RESEARCH ARTICLE

Serum MicroRNA-21 Negatively Relates to Expression of Programmed Cell Death-4 in Patients with Epithelial Ovarian Cancer

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Abstract

Background: Ovarian cancer is the third most common cancer of the female genital tract and the leading cause of cancer death associated with gynecologic tumors. MicroRNAs regulate at least 60% of human genes, including tumor suppressor genes and oncogenes and, thereby, can affect cancer risk. **Aim of the work:** We aimed to assess any diagnostic role for serum miR-21 as a biomarker in human ovarian cancer and to study relations with programmed cell death-4 (PDCD4), one of its target proteins, hoping to help explain heterogeneity of this cancer type and facilitate stratification of regimens for therapy. **Subjects and Methods:** A total of 60 newly diagnosed ovarian cancer cases and 30 apparently healthy females were recruited. Serum microRNA-21 levels were measured by TaqMan- Real time PCR assay and PDCD4 by ELISA. **Results:** Significant over-expression of serum miR-21 and lower serum PDCD4 levels were observed in ovarian cancer patients as compared to the control group. A statistically significant inverse correlation was also evident between miR-21 and PDCD4. However, no significant links were noted observed between miR-21 and tumor grade, stage or histopathological type. **Conclusion:** The present work showed significantly up-regulation of serum miR21 in the recruited group of patients and a significant inverse relation association between miR-21and PDCD4. These findings suggest that miR-21 may be used as a diagnostic biomarker for human ovarian cancer.

Keywords: Ovarian cancer- miR-21- PDCD4

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Introduction

Ovarian cancer is classified as the eighth most common cancer among women, and it represents 4% of all women's cancers (Ferlay et al., 2008). Its incidence rates are greater in high than in middle- to low-income countries. Despite improvements in diagnostics and clinical methods, poor understanding of the malignant biological behavior and pathogenesis of ovarian cancer is a major hurdle in devising an effective strategy for the treatment of this cancer (Lou et al., 2010).

Ovarian cancer (OC) showed histological heterogeneity. They were histologically classified according to the WHO based on their derivation from germ cells, coelomic surface epithelium, and mesenchyme (Scully et al., 1998). Epithelial ovarian cancer represents the major form of the tumors, and it was further subdivided into many histological types as follows: serous, mucinous, clear cell, endometrioid, transitional cell tumors (Brenner tumors), mixed epithelial tumor, and others.

In 1993, microRNAs (miRNAs) were discovered by Lee and his colleagues (Lee et al., 1993). They are small non-coding RNA molecules that affect gene expression in a negative way.

Studies showed that miRNAs circulate in the blood stream in a stable, cell-free form, they play important roles in gene regulatory networks through binding to and repressing the activity of specific targets by recognizing its complementary sequences in the 3' untranslated region (UTR) of target mRNAs through seed region, typically positions 2–7 in the miRNA (Qin et al., 2010). Micro-RNAs have a role in regulating cell proliferation and differentiation processes and their dysregulation appears to play a major role in the onset, progression and dissemination of many tumors including ovarian cancer hence the name, oncomir. MiR-21 is one of the most prominent oncomirs which disturbs cell survival mechanisms. It is located on chromosome 17q23.2 which is dramatically up-regulated in most of human cancer of different origins and is tightly associated with carcinogenesis .The genes targeted by miR-21 have been under intense study (Jazbutyte and Thum, 2010).

Programmed cell death-4 (PDCD4) is a tumor suppressor gene localized on chromosome 10q24. It is expressed in all normal tissues with highest levels in the liver. It is involved in apoptosis that affects cell

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transformation, oncogenesis, and tumor invasion (Yang et al., 2001; Mudduluru et al., 2007). PDCD-4 is one of the miRNA-21 targets, it has a single highly conserved miR-21 target site within its 3'UTR, and its regulation by miR-21 has been reported in a number of human cancer cells including breast cancer, colorectal cancer (Asangani et al., 2008) and glioma (Gabriely et al., 2008), as well as in a murine JB6 epidermal model of neoplastic transformation (Lu et al., 2008).

