Circulating miRNA 27a and miRNA150-5p; a noninvasive approach to endometrial carcinoma

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Abstract

Background

The search for novel non-invasive biomarkers such as epigenetic molecular markers is new hope for common & burdensome cancers. We aim to assess serum expression of miRNA 27a and miRNA150-5p in endometrial cancer patients.

Methods

Serum was drawn for 36 un-intervened endometrial cancer patients scheduled for hysterectomy & 35 controls. miRNA 27a and miRNA150-5p were measured by real time reverse transcription polymerase chain reaction.

Results

Significant overexpression of both miRNA in patients ($p < 0.001$). At cutoffs 0.2872 & 1.02, miRNA 27a showed 100%sensitivity, specificity, positive & negative predictive values. miRNA150-5p showed 88.89%sensitivity, 100%specificity, 100%positive and 78.9%negative predictive values. Areas under curve were 1.0 for miRNA 27a, 0.982 for miRNA 150 performing much better than Ca125. miRNA 27a was significantly associated with type I endometroid endometrial cancer.

Conclusion

We suggest miRNA 27a and miRNA-150-5P as promising biomarkers of endometrial cancer possibly part of a miRNA panel for management.

Introduction

Endometrial cancer (EC) incidence has doubled in the last 20 years with the increasing burden of obesity and is also expected to increase in developing countries. Incidence has increased steadily due to changes in lifestyle of women, delayed marriage and decreased gravidity, which make it one of the major lethal cancer of all gynecologic malignancies. It is the most common female genital tract malignancy worldwide, and its prevailing histological type is endometrial endometrioid adenocarcinoma. [1, 2]

EC diagnosis depends on the combination of ultrasound, MRI, and serological markers but none of them are completely satisfactory. Ca125 is as a serum marker for EC diagnosis and screening but is now recognized to have poor specificity as other gynecologic malignancies such as ovarian cancer can also show the raise of Ca125. Thus, the identification of accurate and validated biomarkers for EC is needed to improve diagnosis. [3]

Epigenetic molecular regulations include RNA-based machinery. Regulatory noncoding RNAs including microRNAs (miRNAs) constitute the most important class in most tissues. Most miRNAs are located within cells but have also been found circulating in body fluids. [4] MiRNA synthesis begins with the transcription of primary RNA transcript (pri-miRNA) which is then processed to pre-miRNA (a 70–100 nucleotide precursor miRNA).[5] which in turn is exported from the nucleus to the cytoplasm through exportin-5.[6] Mature miRNAs form an RNA-induced silencing complex (miRISC) along with Dicer, RNA-binding protein, and Argonaut proteins. [7] The miRISC targets mRNA and regulates their translation [8]. MiRNAs target messenger RNAs (mRNAs) via sequence specific interaction to repress translation or degrade target mRNA [9]. MiRNAs encompass nuclear functions involving the regulation of gene expression at transcriptional level. [10]

MiRNAs play a role in the human endometrium development and in endometriosis [11]. Changes in the expression of uterine miRNAs have been postulated to play a role in endometrial pathologies [12]. Many factors have been established for endometrial cancer including oncogenic pattern, factors for metastasis and invasion and the difference in expression pattern of some miRNAs from that of normal endometrium [13] In endometrial cancer, miRNAs including miR-185, miR-106a, miR-181a, miR-210, miR-423, miR-429 and others are upregulated and involved in oncogenesis, invasion and metastasis. [14–16] Other miRNAs including miR-let7e and miR-30c are downregulated in endometrial cancer. [14, 15, 17]

MiRNA 27a is a family member of miR-23a ~ 27a ~ 24 − 2 cluster. Expression levels of the three family members within the cluster varies in different pathological conditions. All the three members are highly expressed in acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML), while in they are down-regulated in acute promyelocytic leukemia (APL). [18] MiRNA 27a has been confirmed as an important regulator in important pathological processes including osteoarthritis [19], viral infections [20], adipocyte differentiation [21] fat metabolism and cell proliferation [22], and multidrug resistance [23] Its oncogenic role has been illustrated by its upregulation in osteosarcoma, [24] colon carcinoma [25] and breast cancer.[26] This role was demonstrated in tumor promoting functions as increasing cancer progression and resistance to chemotherapeutic agents. [27]

