mean value of $\Delta Ct_{miR\text{-}21}$ of the healthy group.

Statistical analysis

Expression level of miR-21 and ages were compared using the Mann–Whitney U test. Genders were compared using the χ^2 test. A multivariate logistic regression model was established for the four risk factors: smoking, alcohol intake, drinking or eating at hot temperatures, and Chaoshanese nationality. The odds ratios (ORs) of the four risk factors were calculated by Forward LR. Each OR was compared using the χ^2 test. Receiver-operating characteristics (ROC) curves were used to evaluate the diagnostic value of each miRNA for differentiation of patients and controls.Statistical analyses were performed with the SPSS software, version 13.0 (SPSS, Inc., Chicago, IL). A p value of <0.05 was considered to indicate statistical significance.

Results

Characteristics of EC patients and healthy controls

In this study, 32 patients with EC were selected, all of whom had squamous cell carcinoma. Cancer staging of the patients with EC was as follows: 0 (0%) were in stage I, 11 (34.4%) in stage II, 9 (28.1%) in stage III, 9 (28.1%) in stage IV, and 3 (9.4%) were unavailable. Data regarding EC patients and healthy controls were presented in Table 1. All patients with EC were >40 years of age, mainly from Chaoshan, Guangdong Province, reported drinking or eating at high temperatures, and alcohol drinkers, and most were male smokers. According to the logistic regression model, the ORs of Chaoshanese individuals and drinking or eating at a high temperature were statistically significant (14.80 and 11.34, respectively).

Relationships between the miR-21 expression levels and the clinical characteristics of EC patients

From Table 2, it can be seen that the expression levels of saliva supernatant miR-21 were not influenced by age, gender, residency, eating habits, smoking status, alcohol consumption, cancer staging, cancer differentiation, or nodal metastasis of patients with EC.

Potential diagnostic value of saliva supernatant miR-21 for EC

The expression levels of saliva supernatant miR-21 were significantly upregulated in EC group (p=0.003). The

Table 1. Characteristics of EC Patients and Healthy Controls

Charateristics	EC (n = 32)	Healthy controls (n = 16)	p value
Gender			0.249
Male	26(81.3%)	13(81.3%)	
Female	6(18.7%)	3(18.7%)	
Age			0.619
Mean ± SD	61.6±9.3	57.5±7.1	
Median (range)	61(43-88)	56(47-73)	
Ethnicity			
Han Chinese	32 (100%)	16 (100%)	
Chaoshanese		OR=14.80, p=0.022	
Yes	20(62.5%)	1(6.2%)	
No	12(37.5%)	15(93.8%)	
Drinking/eating at high temperature		OR=11.34, p=0.041	
Yes	20(62.5%)	1(6.2%)	
No	12(37.5%)	15(93.8%)	
Smoking			
Yes	26(81.3%)	5(31.3%)	
No	6(18.7%)	11(68.7%)	
Alcoholic[1]			
Yes	23(71.9%)	3(18.8%)	
No	9(28.1%)	13(81.2%)	

[1]According to the standard of mainland China, the definition of an alcoholic is as follows: male, ≥40 g/d; female, ≥20 g/d; consumption of alcohol (g) = volume of alcohol (mL) × concentration of alcohol (%) × 0.8

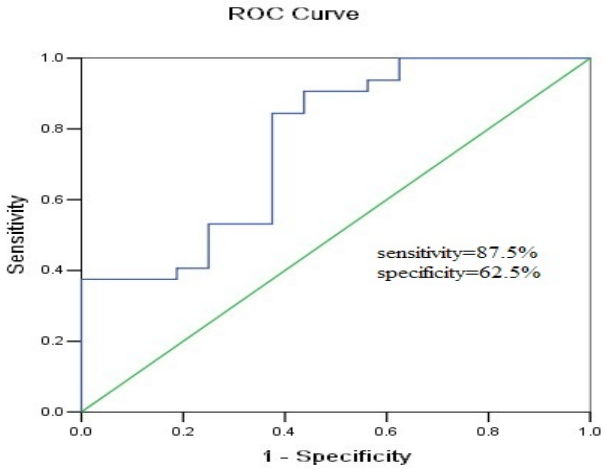

Figure 1. Receiver Operating Characteristic Curve Analysis of Saliva Supernatant miR-21 for Esophageal Cancer Diagnosis

fold-change reached to 14.958 on average. ROC curves were established to evaluate the diagnostic value of saliva supernatant miR-21. The AUC=0.762. An optimum cutoff value is needed for the ROC curve to define the diagnostic value. When the Younden index (Younden index = sensitivity + specificity -1) reaches the maximum value, the corresponding cutoff value will be the optimum cutoff value (Akobeng, 2007). The cutoff value was determined to be 0.8890, Therefore, its sensitivity and specificity for EC detection were 87.5% & 62.5%, respectively (Figure 1).

