RESEARCH ARTICLE

Identification of Serum MicroRNA-21 as a Biomarker for Early Detection and Prognosis in Human Epithelial Ovarian Cancer

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Abstract

Recent investigations have confirmed up-regulation of serum miR-21 and its diagnostic and prognostic value in several human malignancies. In this study, we examined serum miR-21 levels in epithelial ovarian cancer (EOC) patients, and explored its association with clinicopathological factors and prognosis. The results showed significantly higher serum miR-21 levels in EOC patients than in healthy controls. In addition, increased serum miR-21 expression was correlated with advanced FIGO stage, high tumor grade, and shortened overall survival. These findings indicate that serum miR-21 may serve as a novel diagnostic and prognostic marker, and be used as a therapeutic target for the treatment of EOC.

Keywords: miR-21 - real-time reverse transcriptase-PCR - epithelial ovarian cancer - tumor stage - prognosis

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Introduction

Ovarian cancer is the most lethal of all common gynecologic malignancies, with more than 204,000 new cases and 125,000 deaths each year, accounting for 4% of all cancer cases and 4.2% of all cancer deaths in women around the world (Zhang et al., 2011a). Although survival has increased slightly over the past 25 years, 5-year survival remains below 50% (Corney et al., 2010). A major factor for low survival is our poor understanding of the initiating events that lead to ovarian cancer and how the disease progresses. Novel biomarkers with high sensitivity and specificity are urgently needed for the diagnosis and target therapies of ovarian cancer.

One decade ago, microRNAs (miRNAs) were discovered as a novel class of evolutionarily conserved small (18 - 24 nucleotides) non-coding RNA molecules, which are important regulators of gene expression (Bartel, 2004). By targeting the three prime untranslated regions (3' UTRs) of mRNA transcripts, miRNAs influence RNA stability and translational efficiency via degradation or protein translation inhibition, respectively. It is now evident that about 50% of miRNAs are located in cancer-associated genomic regions or at fragile sites (Calin et al., 2004), and they are frequently misexpressed or mutated in cancer patients and regarded as oncogenes or tumor suppressor genes. In addition, it has been shown that miRNAs are present in human serum and plasma in a remarkably stable form (Yang et al., 2008), raising the possibility that unique plasma/serum miRNA patterns might be used as non-invasive disease markers. In support of this, differences have been found between miRNA patterns in serum or plasma of patients with a number of malignancies and healthy controls (Zhou et al., 2012).

One of the founding members of miRNA family, miR-21, has been reported to be up regulated in various tumor types including ovarian cancer (Iorio et al., 2007). However, the actual role of serum miR-21 expression in ovarian cancer has not been systematically studied yet. In this study, we investigated the serum levels of miR-21 in patients with epithelial ovarian cancer (EOC), and evaluated the feasibility of using serum miR-221 as a noninvasive diagnostic and prognostic biomarker for EOC.

Materials and Methods

Patient samples

A total of 94 patients with primary EOC (mean age, 59.2 ± 6.8 y) who were treated in Affiliated Hospital Of NanTong Univesity, NanTong, China between January 2006 and October 2007 were included in the present retrospective study. All patients received standard operation aiming at a maximal tumor resection, including hysterectomy, bilateral salpingo-oophorectomy, pelvic and/or paraaortic lymphadenectomy, and omentectomy. After resection, a platinum-based chemotherapy was administrated for at least six cycles. None of the patients received chemotherapy, radiotherapy, or immunotherapy before the surgical operation. Surgical staging was determined based on the International Federation of Obstetrics and Gynecology (FIGO) criteria. Stage breakdown was: n=9 for stage I, n=23 for stage II, n=42 for stage III, and n=20 for stage IV. Tumor grade was

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Table 1. Serum miR-21 Expression and Clinicopathological Factors