MiRNA150-5p serve as a biomarker of human lymphocyte activation in health and disease conditions by promoting lymphocyte activation. It has been previously reported to be downregulated in various types of hematopoietic malignancies, including leukemia, lymphoma and myelodysplastic syndrome [28] MiRNA150-5p aberrant expression has been associated with tumorigenesis, cancer development by influencing oncogenes and/or tumor suppressor genes [29] The functions and regulatory mechanism of miRNA150-5p as an oncogene or tumor suppressor gene in certain solid tumors is variable. High expression levels have been identified in gastric and breast cancer while its expression was found to be decreased in oesophageal squamous cell carcinoma [30–32] In gastric cancer, miRNA150 overexpression downregulated the expression of the proapoptotic gene, early growth response factor 2 [33] While in breast cancer, blocking miRNA150 action with inhibitors in cell lines resulted in cell death and ectopic expression of miRNA150 promoted growth and clonogenicity, and reduced apoptosis.[30]

The aim of the current work was to study the serum expression level of miRNA 27a and miRNA150-5P in endometrial cancer patients and to correlate their levels with tumor staging as well as different clinical and laboratory findings of the studied patients.

Materials And Methods

Subjects. Thirty-six un-intervened patients who were diagnosed with endometrial cancer attending Shatby Alexandria University hospital in the period between July 2018till February 2019 and scheduled for surgery were included in this study. Also, 36 age and sex matched healthy subjects were included as a control group. Patients with other malignancies, systemic diseases (as hepatic; renal; cardiac or respiratory diseases), sepsis, and collagenic diseases (as systemic lupus erythematosis, rheumatoid arthritis and multiple sclerosis) were excluded. Serum expression level of miRNA 27a and miRNA150-5p were measured by real time polymerase chain reaction RT-PCR.

Gene expression study. Total serum RNA was isolated using miRNeasy Mini Kit (QIAGEN) Cat No./ID: 217004, according to the manufacturer's protocol. Briefly, 5 µl of 5 nM Syn-cel-miR-39 (miScriptmiRNA Mimic) was added to each sample as a spike-in control, and then total RNA was purified from 400 µl of sample. The miRNeasy Mini Kit contains phenol/guanidine-based lysis of samples and silica membrane–based purification of total RNA. Qiazol Lysis Reagent was used to homogenize samples. Addition of chloroform and centrifugation separated the homogenate into aqueous and organic phases. The upper aqueous phase containing RNA was extracted, and ethanol was added to provide appropriate binding conditions for all RNA. The sample was then applied to the RNeasy Mini spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. RNase-free water elutes high quality RNA. The concentration of total RNA was quantified by a Nanodrop 2000 (Nanodrop, USA). The range of results was 11–73. ng/µl. TaqMan MicroRNA Reverse Transcription (RT) Kit, Applied Biosystem was used for the reverse transcription reaction. The recommended reaction volume was 20 µL. The plate was prepared and ABI prism 7900 sequence detection system (Ambion, USA) was used for amplification and detection by RT-PCR. Differences in serum miRNA 27a and miRNA150-5p expression were normalized to cel-miR-39, determined with the Livak ΔCt method, and reported as $2^{-\Delta\Delta Ct}$.

Statistical analysis. Quantitative data of the present work was analyzed, using F-test (ANOVA) and post hoc test (Scheffe) for pair wise comparison. All statistical calculations were performed using IBM SPSS software package version 20.0, where $p < 0.05$ was considered statistically significant.

All study participants signed informed consents. The Research Ethics Committee of the Faculty of Medicine, University of Alexandria approved this study and was conducted in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

Results

Characteristics of the study sample are illustrated in *Table 1.* Relative expression of miRNA 27a and 150-5p were both significantly overexpressed in endometrial cancer patients than control serum samples. This suggests the potential role for both micro RNAs in distinguishing between endometrial cancer and healthy females. ($p < 0.001$).