MicroRNA-21 is one of the most encountered overexpressed miRNAs in many cancers, so we were interested to elucidate the correlation between PCDC-4 and miR-21 in a group of Egyptian females with ovarian cancer and to investigate its relation to different clinicopathological characters.

Materials and Methods

Patients and methods

This study was conducted on a total number of 90 participants, 60 patients newly diagnosed with different stages of ovarian cancer. The patients' median age was 55.5 year (ranged from 18 to 75). They attended the surgical oncology outpatient clinics at National Cancer Institute (NCI), Cairo University during the time period from April 2015 till April 2016. Thirty age-and sex-matched apparently healthy females were included as controls. All patients had not received any therapies and had been diagnosed by histopathology. The study was permitted by the Institutional Review Board (IRB) of the NCI, Cairo University. It was permitted according to the Helsinki guidelines of studies performed on human beings and all participants signed the informed written consent (Institutional Review Board Decision; Approval No: 201516014.2).

All patients were subjected to; Full history taking and clinical examination, Imaging techniques in the form of: Abdominal and pelvic U/S, CT scan. Radiological Investigations in the form of Chest X-rays to exclude lung metastasis. Histopathological study of the ovarian specimen after surgical excision and surgical staging determined based on International Federation of Gynecology and Obstetrics (FIGO) criteria.

Blood sampling

Four milliliters of venous blood samples were collected on serum vacutainer tubes under complete aseptic precautions. Immediately after sample collection, the blood samples were centrifuged at 5,000 rpm for 10 minutes at 4°C to spin down the blood cells; the supernatants containing the serum samples were transferred into two micro centrifuge tubes and stored at -80°C until further processing.

Detection of serum miR-21 by Real-Time RT-PCR

• MicroRNA Isolation and Real-Time RT-PCR Assay

Total RNA was isolated from 200 µL of serum using the miRNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. The RNA was eluted in 40 µL of RNAse-free water and was stored at −70 °C until RT-PCR reaction. Concentrations of all RNA samples were measured using NanoDrop spectrophotometer (Thermo Scientific, USA).The expression of miR-21(gene of interest) was measured by qRT-PCR using TaqMan microRNA assay kit (Applied Biosystems, Foster City, CA). We amplified 5 µl of the cDNA template using 10 ul of TaqMan® Universal PCR Master Mix II (2×), 1 µl of gene-specific TaqMan primer primers/ probe mix , and 6.5 µl of nuclear-free water to reach a final volume of 20 µl. qRT-PCR was run on Applied biosystems StepOne™ detection system (Applied Biosystems, USA). The previous mixture was incubated at 95°C for 5 minutes, then 40 cycles of 95°C for 10 seconds, followed by 30 seconds at 60°C, and finally 72°C for 1 second. TaqMan qRT-PCR was performed in duplicate, and U6 snRNA was used as internal control. We calculated the relative miR-21 expression using the equation $2^{-\Delta\Delta CT}$ where ΔΔCT=ΔCT (target sample) - ΔCT (reference sample).

Detection of serum protein PDCD4 by ELISA technique

PDCD4 protein in serum was measured using sandwich Enzyme-linked Immunosorbent Assay Kit for in vitro quantitative measurement of PDCD4 supplied by Cloud-Clone Corp. (Houston,USA) according to the manufacturer's instructions.

Statistical Methods

Data was tested using SPSS version 22 (SPSS Inc., Chicago, IL). Numerical non-parametric variables were expressed as median and range. Qualitative variables were expressed as frequency and percentage. To examine the relation between qualitative variables, Chi-square test or Fisher's exact test was used. For non-parametric quantitative variables Mann-Whitney test (non-parametric t-test) was used. The Receiver Operating Characteristic (ROC) curve was done to determine the performance of the examined markers. All tests were two-tailed. The criterion for statistical significant result was p value < 0.05.

Results

Characteristics of the ovarian cancer group.

The age and histopathological characteristics of the cancer group were illustrated in Table 1.