Clinicopathological features	No. of patients	Serum miR-221(ΔCt)	*P* value
Age (years)			
<59.2	45	5.81 ± 0.48	NS
≥59.2	49	5.47 ± 0.65	
Tumor stage			
FIGO I-II	32	6.65 ± 0.73	0.002
FIGO III-IV	62	4.48 ± 0.34	
Tumor grade			
Low	39	6.18 ± 0.61	0.015
High	55	5.04 ± 0.43	
Histologic type			
Serous	68	5.93 ± 0.52	NS
Others	26	5.26 ± 0.55	

$\Delta Ct = Ct_{miR\text{-}21} - Ct_{U6}$; Data were expressed as the mean ± standard deviation

designated as low or high according to the M.D. Anderson grading criteria (Malpica et al., 2004).

Patients' blood was obtained before surgery by peripheral venous puncture, 5ml for each patient. Blood samples from 40 healthy age-matched volunteers were used as control. Immediately after sample collection, the blood samples were centrifuged at 5000 rpm for 10 min at 4°C to spin down the blood cells; the supernatants containing the serum samples were transferred into fresh tubes and stored at –80°C until further processing. Ethical approval was obtained from the Institutional Review Boards, and informed consent for the use of serum samples was obtained for all individuals.

MicroRNA Isolation and Real-Time RT-PCR Assay

Total RNA was isolated from 400 ul of serum sample by using the mirVana kit® (Ambion, Austin, TX, USA), according to the manufacturer's instructions. The expression of miR-21 was determined by qRT-PCR using TaqMan microRNA assay kit® (Applied Biosystems, Foster City, CA). For synthesis of cDNA, 10 ng of total RNA for each serum sample was used for the individual assays in a 15-ul reaction mixture containing 5 ul of RNA extract, 0.15 ul of 100 mM dNTPs, 1 ul of multiscribe reverse transcriptase (50 U/ul), 1.5 ul of 10 × reverse transcription buffer, 0.19 ul of RNase inhibitor (20 U/ml), 1 ul of gene-specific TaqMan primer, and 4.16 ul of nuclear-free water. The reaction mixture was incubated at 16°C for 30 min, 42°C for 60 min, and 85°C for 5 min. Subsequently, 5 ul of the DNA template was amplified using 10 ul of LightCycler 480 Probes Master® (Roche Diagnostics, Manniheim, Germany), 3 ul of nuclear-free water, and 2 ul of gene-specific TaqMan primer primers/probe mix in a final volume of 20 ul. qRT-PCR was run on the LightCycler 480 System II® (Roche). The reaction mixture was incubated at 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec, and 72°C for 1 sec. TaqMan qRT-PCR was performed in triplicate, and U6 snRNA was used as normalizer. The relative miR-21 expression was calculated using the equation $2^{-\Delta Ct}$ where $\Delta Ct = (Ct_{miR\text{-}21} - Ct_{U6})$ (Lawrie et al., 2008), and the ΔCt value negatively correlated with the serum level of miR-21.

Table 2. Univariate and Multivariate Survival Analysis in Patients with Ovarian Cancer

Variable	Subset	Hazard ratio	*P* value
Univariate analysis (n=94)			
Age	≥59.2 versus <59.2	0.528	NS
Tumor stage	FIGO I-II versus FIGO III-IV	5.335	< 0.001
Tumor grade	Low versus high	3.267	0.006
serum miR-21 levels	ΔCt	2.412	0.018
Histological type	Serous versus others	2.036	0.035
Multivariate analysis (n=94)			
Age	≥59.2 versus <59.2	0.622	NS
Tumor stage	FIGO I-II versus FIGO III-IV	5.075	< 0.001
Tumor grade	Low versus high	2.158	0.032
serum miR-21 levels	ΔCt	2.327	0.019
Histological type	Serous versus others	0.796	NS

Statistics

All statistical analyses were carried out using SPSS version 17.0 statistical software (SPSS Inc., Chicago, IL, USA). *P* values < 0.05 were considered statistically significant and all of them are two-sided. Data were expressed as the mean ± SD. Differences in miR-21 expression levels between groups were compared using the Student's t-test. Overall survival (OS) was defined as the time interval between the start of the treatment and the date of death or end of follow-up. The association between expression levels of miR-21 and OS was analyzed by Spearman correlation coefficient. Multivariate analysis of the prognostic factors was performed with Cox regression model.