For diagnosis of endometrial cancer, the overall accuracy of miRNA 27a relative expression at a cutoff of **0.2872** was surprisingly 100%. miRNA 150-5p relative expression had an overall accuracy of 98.2% at a cutoff **> 1.02** while the traditional Ca 125 serum marker had an overall accuracy of 82.2%. At the above mentioned cutoffs, miRNA 27a showed 100% sensitivity and specificity, positive and negative predictive values of 100%. miRNA150 -5p showed 88.89% sensitivity and 100% specificity, 100% positive and 78.9 % negative predictive values. Areas under the curve were 1.0 for miRNA 27a, 0.982 for miRNA150-5p which were higher than serum Ca 125.

Table 2 and *Fig. 2* show the accuracy measures (at cutoff point with highest sensitivity and specificity) of the conventional serum parameter Ca 125 and miRNA 27a and miRNA150-5p as a proposed new marker.

A significant association between miRNA 27a was found; as it was overexpressed in endometrial cancer type I than type II. miRNA150- 5P was overexpressed in postmenopausal when compared to premenopausal endometrial cancer patients. No other associations could be detected as regards tumor grade, stage, depth of myometrial invasion or lymphovascular space or lymph node involvement. Table 3 describes the tumor related variables of the studied endometrial cancer patients. The association of these characteristics with miRNA 27a was described in Table 4 and with miRNA-150-5P in Table 5.

Discussion

Endometrial cancer (EC) is a common malignant gynecological tumor where few biomarkers are useful for early and accurate diagnosis. Serum miRNAs, considering their stability, maybe an effective and minimally invasive diagnostic method. Circulating miRNAs have been reported to have diagnostic significance in cases of EC. Jia W. and his colleagues were the first study using a genome wide serum miRNA expression profiling analysis who found a four-miRNA signature, including miR-222, -23, -186, and – 204 that might serve as a non-invasive approach for EC diagnosis. [34]

In the current study, relative expression of miRNA 27a and miRNA150-5p were both significantly relatively overexpressed in serum of endometrial cancer patients than serum of control. This suggests the potential role for both micro RNAs in distinguishing between endometrial cancer and healthy females. ($p < 0.001$).

Upregulation of miRNA 27a in endometrial cancer patients is in accordance with previous studies. Mozos et al., observed upregulation of miRNA 27a expression in invasive endometrioid adenocarcinoma tissue [35]. Our findings further complete this work as we confirm the upregulation of miRNA 27a in the serum of these patients which proposes it as a strong non-invasive biomarker. Also, we note that miRNA 27a was significantly associated with the same type of endometrial cancer (type I endometroid). [35] reported

that miRNA 27a is one of three miRNAs miR-15b, -27a, and – 22 that might have their unique pathway to regulate the development of EC having diagnostic role [36]

On the other hand, in cervical cancer, decrease in expression of miRNA 27a was documented. Using miRNA array and small RNA sequencing, host miRNAs were specifically regulated by viruses, HPV16 and HPV18 in organotypic raft cultures vaginal keratinocytes. Viral oncoprotein E6, E7 decreased miRNA 27a expression [37]

In a trial to understand the role of miRNA 27a as an oncogenic miRNA, it was demonstrated that activated estrogen receptor can suppress the expression of proapoptotic protein BAX through upregulating miRNA 27a. Thereby increased BCL2/BAX ratio may promote survival and proliferation leading to precancerous lesions and type I endometrial adenocarcinoma.[38] Moreover, miRNA 27a target the expression of FOXO1 (apoptosis factor) resulting in tumor cells survival by apoptosis inhibition. [35]

The current study finding can be explained also by the function of miRNA 27a as a tumor promoter in various other cancers through targeting MAP2K4 as in osteosarcoma(Pan et al. 2014), AGGF1 in bladder carcinoma [39], prohibitin in gastric carcinoma [40], and other genes that control protein transcription factors at G2-M checkpoint demonstrated in breast cancer cells.[41] Genistein anticancer agent studied in colon cancer targeted miRNA 27a also. [42]

Serum miRNA150-5p was also significantly upregulated in patients in comparison to controls. This was in accordance with previous miRNA-based on TCGA-UCEC project [43] focused on miRNA sequences downloaded from The Cancer Genome Atlas Project. Difference of miRNA profile between metastatic and nonmetastatic ECs was studied using bioinformatics technique and miRNA150-5p was differently expressed. It was demonstrated to regulate multiple pathways of cancer, including the Wnt, NOTCH, and TGF-β signaling by functional enrichment analysis.

