miR-199a and miR-125b expression levels in serum of women affected by epithelial ovarian cancer

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Questo lavoro è stato in parte presentato al 47° Congresso Nazionale SIBioC, 26-28 ottobre 2015, Firenze, sotto forma di poster, ricevendo, nella persona del suo primo autore (M. Benati), il premio SIBioC destinato ai 4 migliori poster presentati.

ABSTRACT

Recent studies show that microRNA (miRNAs) are involved in cancer by regulating cell proliferation, apoptosis and angiogenesis. Accordingly, their deregulation could contribute to cancer development and progression. It has been demonstrated that in ovarian tissue the over-expression of miR-199a and miR-125b inhibits tumor angiogenesis, a fundamental process for cancer development and growth. Aims of our study were to investigate the expression levels of miR-199a and miR-125b in serum of patients with ovarian cancer (OC) and to evaluate the correlation between miRNAs expression and traditional biomarkers [CA125 and human epididymis protein 4 (HE4)]. 32 patients with epithelial OC (54±14 years old) and 31 healthy controls (55±17 years old) were enrolled. Serum samples were collected prior to definitive surgical treatment and RNA extraction was performed by using the miRNeasy Serum/Plasma kit (Qiagen GmbH). miR-199a and miR-125b expression was determined by quantitative real time-polymerase chain reaction (TaqMan MicroRNA Assay, Applied Biosystems). The expression levels of miRNAs were normalized to miR-16 and calculated utilizing the $2^{-\Delta Ct}$ method. Serum levels of miR-199a and miR-125b were significantly higher in OC patients compared to controls (P=0.007 and P=0.002, respectively). A marginally statistically significant correlation was found between miR-199a and miR-125b expression levels (r=0.38, P=0.03). The ROC curve analysis of the diagnostic performance between healthy controls and OC patients revealed that HE4 had a significantly higher area under the curve (AUC=0.90) when compared to CA125 (AUC=0.85), miR-199a (AUC=0.70) and miR-125b (AUC=0.67). Anyway, the determination of circulating miRNAs may be relevant, since their expression is known to be aberrant in cancer, having potential ability to monitor tumor dynamics.

INTRODUCTION

The epithelial ovarian cancer (EOC) is the leading cause of gynecological cancer-related mortality worldwide, since it is commonly detected at an advanced and scarcely curable stage (1). Age-standardised incidence rate is >9.1 per 100,000 women in Central and Eastern Europe (2). The crude incidence rate in Europe changes from 12.4 per 100,000 in the age group of 40-44 years to 35.9 per 100,000 in the age group of 60-64 years. Since EOC is generally asymptomatic in the early stages, about 75% of patients are diagnosed at an advanced stage (3).

The carbohydrate antigen 125 (CA125) is the widely used biomarker for monitoring patients affected by EOC, although being characterized by low sensitivity and specificity (4). Accordingly, elevated serum CA125 concentrations can be found not only in a broad range of benign gynecologic disease, but also in malignancies of different origin, including non-ovarian gynecologic

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Ricevuto: 18.01.2016 Revisionato: 22.03.2016 Accettato: 24.03.2016

Pubblicato on-line: 16.11.2016 DOI: 10.19186/BC_2016.035

cancers, other epithelial tumors and non-epithelial malignancies (5). Another serum biomarker more recently proposed for monitoring patients with EOC is human epididymis protein 4 (HE4). Serum HE4 shows advantages over CA125 in terms of specificity, as it is less frequently increased in patients with non malignant ovary diseases. However, guidelines do not recommend the use of this biomarker to screen the malignancy of a pelvic mass (6). Although recent meta-analyses have reported that HE4 exhibits higher rule-in capability for EOC than CA125, more studies focusing on early tumour stages are required (7-9).