Results

miR-21 is upregulated in EOC serum samples

A stem-loop reverse transcription polymerase chain reaction assay was used to examine miRNA21 expression in serum. The results showed that serum miR-21 was significantly upregulated in EOC patients. The mean ± SD ΔCt value of miR-21 was 5.65 ± 0.68 in the 94 EOC samples, and 7.41 ± 0.86 in healthy controls ($P < 0.001$, Student's t-test).

Serum miR-21 correlates with clinicopathological features of EOC

For better understanding of the potential roles of serum miR-21 in EOC development and progression, we observed the relationship of miR-21 expression with various clinical features of EOC. The results revealed that miR-21 expression of FIGO III-IV stages (n=62) was significantly higher than FIGO I–II stages (n=32, P=0.002). Additionally, the miR-21 expression also correlated with tumor grade (P=0.015). However, no significant correlation between miR-21 levels and pathological type or patients' age was found in our study (Table 1).

Serum miR-21 correlates with prognosis of EOC patients

At the time of scheduled analyses (July 2012), 36 of the 94 patients were still alive, including 17 patients alive with no evidence of disease. Fifty two patients died as a result of EOC and 6 patients died as a result of other causes. Spearman correlation coefficient analysis showed

significant correlation between serum miR-21 levels (ΔCt) and survival time (r = 0.565, $P = 0.006$).

Univariate analysis and multivariate Cox regression were used to analyze whether plasmamiR-21 and other clinical parameters could be independent prognostic factors for EOC. In a univariate survival analysis, FIGO stage (HR 5.335; $P < 0.001$), tunor grade (HR 3.267; $P = 0.006$), histologic type (HR 2.036; $P = 0.035$), and serum miR-21 levels (HR 2.412; $P = 0.018$) were associated with overall survival. In the multivariate Cox regression model, high-level of serum miR-21 expression ($P = 0.019$, HR = 2.327) was an unfavorable prognostic factor independent of other clinicopathological factors, including high FIGO stage (HR 5.075; $P < 0.001$) and high tumor grade ($P = 0.032$, HR = 2.158; Table 2).

Discussion

Ovarian cancer is a common gynecologic malignancy and a leading cause of cancer death among women. Elucidation of factors affecting behavior of ovarian cancer is indispensable to improve prognosis. The discovery of miRNAs has broadened our understanding of carcinogenesis. It has been proposed that there are more than 1000 miRNAs in the human genome (Friedman et al., 2009), and these miRNAs have recently been reported to play important roles in vivo, including roles in cancer development, angiogenesis and the immune response (Kuehbacher et al., 2008; Davidson-Moncada et al., 2010; Song et al., 2010; Liu et al., 2011). Although, many miRNAs are expressed in tissues and tumor cells, their development as biomarkers requires tissue collection by invasive methods as opposed to the more convenient approach of studying peripheral blood. Since miRNA levels were first measured in serum in 2008 (Lawrie et al., 2008), researchers have focused on investigating circulating miRNAs in cancer and have begun to elucidate their diagnostic and prognostic significance. In this study, we show that patients with EOC had significantly elevated levels of serum miR-21 compared to healthy controls, and serum miR-21 expression was correlated with FIGO stage, tumor grade, and overall survival. These results suggest that serum miR-21 may serve as a useful diagnostic and prognostic biomarker for EOC.