In cancer cervix also miRNA150-5p played an important role. In invasive cervical squamous cell carcinomas 68 up-regulated miRNAs were identified including miRNA150-5p.[44] Li et al. (2015) [45] demonstrated that the level of miRNA150-5p expression was higher in the advanced stage of cervical cancer and in cervical intraepithelial neoplasia which is a well-defined precursor stages of squamous cell carcinomas.[46] In serum samples from cervical cancer patients expression of miRNA150-5p patients was also increased. MiRNA150-5p promoted the proliferation, migration and invasion of human cervical cancer cells HeLa and SiHa cells. [47]

These findings can be explained by the action of miRNA150-5p as it has multiple targets involved in the cell proliferation, apoptosis, and metastasis including p53, P2X purinoceptor 7 (P2X7 mucins 4 (MUC4), BRI1associated receptor kinase 1 (BAK1), C-Myb. zincfinger Ebox binding homeobox 1 (ZEB1), EGR2, and SRC kinase signaling inhibitor 1 (SRCIN1). Significant downregulation of FOXO4 in C-33A cells expressing miR-150 mimics and the upregulation of FOXO4 (apoptosis factor) in the cells expressing miRNA150-5p inhibitors was also found.[45]The same family of FOX protein was targeted by miRNA27-a as demonstrated by Mozos et al. (2014).[35] MiRNA150 induces the arrest of FOXO4 transcription by

binding to 3'-UTR of its mRNA, therefore reduces p27 and pRb activation, and increases CyclinD1, leading to cell cycle progression and survival.MiRNA150-5p enhances the cell cycle progression from the G1/G0 toS phase due to decrease of p27 and the increase of CyclinD1(Zhang et al. 2018).[43] Cell cycle protein transcription factors act also as a common target also for miRNA 27a [41]. MiRNA150-5p targets PDCD4 gene which is a direct suppressor of NF-κB. It can also suppress AKT pathway as well as the expression of matrix metallopeptidase 9 (MMP-9) which facilitates cancer cell migration.[48] Allgayer et al. have demonstrated that PDCD4 could inhibit the invasion of cells through also regulating the expression of urokinase receptor (u-PAR) which is one of the major invasion-related genes in various cancers. [49]

We found a significant association between miRNA 27a and endometrial cell type; it was overexpressed in endometrial cancer type I than type II. Endometrial adenocarcinoma (EC) has two basic clinicopathologic forms, type I and type II. Type I EC is usually a well to moderately differentiated cancer and accounts for 80–85% of all ECs and includes tumors of endometrioid histology.[50] Type II is poorly differentiated, usually of a nonendometrioid histological subtype, frequently lack steroid receptors. Type I tumors generally arise on a background of endometrial hyperplasia and have a good prognosis survival. It develops in a steroid environment, associated with high levels of hormone receptors and usually responds to hormonal therapy.[51] In a trial to explain the current study finding miRNA 27a expression in breast cancer was demonstrated as an example.[52] A previous study showed that PR + versus PR- breast tumors had higher expression of miRNA 27a.[53] This can indicate that miRNA 27a may be regulated by the ovarian steroids estrogen and progesterone in endometrial epithelium.

Moreover, miRNAs are differentially expressed in noninvasive (stage IA) and myoinvasive adenocarcinomas (stage IB and IC), miRNA 27a was overexpressed in invasive adenocarcinomas, and its expression increased linearly according to tumor stage. Results were validated by RT-PCR in an independent series of EC. The expression of FOXO1 (miRNA 27a main target) was down-regulated in invasive compared with noninvasive tumors. Nonmutated adenocarcinomas showed miRNA 27a overexpression. It was concluded that the miRNA 27-FOXO1 tandem inhibits apoptosis and enhances tumor cell survival in-nonmutated EC. [35]