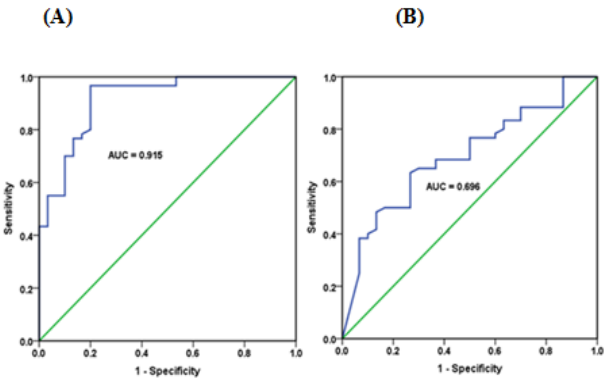

Figure 1. (A), ROC curve of miR-21relative expression for diagnosis of ovarian cancer; (B), ROC curve of PDCD4 levels for diagnosis of ovarian cancer.

Table 1. The Age and Histological Characteristics of the Cancer Ggroup

Variable	
Age in years	
(median , range)	55.5 (18-75)
Histopathological type	
Papillary Serous cystadenocarcinoma	19 (31.7%)
Serous Cystadenocarcinoma	12 (21.1%)
Mucinous cystadenocarcinoma	13 (22.8%)
Adenocarcinoma	8 (14%)
Endometrioid Adenocarcinoma	5 (8.8%)
Pathological Grade	
G1	5 (11.1%)
G2	23 (51.1%)
G3	17 (37.8%)
FIGO staging	
IA	12 (20.3%)
IB	3 (5.08%)
IC	6 (10.1%)
IIIB	2 (3.38%)
IIIC	25 (42.3%)
IV	13 (18.6%)
Metastasis	
No Metastasis	21 (35.6%)
Peritoneal and/or Omental	27 (45.8%)
Distant	11 (18.6%)
Family history of Ovarian cancer	
Positive	0 (0%)
Negative	60 (100%)

Qualitative data are represented as frequency (percentage).

Table 2. Level of miR-21 and PDCD4 in Ovarian Cancer Patients and Control Group

Variable	Ovarian Cancer group (n=60) median (Range)	Control group (n=30) median (Range)	P value
Serum miR-21	3.05 (0.59-60.19)	0.66 (0.01-3.82)	0.001*
Serum Pdcd4	0.64 (0.06-1.9)	0.93 (0.06-4.69)	0.002*

*P value <0.05 is significant

Up – regulation of serum miR-21 in ovarian cancer serum samples

MicroRNA-21 was significantly up – regulated while PDCD4 was down regulated in the serum of ovarian cancer patients, Table 2.

Serum miR-21 inversely correlates with PDCD4 in the studied groups

Data of the relative expression of miR-21 and serum PDCD4 are non-parametric distributed .So spearman; s test was applied to analyze the correlation between the two markers. There was a significant negative correlation between miR-21 and serum pdcd4, r = -0.23 and p = 0.03.

Serum miR-21 and pdcd4 did not correlate with clinicopathological characteristics of Epithelial ovarian cancer (EOC) patients (histopathology, grade, stage and metastasis)

Data are illustrated in Table 3.

Receiver Operating Characteristic (ROC) Curve

The diagnostic value of miR-21 and PDCD4 were evaluated by ROC analysis. The results shown in Figure. (A) and Figure. (B), suggested that ovarian cancer patients

Table 3. The Relation between Serum miR-21 Level, PDCD4 Level and Clinicopathological Characteristics of Ovarian Cancer Group