MiR-21 has been confirmed to be overexpressed in many malignancies such as gastric cancer (Chan et al., 2008), pancreatic cancer (Roldo et al., 2006), hepatocellular cancer (Meng et al., 2007), colon cancer (Slaby et al., 2007), breast cancer (Iorio et al., 2005), prostate cancer (Volinia et al., 2006), brain tumor (Chan et al., 2005), cholangiocarcinoma (Meng et al., 2006), lung cancer (Markou et al., 2008), esophageal cancer (Feber et al., 2008), head and neck cancer (Tran et al., 2007), and ovarian cancer (Iorio et al., 2007). In addition, clinical significance of serum miR-21 expression in human cancers has been reported in recent investigations. Lawrie et al. were the first to discover tumor specific deregulation of circulating miRNAs. Their study demonstrated that miRNA-21 was highly abundant in the sera of diffuse large B-cell lymphoma patients (Lawrie et al., 2008). Serum miR-21 was also found to be significantly higher in patients with hepatocellular carcinoma (HCC) than in healthy controls (Xu et al., 2011). Asaga et al. found that serum miR-21 has diagnostic and prognostic potential in breast cancer (Asaga et al., 2011). In Zheng's study, patients with gastric cancer display a significantly higher level of miR-21 in peripheral blood than those from controls. The miR-21 level was associated with the tumor node metastasis (TNM) stage, tumor size and tissue categories (Tsujiura et al., 2010). Wang and his colleagues revealed that serum miR-21 was elevated in patients with non-small cell lung cancer, and high serum miR-21 was significantly correlated with tumor-node metastases stage, lymph node metastasis, and shorter survival (Wang et al., 2011). Kurashige et al reported up-regulation of serum miRNA-21 in esophageal squamous cell carcinoma patients and its correlation with chemosensitivity (Kurashige et al., 2012). Zhang et al. (2011b) confirmed elevated serum miR-21 levels in patients with hormone-refractory prostate cancer and its potential value in predicting the efficacy of docetaxel-based chemotherapy. Wang et al. (2012) tested the levels of serum miR-21 in advanced pancreatic cancer patients who received gemcitabine-based palliative chemotherapy, and the results showed serum miR-21 level was an independent prognostic factor for both the time-to-progression (TTP) and the OS. So, miR-21 may play an important role not only in tumor initiation but also in development and drug resistance, and serum miR-21 would offer great hope for the diagnosis and prognosis of many human malignancies..

The mechanism by which miR-21 affects tumor behavior has been described in a few published reports. Up to now, it has been elucidated that miR-21 can modulate growth, cell cycle progression, metastasis, and chemosensitivity of tumor cells by targeting several oncogenes or tumor suppressor genes. For example, miR-21 could target PDCD4 at the posttranscriptional level and regulate cell proliferation and invasion in esophageal squamous cell carcinoma (Hiyoshi et al., 2009). Knockdown of miR-21 in gastric cancer cell lines could dramatically decrease cell invasion and migration by regulating RECK (Zhang et al., 2008). When transfected with anti-miR-21, cell growth of breast cancer cell line MCF7 was inhibited, partly owing to the downregulation of Bcl-2 (Si et al., 2007). By targeting RhoB expression, microRNA-21 exhibited antiangiogenic function in endothelial cells (Sabatel et al., 2011). Furthermore, some other tumor related genes, such as PTEN, MARCKS, maspin, Cdc25A, and TMP1 have also been proven regulated by microRNA-21 in recent studies, However, an average miRNA can have more than 100 targets (Brennecke et al., 2005). On the other hand, several miRNAs can converge on a single transcript target (Krek et al., 2005). So the potential regulatory circuitry afforded by miR-21 may be enormous, and identification of the complex molecular network involved in its function remains an important facet in future investigations.

In conclusion, our analysis showed that level of serum miR-21 is elevated in EOC patients, and associated with FIGO stage and tumor grade. Furthermore, a higher plasma miR-21 level correlates with a less favorable long term outcome. Based on these results, serum miR-21 may

serve as a diagnostic and prognostic marker, and be used as a therapeutic target for the treatment of EOC.

Acknowledgements

The author(s) declare that they have no competing interests.

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