The current study also demonstrated that miRNA150-5p was overexpressed in postmenopausal more than premenopausal endometrial cancer patients. Since endometrial cancer predominantes in postmenopausal women reflecting the contribution of several extraovarian tissues to circulating estrogens pool by the production of estrogen from adrenal steroids in absence of ovarian synthesis. Estrogen can also be formed from conjugate estrogens such as E_1-S. The sulfatase enzyme, which produces E_1 from high circulating E_1-S metabolite, also contributes significantly to estrogen synthesis in malignant endometrium.[54, 55] In vitro studies have demonstrated the modulation of uterine miRNAs by estrogen using isolated endometrial epithelial and stromal cell cultures through their respective receptor-mediated pathways. In addition to modulating miRNA expression at the level of transcription, steroids may also influence the expression of the miRNA biogenesis components necessary for their processing to the mature cytoplasmic form. Among miRNA biogenesis components, Exportin-5 and Dicer1 expressed in the mouse uterus and appear to be the major steroid regulated components in the miRNA biogenesis

pathway.[56] In postmenopausal women the expression of p53, which is targeted by miRNA150-5p, was the highest in adenocarcinoma samples when compared to endometrial polyp and atrophic endometrium. [57, 46]

In the current study miRNA 27a showed 100% sensitivity and specificity, positive and negative predictive values of 100%. MiRNA150-5p showed 88.89% sensitivity and 100% specificity, 100% positive and 78.9 % negative predictive values. Areas under the curve were 1.0 for miRNA 27a, 0.982 for miRNA150-5p which were higher than serum Ca 125. A similar study showed that combination of miR27-a and Ca125 had an AUC of 0.894 (95% CI, 0.807, 0.980; sensitivity = 0.774, specificity = 0.970), which makes miRNA 27a an optimal non-invasive biomarker to diagnose EC.[36] A recent study identified a series of miRNA/mRNA pairs (miR-497/EMX1, miR-23c/DMBX1, and miR-670/KCNS1) to be associated with survival in EC.[58] In light of the above we suggest a similar diagnostic panel including miRNA 27a and miRNA150-5p to be considered for larger scale studies and further evaluation.

Declarations

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- Authors' contributions : All authors whose names appear on the submission

    1. made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work;
    2. drafted the work or revised it critically for important intellectual content;
    3. approved the version to be published; and
    4. agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
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Tables

Table (1): Comparison between the two studied groups according to different parameters

Characteristics	Endometrial Cancer patients (n = 36)	Controls (n = 36)	Test of sig.	p
Age (years)				
Mean ± SD.	57.6 ± 8.3	53.7±9.4	t= 1.488	0.143
Median (Min. – Max.)	59.5 (40 – 70)	54 (39 – 67)		
Ca125 (U/mL)				
Mean ± SD.	25. 5 ± 15.8	11.3 ± 4. 5	U=96.0*	<0.001*
Median (Min. – Max.)	19.1 (3.4 – 56.3)	9.9 (5. 6 – 19.2)		
miRNA Relative expression				
miRNA 27-a				
Mean ± SD.	1.1 ± 0.5	0.1 ± 0.1	U=0.0*	<0.001*
Median (Min. – Max.)	0.9 (0.5 – 2.4)	0.1 (0 – 0.3)		
miRNA 150-5p				
Mean ± SD.	1.7 ± 0.7	0.6 ± 0.3	U=9.50*	<0.001*
Median (Min. – Max.)	1. 5 (0. 9 – 3.4)	0.6 (0.1 – 1)		

U: Mann Whitney test t: Student t-test

p: p value for comparing between the two studied groups

*: Statistically significant at p ≤ 0.05

Table (2): Accuracy measures with cut-off points of serum miRNA 27a and miRNA-150-5p relative expression in prediction of endometrial cancer as opposed to Ca 125 as an old marker.