Clinicopathological Features (n)	Serum miR-21 median (Range)	P value	Serum PDCD4 (ng/ml) median (Range)	P value
Age in years (number)				
<55 (24)	4.08 (1.3-60.19)	0.135	0.69 (0.06-1.9)	0.166
>55 (36)	2.01 (0.59-59.84)		0.53 (0.06-1.9)	
Pathology				
Serous (31)	2.84 (0.59-60.19)		0.68 (0.06-1.58)	
Mucinous (13)	4.26 (1.28-52.74)	0.643	0.5 (0.06-1.18)	0.583
Other adenocarcinomas(13)	2.77 (1.2-14.51)		0.33 (0.06-1.9)	
Grade				
Low Grade (I &II) (28)	3.51 (0.59-59.84)	0.206	0.6 (0.06-1.58)	0.686
High Grade(III) (17)	1.42 (0.59-60.19)		0.68 (0.06-1.9)	
FIGO Stage				
Stage I&II (21)	2 (1.22-59.84)	0.486	0.47 (0.06-1.66)	0.135
Stage III&IV (38)	3.51 (0.59-60.19)		0.71 (0.06-1.9)	
Metastasis				
No metastasis (21)	2 (1.22-59.84)		0.47 (0.06-1.66)	
Local metastasis (27)	3.36 (0.59-60.19)	0.639	0.71 (0.06-1.9)	0.295
Distant metastasis (11)	5.59 (0.59-52.74)		0.59 (0.06-1.9)	

*P value <0.05 is significant

could be distinguished from apparently healthy controls according to miR-21 expression at a cut-off value ≥1.005. The AUC was 0.915 with P value 0.001, sensitivity was 96.7% and specificity was 80%, positive predictive value (PPV) was 90%, negative predictive value (NPV) was 92.3% and total accuracy was 91.1%. For PCDC4, at a cut-off value ≤ 0.75 ng/ml, sensitivity was 65% and specificity was 70, PPV was 81.3%, NPV 50% and total accuracy was 95.5%. The AUC was 0.67, p value was 0.002.

Discussion

Micro RNA-21 (miR-21) is one of the early identified miRNA (Lagos-Quintana et al., 2001). It is one of the most prominent miRNAs implicated in the genesis and progression of human cancer. It has been involved in many tumor genesis processes including antiapoptosis (Chan et al., 2005), proliferation (Roldo et al., 2006), tumor growth (Si et al., 2007), and response to gemcitabine-based chemotherapy (Meng et al., 2006). Additionally, it has been proposed as a biomarker of malignancy in many body fluids e.g. circulation (Xu et al., 2011), sputum (Yu et al., 2010), and cerebrospinal fluid (Baraniskin et al., 2011).

In this study we found that serum miR-21 was significantly up-regulated in the serum of ovarian cancer patients when compared to control group. This was in concordance with other studies (Xu et al., 2011); (Cappellesso et al., 2014) and (Chen et al., 2016). It is tightly associated with cancerogenesis as it disturbs cell survival mechanisms by regulating cell cycle , several apoptotic proteins, negative regulators of metalloproteinases proteins and others (Jazbutyte and Thum ,2010).Also, it was reported to have diagnostic and prognostic potential in breast cancer (Asaga et al., 2011) .

In the present study most of our patients were diagnosed in late stages, 56.7% of our cancer group was in stages III and IV and they presented with low histological grade (62.2%). This was with in agreement with (Lou et al., 2010). They reported that 66.6% of their patients were stage III and IV and 60.4% of patients were of Grade I and II.

Epithelial ovarian cancer (EOC) is a heterogeneous group of neoplasms that comprise 90% of ovarian cancer. There are five major subtypes; Endometrioid, mucinous, Brenner, clear cell, and undifferentiated carcinomas. Upon these types, the serous form accounts 75% to 80% of epithelial ovarian carcinomas (Seidman and Kurman, 2003). We found that 54.4% of patients enrolled in our study were of the serous type, similar to other study, they reported that nearly 72% of patients were of the serous type (Xu et al., 2011).

In the present study, serum PDCD4 was significantly down-regulated in ovarian cancer patients when compared to healthy subjects. In accordance with our results the studies done by Wei et al., 2009a; Wang et al., 2013) reported that PDCD4 was down-regulated in ovarian cancer tissues and cell line. These can be referred to its role in the initiation and progression of ovarian cancer as it inhibits proliferation and cell cycle progression, and induces apoptosis in ovarian cancer cells. Thus, it is reasonable that loss of PDCD4 is a common abnormality at molecular level in ovarian cancer. Also, a frequent down-regulation or loss of PDCD4 was found in other cancers: different skin tumors (Matsuhashi et al., 2007) colorectal normal adenoma-carcinoma sequence (Lee et al., 2006; Mudduluru et al., 2007. The decreased level of PDCD4 observed can allow it to be a potential molecular marker of tumors and can be used as an indicator of neoplastic changes.