Discussion

miRNAs fall into two categories: cellular and extracellular. Extracellular miRNAs are present in plasma or other body fluids; they are also called secretory miRNAs. Several studies have found charateristic and stable miRNA profiles in bodily fluids. Extracellular miRNAs may be intercellular signaling molecules and transduct intercellular signals when flowing (Iguchi et al., 2010).

The mechanisms of how miRNA enters body fluids from cells are not clear. Kaosuka et al. (2010) found that miRNA released from cells is regulated by neutral sphingomyelinase 2 (nSMase2). When ceramide increases in cells, nSMase2 promotes miRNA release. Inhibition of nSMase2 by the chemical inhibitor GW4869 impedes miRNA release from cells.

RNase is present in plasma, saliva, urine, and other body fluids. However, several studies have found that miRNAs in body fluids are stable and can resist degradation at high and low temperatures, in strong acids and bases, and by RNase (Gilad et al., 2008; Chen et al., 2008; Li et al., 2010). This is likely because free miRNAs in bodily fluids are wrapped up by proteins or stored within vesicles which help resist the digestion of RNase or the

Table 2. Relationships Between miR-21 Expression Levels (means ± SD) and Clinical Characteristics of EC Patients

Feature	Number	miR-21	p value
Gender			p=0.381
Male	26	98.65±205.08	
Female	6	65.05±149.23	
Age (years)			
≤61	17	35.30±77.40	
>61	15	157.00±261.10	
Chaoshanese			p=0.216
Yes	20	80.75±77.40	
No	12	121.98±164.01	
Drinking or eating at high temperature			p=0.515
Yes	20	122.94±227.57	
No	12	23.64±40.64	
Smoking			p=0.209
Yes	26	107.96±217.77	
No	6	24.67±51.68	
Alcoholic			p=0.285
Yes	23	119.59±222.91	
No	9	22.74±42.55	
Cancer staging			p=0.776
II	11	67.97±118.16	
III	9	107.27±204.71	
IV	9	136.29±283.87	
N/A	3		
Nodal metastasis			p=0.529
No	11	67.97±118.16	
Yes	18	105.11±225.69	
N/A	3		
Differentiation			p=0.196
Moderate	14	37.52±106.14	
Poor	3	210.96±323.78	
Moderate-poor	6	83.35±120.78	
N/A	3		

[2]N/A = not available due to patients' refusal of further tests or treatments; meanwhile, some patients in stage IV could not undergo operations and resected tumors were not available, so the differentiation of the tumors in these patients could not be determined

damages of other factors (Zampetaki et al., 2012).

Saliva is a complex liquid that comprises secretions from the major and minor salivary glands. There are 450 to 750 minor accessory salivary glands situated on the tongue, buccal mucosa, and palate excluding the anterior part of the hard palate and gums (Schipper et al., 2007). There is also an extensive blood supply to these glands; therefore, molecules present in plasma are also present in saliva, such as proteins, DNA, RNA, etc. Several studies have reported that salivary molecules can detect oral and other organic and systemic diseases. Lee et al. (2006) reported that salivary mRNAs of DUSP1, H3F3A, OAZ1, S100P, SAT, IL-8, and IL-1 can detect oral cancer more accuately than can plasmatic mRNAs of these genes. Mbulaiteye et al. (2006) found that salivary tests could replace blood tests to detect EB virus infection. Also, salivary C-erbB-2 and CA153 proteins could be new biomarkers of breast cancer (Streckfus et al., 1999; 2000; 2001).

Weber et al. (2010) reported the miRNA profiles of 12 bodily fluids and found a maximum of 458 miRNAs in saliva. Patel et al. (2011) found that the expression levels of salivary miRNAs were stable and did not change with time in the same person. Park et al. (2009) evaluated both whole saliva and saliva supernatant miRNAs in patients with oral cancer, and found similar miRNA profiles. They also reported that salivary miR-125a and miR-200a were significantly downregulated and that both might be new biomarkers of oral cancer.