	AUC	p	95% C.I		Cut off	Sensitivity	Specificity	PPV	NPV
			L.L	U.L					
Ca125 (U/mL)	0.822*	<0.001*	0.701	0.943	**>12.23**	83.33	73.33	88.2	64.7
Relative expression									
miRNA 27a	1.000*	<0.001*	1.000	1.000	**0.2872**	100.0	100.0	100.0	100.0
miRNA-150-5P	0.982	<0.001*	0.955	1.009	**>1.02**	88.89	100.0	100.0	78.9

AUC: Area under a Curve

P value: Probability value

CI: Confidence Intervals , L.L (lower limit), U.L (upper limit)

PPV positive predictive value, NPV negative predictive value

*: Statistically significant at p ≤ 0.05

Table (3): Tumor related variables in the studied cases (n = 36)

	No. (%)
Menopause	
Pre	9(25%)
Post	**27(75 %)**
Tumour	
Type	
I	28 (77.8%)
II	8 (22.2%)
Grade	
I	9 (25%)
II	22 (61.1%)
III	5 (13.9%)
Stage (n = 34)	
I	**20 (55.6%)**
Ia	13 (38.2%)
Ib	7 (20.6%)
II	**10 (29.4%)**
IIIc	**4 (11.8%)**
Depth of myometrial invasion (n = 34)	
< 50%	17 (50%)
> 50%	17 (50%)
Lymph vascular invasion(n = 34)	
Space	
Negative	23 (67.6 %)
Positive	11 (32.4%)
Nodes	
Not done	1 (2.9%)
Negative	27 (79.4%)
Positive	6 (17.6%)

Table (4): Association of miRNA 27a relative gene expression with tumor related variables (n=36)

	No.	miRNA 27a			Test of sig.	p
		Min. – Max.	Mean ± SD.	Median		
Menopause						
Pre	9	0.5 – 1.4	1 ± 0.3	0.9	U= 113.00	0.774
Post	27	0.5 – 2.4	1.1 ± 0.6	0.8		
Tumor						
Type						
I	28	0.5 – 2.4	1.2±0.6	0.9	U=54.0*	0.027*
II	8	0.6 – 1.3	0.8 ± 0.3	0.7		
Grade						
I	9	0.5 – 1.1	0.8 ± 0.2	0.7	H= 5.583	0.061
II	22	0.6 – 2.4	1.2 ± 0.6	0.9		
III	5	0.6 – 1.3	1.0 ± 0.4	0.9		
Stage (n = 34)						
Ia	13	0.5 – 2.4	1.2 ± 0.6	1.1	H= 4.469	0.215
Ib	7	0.6 – 2.2	1.4 ± 0.7	1.7		
II	10	0.6 – 0.9	0.8 ± 0.1	0.8		
IIIc	4	0.7 – 1.3	1.0 ± 0.4	1.0		
Depth of myometrial invasion						
< 50%	17	0.5 – 2.4	1.3 ± 0.6	1.1	U= 94.0	0.085
> 50%	17	0.6 – 1.7	0.9 ± 0.4	0.8		
Lymph vascular space						
Negative	23	0.5 – 2.4	1.1 ± 0.6	0.8	U=119.0	0.800
Positive	11	0.6 – 1.7	1.1 ± 0.5	0.9		
Lymph Nodes						
Negative	27	0.5 – 2.4	1.1 ± 0.6	0.8		
Positive	6	0.7 – 1.3	1.0 ± 0.3	0.9		

U: Mann Whitney test H: Kruskal Wallis test

p: p value for comparing between the different categories

*: Statistically significant at $p \leq 0.05$

Figures

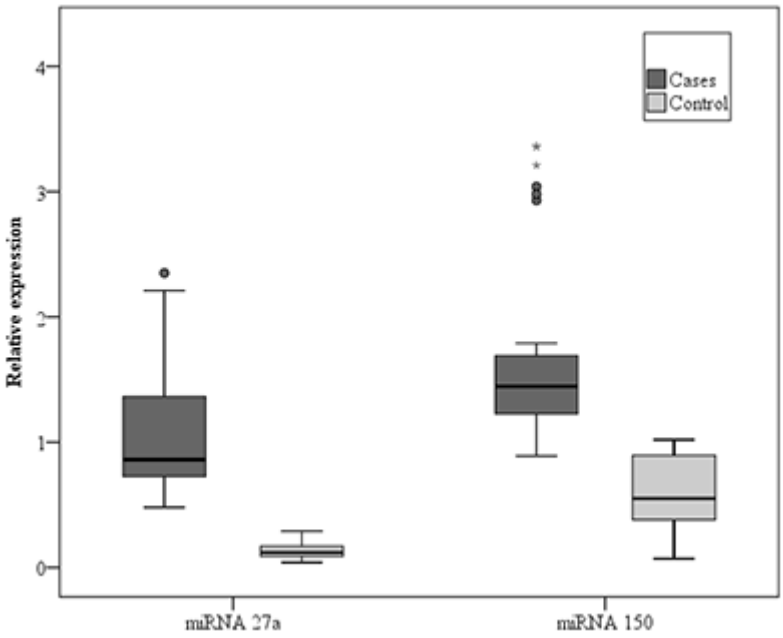

Figure 1

Comparison between the two studied groups according to miRNA 27-a and miRNA 150-5p