Carcinogenesis is characterized by the accumulation of both genetic and epigenetic alterations, which can be used as disease biomarkers to provide important information for early detection of malignant diseases usually asymptomatic at early stages (10, 11). Notably, the number of genes involved in development and progression of tumours that are epigenetically silenced probably overcomes the number of genes inactivated by mutation (12). Recent reports showed that methylated DNA and microRNAs (miRs) expression could be readily detected in a wide variety of tissues, as well as in various body fluids, indicating that these epigenetic biomarkers could represent the next generation biomarkers for cancer detection (13, 14). miRs, a class of small non-coding RNAs involved in regulating a variety of biologic processes (15, 16), have been reported to be stably detectable in plasma and serum (17, 18) and to exhibit resistance to endogenous ribonuclease activity (19). By targeting multiple transcripts, a single miR can regulate many fundamental cellular processes, such as cell proliferation, apoptosis, differentiation and migration. On the other hand, any gene can be regulated by multiple miRs (20). Alterations in miR expression are not simply an effect of tumorigenesis and may have a causative role in cancer development. They are involved in the initiation, progression and metastasis of human tumors.

Iorio et al. (21) first evaluated genome-wide miR expression profiles of ovarian cancer tissues and normal ovary tissues, concluding that miRs expression was differentially regulated in the two groups. More specifically, miR-200a, miR-141, miR-200c and miR-200b were found to be over-expressed in ovarian cancer, while miR-199a, miR-140, miR-145, and miR-125b were down-regulated. Both miR-199a and miR-125b are involved in many biological processes including inhibition of tumor angiogenesis, a fundamental process for cancer development and growth. These miRs mainly act by targeting the 3' untranslated region of vascular endothelial growth factor (VEGF) (22), by increasing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity (23) and by negatively regulating tumor suppresor p53 (24).

The aim of our study was to investigate the serum expression levels of miR-199a and miR-125b in EOC patients in comparison to healthy controls and to evaluate the correlation between miRs expression and concentrations of CA125 and HE4 in serum.

MATERIALS AND METHODS

Patients and samples

The study population consisted in 32 consecutive women (54±14 years old) with EOC, enrolled between December 2007 and February 2013 at the Obstetrics and Gynecology Clinics of the University Hospital of Verona. Blood was drawn in vacuum tubes containing no additives (Becton-Dickinson) prior to any therapeutic procedure. After centrifugation at 1,500g for 10 min at room temperature, serum was separated, stored in aliquots and kept frozen at -80 °C until measurement. The histopathology results were confirmed by surgical resection of the tumors and the tumor stage was defined according to the International Federation of Gynecology and Obstetrics (FIGO) system criteria (25). The demographics and clinical features of the patients are listed in Table 1. The control population consisted of 31 apparently healthy females (55±17 years old), matched by age and ethnicity. The study was carried out in accordance with the ethical standards of the revised Declaration of Helsinki.

Laboratory methods

Serum CA125 was measured using a chemiluminescent enzyme immunoassay on the Liaison platform (DiaSorin). Inter-assay CV was 4.6-5.8%. Serum HE4 was determined using an ELISA kit developed by Fujirebio Diagnostic, Inc. Total CV quoted by the manufacturer is <10%.

The isolation of miRNA from serum samples was

Table 1
Demographics and clinical features of enrolled patients

Age, years (±DS)	54.1 (±14.3)
≥55 years, n (%)	15 (46.9)
<55 years, n (%)	17 (53.1)
FIGO stage, n (%)	
I	4 (12.5)
II	2 (6.3)
III	26 (81.3)
Histological grade, n (%)	
1	4 (12.5)
2	7 (21.9)
3	21 (65.6)
Histology, n (%)	
Endometrioid	4 (12.5)
Clear cells	4 (12.5)
Undifferentiated	2 (6.2)
Mixed epithelial tumor	3 (9.4)
Serous	18 (56.2)
Transitional	1 (3.1)

FIGO, International Federation of Gynecology and Obstetrics.