Esophageal cancer is the 8th most common cancer and the 6th most common cause of cancer death in the world. The proportion of EC in China comprises 70% of all cases of the disease in the world (Li et al., 2011). The EC incidence in the Taihang mountain area and Chaoshan area of China exceeds 100/10 0000 (Su et al., 2007; Zhou et al., 2010) with survival rate of 5 years 3-5% (Kim et al., 2011). The pathological types of EC are mainly squamous cell carcinoma and adenocarcinoma. More than 80% of EC cases in China are squamous cell carcinoma, but the majority in the West are adenocarcinoma (Lambert et al., 2007). The risk factors for EC in Chaoshan are smoking, alcohol consumption, and drinking or eating at high temperatures (Xu et al., 2009; Lin et al., 2011). The risk factors for EC in the West are gastroesophageal reflux disease and Barrett's esophagus (Rastogi et al., 2008). In our study, the majority of the EC patients were from Chaoshan area, which might be associated with Chaoshanese habit of drinking or eating at high temperatures.

Until now, miR-21 has been found to be significantly upregulated in EC, gastric cancer, liver cancer, pancreatic cancer, bile duct cancer, colorectal cancer, etc. Furthermore, miR-21 may act as oncomir, and is closely associated with the pathogenesis, development, and metastasis of cancers. Recent studies have found that miR-21 is related to cancer target therapy, chemotherapy sensitivity, and drug resistance (Bonci et al., 2010; Jazbutyte et al., 2010). miR-21 was upregulated in both esophageal squamous cell carcinoma and adenocarcinoma tissue, which suggests that its expression level is not influenced by pathology type. Komatsu et al. (2011) reported that plasma miR-21 can detect esophageal squamous cell carcinoma and that patients with a high plasma miR-21 level tend to have greater vascular invasion and more frequent recurrence. Patients with Barrett's esophagus have a 30-fold higher risk of developing esophageal adenocarcinoma (Soest et al., 2005). Smith et al. (2010) reported that upregulation of miR-21 in the lesional epithelium of patients with Barrett's esophagus predicted development of esophageal adenocarcinoma. Therefore, miR-21 may be an appropriate marker in patients with Barrett's esophagus. Hence saliva supernatant miR-21 might take the place of tissue and plasma miR-21 to evaluate the prognosis, target therapy, chemotherapy sensitivity, and drug resistance of the patients with EC or Barrett's esophagus.

Saliva collection is more convenient, noninvasive, and cheaper than blood collection, so shows great promise in disease screening in poor nations. We analyzed the expression level of saliva supernatant miR-21 and identify whether it can serve as a novel biomarke for EC detection to our knowledge for the first time worldwide. Our data suggest saliva supernatant miR-21 to be a new biomarker of EC. In addition, the expression levels of saliva supernatant miR-21 were not influenced by cancer staging, differentiation, nodal metastasis, etc. Thus aberrant expression of saliva supernatant miR-21 may be an early molecular event in the pathogenesis of EC. Because detection of the expression level of saliva supernatant miR-21 is convenient and noninvasive, it may be an option for early screening and secondary prevention of EC. In conclusion, the role of saliva supernatant miR-21 in EC is worthy of further study.

References

Akobeng AK (2007). Understanding diagnostic tests 3: Receiver operating characteristic curves. *Acta Paediatr*, **96**, 644-7.

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

Bonci D (2010). MicroRNA-21 as therapeutic target in cancer and cardiovascular disease. *Recent Pat Cardiovasc Drug Discov*, **5**, 156-61.

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.

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.

Iguchi H, Kosaka N, Ochiya T (2010). Secretory microRNAs as a versatile communication tool. *Commun Integr Biol*, **3**, 478-81.

Jazbutyte V, Thum T (2010). MicroRNA-21: from cancer to cardiovascular disease. *Curr Drug Targets*, **11**, 926-35.

Kim T, Grobmyer SR, Smith R, et al (2011). Hochwald SN. Esophageal cancer--the five year survivors. *J Surg Oncol*, **103**, 179-83.

Komatsu S, Ichikawa D, Takeshita H, et al (2011). Circulating microRNAs in plasma of patients with oesophageal squamous cell carcinoma. *Br J Cancer*, **105**, 104-11.