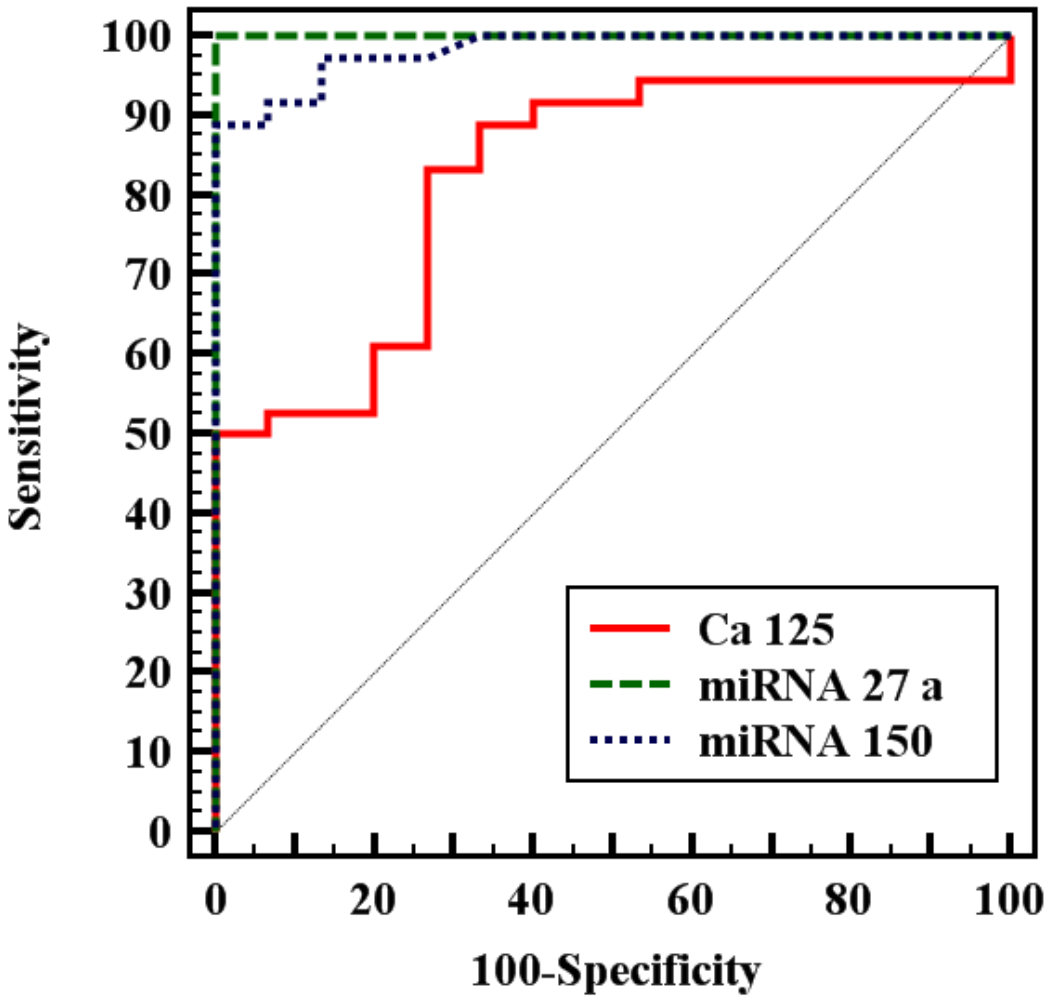

Figure 2

Receiver Operator Characteristics curve of serum levels miRNA 27-a, miRNA-150-5p and Ca 125 in prediction of endometrial cancer.