performed with the miRNeasy RNA isolation kit (Qiagen), according to the manufacturer's instructions. Briefly, 200 μL of serum sample was added with 1 mL of QIAzol Lysis Reagent (Qiagen) and mixed by gentle vortexing. Aqueous and organic phase separation was achieved by addition of chloroform. The aqueous phase was applied to an RNeasy spin column. The miR was eluted from the column with 14 μL of nuclease-free water. Quantity of RNA was assessed using small-RNA chip on Bioanalyzer 2100 (Agilent). RNA aliquots were stored at -80 °C. For miR quantitative polymerase chain reaction (qPCR), reverse transcription was performed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) in a total volume of 10 μL containing 3.33 μL RNA, 1 μL 10x reverse transcription buffer, 0.67 μL mutiscribe reverse transcriptase, 0.13 μL RNase inhibitor. The reaction mixture was incubated for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C and then held at 4 °C. The complementary DNA (cDNA) served as the template for real-time PCR. The reactions were performed in triplicate on a 7500 Real-Time PCR system (Applied Biosystems) using TaqMan MicroRNA assay (Applied Biosystems). Briefly, qRT-PCR was carried out in a total of 20 μL volume containing 1.33 μL cDNA, 1x universal PCR master mix and 1 μL gene-specific primers and probe. PCR parameters were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The comparative cycle threshold (Ct) method was applied to quantify the expression levels of miRNAs. The relative amount of miR-199a and miR-125b to small nuclear miR-16 was calculated using the equation $2^{-\Delta Ct}$, where ΔCt = (Ct miR-199a/miR-125b - Ct miR-16) (26).

Statistical analysis

Data were tested for normality using the D'Agostino and Pearson omnibus normality test. Non-normally and normally distributed variables were reported as median (range) or mean ± SD, respectively. The concentrations of different biomarkers were compared between cases and controls using Kruskall-Wallis and Mann-Whitney tests according to their value distribution. The correlation between variables was assessed with Spearman's correlation coefficient. For each miR and serum biomarkers, the diagnostic performance in terms of discriminatory capability was calculated by means of ROC curves. The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software) and the level of statistical significance was set at P <0.05.

RESULTS

Serum concentrations of CA125 and HE4 were found to be significantly higher in EOC patients than in controls (Table 2). However, the median CA125 and HE4 concentrations were not statistically different between advanced (stage III) and early EOC stage (I-II). In EOC patients, a statistically significant correlation was found between CA125 and HE4 levels (r=0.46, P=0.009). The serum concentrations of miR-199a and miR-125b were found to be significantly higher in EOC patients compared to healthy controls (P=0.007 and P=0.002, respectively) (Figure 1). However, the concentrations of miR-199a and miR-125b were not significantly higher in patients with advanced cancer (FIGO stage III) in comparison to early stages (I and II) (P=0.72 and P=0.12). Moreover, miR-199a and miR-125b serum concentrations were found to be not significantly different in patients with grade 3 EOC compared to those with grade 1 and 2 (P=0.23 and P=0.35, respectively). A significant correlation was found between miR-199a and miR-125b serum concentrations (r=0.38, P=0.03). Serum concentrations of miR-199a were not significantly correlated with CA125 or HE4 values, whereas miR-125b expression levels correlated significantly with CA125 (r=0.33, P=0.007), but not with HE4.

The ROC curve analysis evaluating the capability to discriminate EOC from healthy, revealed that the single measurement of HE4 exhibited a significantly higher area under the curve (AUC) compared to CA125, miR-199a and miR-125b (Figure 2).

DISCUSSION

Survival of women affected by EOC is higher when the diagnosis is made at an early stage, but it dramatically drops when the cancer has spread to the pelvis and peritoneum. Therefore, a better diagnostic approach is needed to improve the clinical outcome (27). Several studies have demonstrated the limitations of using CA125 for detecting EOC. CA125 may increase in patients with other malignant or gynecological benign disease (e.g., endometriosis), resulting in a poor diagnostic specificity and in a high rate of false-positive results (28). Furthermore, a low sensitivity is generally reported in all stages of ovarian cancer and particularly in early stages (29). For these reasons, CA125 is not useful to rule-out or rule-in patients for EOC.