In the present study no significant difference was observed in miR-21and PDCD4 levels when comparing different stages, grade, Histopathological types of ovarian cancer, and these may be due to the small sample size compared to other studies.

There was a discrepancy in the results of association between miR-21 and PDCD4 and different clinico-pathological parameters in ovarian cancer patients. In our study no significant association was found between miR-21 and PDCD4 with the clinico-pathological parameters examined in patients such as FIGO stage, grading and pathological types. On the other hand Xu and his colleagues reported an association between miR-21 and tumor stages (Xu et al., 2011). Another study in 2016 reported miR-21 and tumor grades (Chen et al., 2016).

Up to our knowledge, this is the first study that correlates serum PDCD4 measured by ELISA with serum miR-21 by RT-qPCR in cancer ovaries. It revealed a significant inverse correlation between miR-21 and PDCD4 in whole groups (cancer and healthy) with P value =0.03 and correlation coefficient was (-0.23). This was in concordance with another study, they found a reduction in PDCD4 levels in Ovarian serous carcinoma (OSC) specimens compared with normal controls and serous cystadenoma (CA) specimens, at both the protein level measured by immunohistochemistry and mRNA level measured by RT-qPCR, also they highlighted miR-21 overexpression in OSC specimens (Chen et al., 2016). PDCD4 expression level was inversely correlated with miR-21 in a variety of tumor tissues and cells, including breast cancer cells ,colorectal cancer cells and NK-cell lymphoma/leukemia cells (Asangani et al., 2008; Yamanaka et al., 2009), This could be explained by the ability of miR-21 to target multiple genes including PTEN, PDCD4 and BCL-2, so it is potentially a key factor not only in tumor growth but also in initiation, progression, invasion and metastasis of a wide variety of tumor types including ovarian cancer (Lou et al., 2010). In addition, miR-21 is induced by AP-1 as a response to the Ras oncogene expression and accordingly down-regulates two target genes, phosphatase and tensin homologue (PTEN) ,and programmed cell death 4 (PDCD4). PDCD4 is required in return for stimulation of AP-1 in response to the Ras oncoprotein. This could be a proof for the autoregulatory loop between PDCD4 and miR-21 for controlling AP-1 activity in Ras-transformed cells (Talotta et al., 2009).

Considering the complexity of the regulation network between miRNA and protein coding genes, some authors reported that miR-182 may also target and regulate PDCD4 expression, and they observed that the binding

site of miR-21 on PDCD4 3' UTR is different from miR-182 (Wang et al., 2013). Thus, it is possible that these two miRNAs cooperatively regulate PDCD4, which needs to be elucidated in the future.

The diagnostic value of miR-21 was evaluated by ROC analysis. AUC: 0.915 at Cut-off: 1.005, Sensitivity: 96.7% Specificity: 80%. Although other researchers, studied miR-21 in ovarian cancers yet they did not evaluate its diagnostic accuracy (Xu, et al., 2011; Cappellesso et al., 2014 ; Chen et al., 2016) . Others studies including breast cancer reported that sensitivity of miR-21 ranged from (67% to 87.6%) and its specificity ranged from (75% to 87.3%).

Yu et al., (2016) performed meta-analysis on 9 studies concerning Circulating microRNA-21 as a potential diagnostic marker for colorectal cancer. The analysis results showed that the pooled sensitivity and specificity of miR-21 for colorectal cancers (CRC) diagnosis were 72% (range: 62–80) and 85% (Range: 80–88), respectively. The SROC (summarizing ROC) curve of the selected studies shows that The AUC was 0.87 (range: 0.83–0.89).

In summary, miR-21 was up-regulated in ovarian cancer when compared to healthy controls which suggests that miR-21 may be used as a diagnostic biomarker in ovarian cancer. Also its expression was negatively correlated with serum PDCD4, which was down-regulated in ovarian cancer patients who may explain the influence of miR-21 on increased risk of ovarian cancer.

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