Kosaka N, Iguchi H, Yoshioka Y, et al (2010). Secretory mechanisms and intercellular transfer of microRNAs in

living cells. *J Biol Chem*, **285**, 17442-52.
Lambert R, Hainaut P (2007). Esophageal cancer: cases and causes (part I). *Endoscopy*, **39**, 550-5.
Lee YH, Wong DT (2009). Saliva: an emerging biofluid for early detection of diseases. *Am J Dent*, **22**, 241-8.
Li XY, Guo YB, Su M, et al (2011). Association of mitochondrial haplogroup D and risk of esophageal cancer in Taihang Mountain and Chaoshan areas in China. *Mitochondrion*, **11**, 27-32.
Li Y, Elashoff D, Oh M, et al (2006). Wong DT. Serum circulating human mRNA profiling and its utility for oral cancer detection. *J Clin Oncol*, **24**, 1754-60.
Li Y, Jiang Z, Xu L, et al (2011). Stability analysis of liver cancer-related microRNAs. *Acta Biochim Biophys Sin (Shanghai)*, **43**, 69-78.
Lin J, Zeng R, Cao W, et al (2011). Hot beverage and food intake and esophageal cancer in southern China. *Asian Pac J Cancer Prev*, **12**, 2189-92.
Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402-8.
Mbulaiteye SM, Walters M, Engels EA, et al (2006). High levels of Epstein-Barr virus DNA in saliva and peripheral blood from Ugandan mother-child pairs. *J Infect Dis*, **193**, 422- 6.
McDonald JS, Milosevic D, Reddi HV, et al (2011). Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem*, **57**, 833-40.
Michael A, Bajracharya SD, Yuen PS, et al (2010). Exosomes from human saliva as a source of microRNA biomarkers. *Oral Dis*, **16**, 34-8.
Mishima T, Akagi I, Miyashita M, et al (2009). Study of MicroRNA expression profiles of esophageal cancer. *J Nippon Med Sch*, **76**, 43.
Park NJ, Zhou H, Elashoff D, et al (2009). Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. *Clin Cancer Res*, **15**, 5473-77.
Patel RS, Jakymiw A, Yao B, et al (2011). High resolution of microRNA signatures in human whole saliva. *Arch Oral Biol*, **56**, 1506-13.
Rabinowits G, Gercel-Taylor C, Day JM, et al (2009). Exosomal microRNA: a diagnostic marker for lung cancer. *Clin Lung Cancer*, **10**, 42-6.
Rastogi A, Puli S, El-Serag HB, et al (2008). Incidence of esophageal adenocarcinoma in patients with Barrett's esophagus and high-grade dysplasia: a meta-analysis. *Gastrointest Endosc*, **67**, 394-8.
Schipper RG, Silletti E, Vingerhoeds MH (2007). Saliva as research material: biochemical, physicochemical and practical aspects. *Arch Oral Biol*, **52**, 1114-35.
Smith CM, Watson DI, Michael MZ, Hussey DJ (2010). MicroRNAs, development of Barrett's esophagus, and progression to esophageal adenocarcinoma. *World J Gastroenterol*, **16**, 531-7.
Streckfus C, Bigler L, Dellinger T, et al (2001).Reliability assessment of soluble c-erbB-2 concentrations in the saliva of healthy women and men. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, **91**, 174-9.
Streckfus C, Bigler L, Dellinger T, et al (2000). The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. *Clin Cancer Res*, **6**, 2363-70.
Streckfus C, Bigler L, Dellinger T, et al (1999). CA 15-3 and c-erbB-2 presence in the saliva of women. *Clin Oral Investig*, **3**, 138-43.
Su M, Liu M, Tian DP, et al (2007). Temporal trends of esophageal cancer during 1995-2004 in Nanao Island, an extremely high-risk area in China. *Eur J Epidemio*, **22**, 43-8.
Taylor DD, Gercel-Taylor C (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol Oncol*, **110**, 13-21.
van Soest EM, Dieleman JP, Siersema PD, et al (2005). Increasing incidence of Barrett's oesophagus in the general population. *Gut*, **54**, 1062-6.
Weber JA, Baxter DH, Zhang S, et al (2010). The microRNA spectrum in 12 body fluids. *Clin Chem*, **56**, 1733-41.
Wiklund ED, Gao S, Hulf T, et al (2011). MicroRNA alterations and associated aberrant DNA methylation patterns across multiple sample types in oral squamous cell carcinoma. *PLoS One*, **6**, e27840.
Wong DT (2006). Towards a simple, saliva-based test for the detection of oral cancer 'oral fluid (saliva), which is the mirror of the body, is a perfect medium to be explored for health and disease surveillance'. *Expert Rev Mol Diagn*, **6**, 267-72.
Xu XC (2009). Risk factors and gene expression in esophageal cancer. *Methods Mol Biol*, **471**, 335-60.
Zampetaki A, Willeit P, Drozdov I, Kiechl S, Mayr M (2012). Profiling of circulating microRNAs: from single biomarkers to re-wired networks. *Cardiovasc Res*, **93**, 555-62.
Zhou SL, Wang LD (2010). Circulating microRNAs: novel biomarkers for esophageal cancer. *World J Gastroenterol*, **16**, 2348-54.