Among additional putative tumour biomarkers, HE4

Table 2
CA125 e HE4 concentrations in patients with ovarian cancer and healthy controls

	Patients (n=32)	Controls (n=31)	P value	Stage I-II (n=6)	Stage III (n=26)	P value	Grade 1-2 (n=11)	Grade 3 (n=21)	P value
CA125, kU/L	174.6 (3.3-3158)	12.0 (5.0-32.0)	<0.0001	38.1 (6.8-2176)	201.8 (3.3-3158)	0.22	96.0 (6.5-2176)	174.9 (3.3-3158)	0.69
HE4, pmol/L	123.6 (34.1-2300)	37.5 (25.0-121.2)	<0.0001	76.9 (43.6-208)	128.1 (34.1-2300)	0.14	81.6 (43.6-861)	136.8 (34.1-2300)	0.24

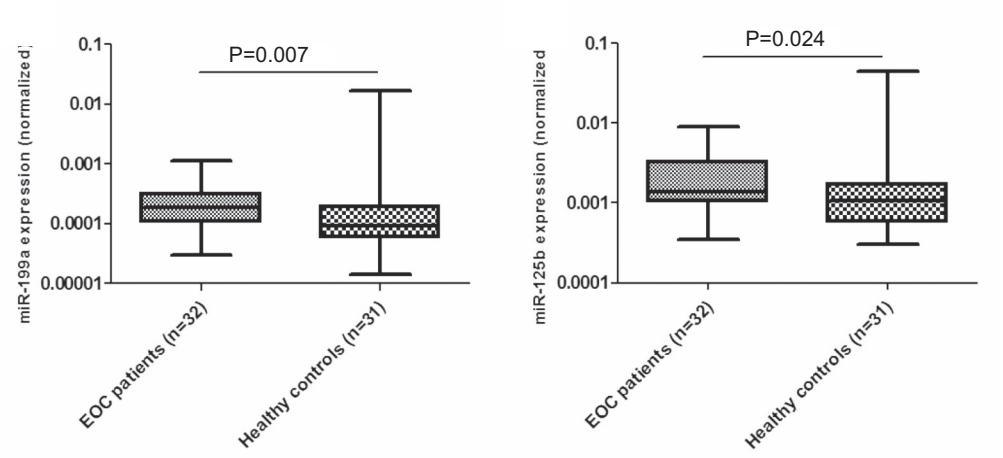

Figure 1
miR-199a and miR-125b expression levels in patients with epithelial ovarian cancer (EOC) and healthy controls.

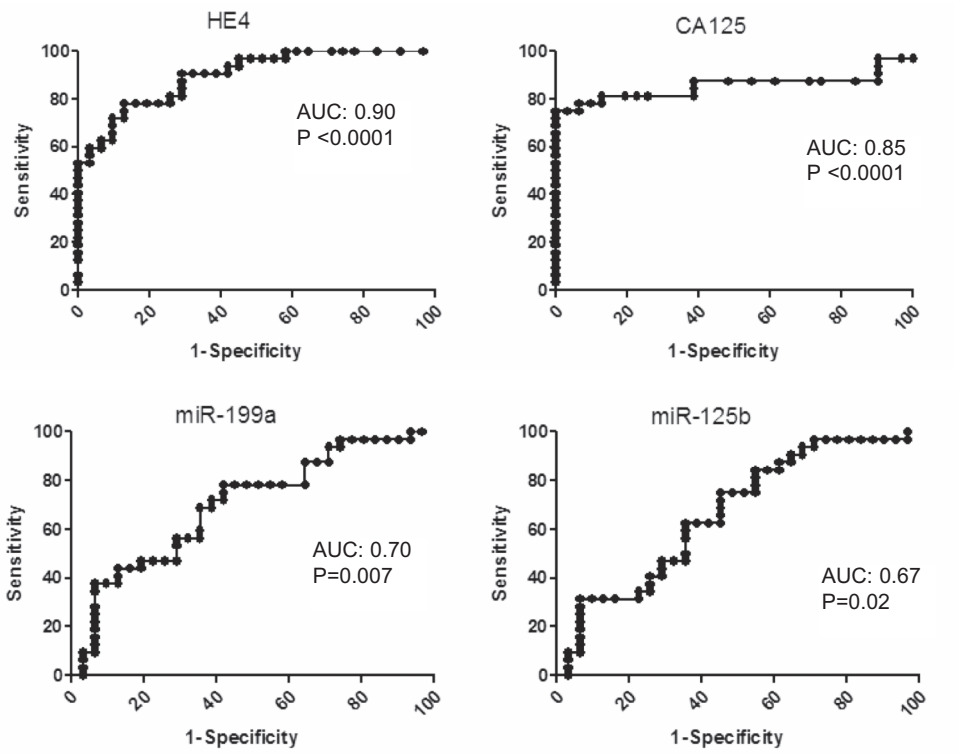

Figure 2
HE4, CA125, miR199a and miR-125b ROC curves obtained on patients with ovarian cancer and healthy controls. AUC, area under the curve.

seems the most promising (30-32). In our study, the AUC for HE4 and CA125 were 0.90 and 0.85, respectively, which agrees with previous evidence published by Ghasemi et al. (33). Anyway, a considerable expression of HE4 in normal tissues and the lack of increase in borderline tumors are the main limitations to HE4 specificity (34, 35). According to previous observations (36), we have also found that no difference exists in the concentrations of HE4 among different EOC FIGO stages. However, this result could be due to the low sample size. Indeed, the major limitation of our study is the small number of investigated subjects and, in particular, the low number of patients in early stages.

More recently, miRs were identified as cancer biomarkers in cell-free serum (37). They are readily detected in blood and they can be measured non-invasively, thus opening new avenues about the clinical usefulness of epigenetic biomarkers for early cancer detection (38, 39). The results of our study suggest that miR-199a and miR-125b were up-regulated in serum of

EOC patients compared to controls. In previous studies based on ovarian cancer tissues/cell lines, both these miRs resulted to be down-regulated (21, 40). In another study by Chen et al., the expression of miR-199a was found to be significantly higher in type II (high-grade, serous EOC) cancer compared with type I EOC (41). Type I tumors comprise low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas. They are generally indolent, present in stage I (tumor confined to the ovary) and are characterized by specific gene mutations, including *KRAS* and *BRAF*, which target specific cell signaling pathways. These tumors rarely harbor p53 mutations and are relatively stable genetically.

The significant difference between data obtained in different studies can be reliably attributed to the different enrolled populations, wherein 65.6% of our patients were diagnosed in the most advanced stage and 56.2% of the EOC were of serous origin. Notably, Chen et al. (41) previously described that hsa-miR-199a has a functional role in carcinogenesis by regulating 3-phosphoinositide-dependent protein kinase-1-mediated IκB kinase β (IKKβ) expression and this evidence is seemingly in support of our finding suggesting that this miR may be somehow involved in the pathogenesis of this type of cancer.

As reported in the scientific literature, miR-125b may be up-regulated in some tumors and down-regulated in others (42). According to its biological activity and function, this miR interplays with many target genes related to tumor growth and metastatic invasion, progression survival and chemotherapy recurrence, but its specific metabolic pathway remains largely unknown (42). It has been recently shown that p53 protein, a well-known anti-tumor molecule, is a putative miR-125b target (43). Le et al. (44) demonstrated that miR-125b may regulate cancer growth by inhibiting p53 expression through direct binding with p53 mRNA 3'UTR.

Taken together, our results show that HE4 retains better diagnostic performance in EOC patients than the conventional biomarker CA125 and also better than two promising miRs, such as miR-199a and miR-125b. Currently, the identification of cancer-specific miR profiles in the circulation is an emerging field of particular interest. Accordingly, the evidence that serum concentrations of these two miRs is considerably increased in patients with EOC underpins that their assessment may retain some biological interest in basic research and for increasing our understanding of the still intriguing EOC carcinogenesis. However, further studies remain to be performed to elucidate the biological significance of these miRs in ovarian cancer.

CONFLICTS OF INTEREST

None.

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