

The Aberrant Expression Levels of miR-423-5p and miR-664b-5p in Peripheral Blood of Patients With Familial and Sporadic Ovarian Carcinoma

BUSRA KURT (✉ bbusrakurt92@gmail.com)

Division of Cancer Genetics, Department of Basic Oncology, Oncology Institute, The university of Istanbul, Fatih, 34093 Istanbul, Turkey. <https://orcid.org/0000-0003-4151-7711>

Seref Bugra TUNCER

Istanbul Universitesi

Demet AKDENIZ ODEMIS

Istanbul Universitesi

Arash ADAMNEJAD GHAFOR

Istanbul Universitesi

Mukaddes Avsar Sarali

Istanbul Aydın Üniversitesi: Istanbul Aydın Universitesi

Seda KILIC

Istanbul Universitesi

Ozge Sukruoglu Erdogan

Istanbul Universitesi

Betul Celik

Istanbul Universitesi

Pinar Saip

Istanbul University Istanbul Faculty of Medicine: Istanbul Universitesi Istanbul Tıp Fakultesi

Hulya Yazici

Istanbul Universitesi

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Abstract

MicroRNAs (miRNAs), are endogenous noncoding single strand RNA molecules with approximate length of 22 nucleotides. Ovarian cancer is a heterogenous disease which includes different biologic behaviors in the clinical, and molecular level. miRNAs have roles in the early diagnosis, prognosis, and chemotherapy sensitivity for ovarian cancer, and in the regulation of reproduction by being expressed in the ovaries. The expression level of two miRNAs of miR-423-5p, and miR-664b-5p selected from the miRNAs which were detected to be important for the ovarian cancer etiology in our previous studies were investigated in the peripheral blood of familial and sporadic ovarian cancer patients, and in healthy individuals. The expression analyses were statistically evaluated with SPSS v21.0 program using the Quantitative Real-Time PCR technique. A statistically significant difference was detected for both miRNAs between the healthy controls and ovarian cancer patient groups ($p:0.000$). The miR-423-5p expression was detected to increase 2.35 fold, and miR-664b-5p was detected to increase 2.47 fold in ovarian cancer patients compared with the levels in the control group. miR-423-5p was reported to be overexpressed in various cancer types, and in different disease stages in previous study, and miR-423-5p was demonstrated to have increased in the lymphocytes in the peripheral circulation of ovarian cancer patients first in our literature. The miR-664b-5p expression level was detected in high levels in ovarian cancer patient group, however was detected to have decreased in 64% of ovarian cancer patients with BRCA mutation carrier. The expressions of both miRNA molecules were detected to have decreased in the ovarian cancer subgroups in patients with ovarian cancer with addition of a second primary cancer of endometrium cancer, however, were detected in the highest level in ovarian cancer patients in addition to another secondary cancer except breast and endometrium cancer. The results obtained from the study showed that the increased expression of both miRNA molecules were suggested to be associated with ovarian cancer, and decrease was suggested to be associated with endometrium cancer. In summary, the miRNA molecules in the present study might be the non-invasive biological indicator in ovarian cancer, and the demonstration of these molecules on the tumor tissue, and their change during treatment in future studies will be beneficial.

Introduction

MicroRNAs are small endogenous non-coding single strand RNA molecules in a length of approximately 22 nucleotides. miRNAs have roles in the regulation of various cellular processes such as cell proliferation, differentiation, apoptosis, hormone biosynthesis, and secretion [1]. miRNAs was suggested to regulate the cellular gene expression in transcriptional and post transcriptional level [2]. The functioning of some miRNAs as oncogene and some RNAs as tumor suppressive gene in various cancers demonstrated that miRNAs had regulatory roles in tumor development, metastasis, and invasion [3, 4].

The changes in the protein coding oncogene and/or tumor suppressive genes are known to cause cancer. The genetic causes of cancer were reported to be as complex with the demonstration in recent years that miRNAs were also effective in tumorigenesis [5]. The detection of more than 50% of cancer associated

genomic regions or fragile regions consisting of miRNA encoding genes revealed that miRNAs were important in cancer pathogenesis [6].

The first study revealing the association of miRNA genes, and cancer was performed by Calin et al. [7]. The 13q14 deletion detected in patients in the occurrence of disease in this first study with CLL patients was shown to be associated with the presence of miR-15, and miR-16 genes rather than the suggestion that developed with the disappearance of a tumor suppressor gene.

Ovarian cancer is the 7th most prevalent malignancy worldwide [8]. miRNAs are expressed in the ovaries, and have roles in the regulation of the reproduction of the mammals. miRNAs have significant role in the early diagnosis, prognosis, and chemotherapy sensitivity in ovarian cancer [1, 9, 10]. Song et al. demonstrated in their study that miR-664b-5p functioned as a tumor suppressive gene, and its overexpression suppressed the cell growth, invasion, and invasion [11].

The expression levels of two miRNA molecules named as miR-664b-5p, and miR-423-5p which were detected in a high risk ovarian cancer family in a previous doctoral thesis study, and were demonstrated as important for ovarian cancer etiology however yet not associated with ovarian cancer in the literature, were investigated in 150 familial and sporadic ovarian cancer patients, and in 100 healthy individuals who were matched for age, sex, and ethnicity with the patients in the present study. We investigated the place of miRNAs in ovarian cancer etiology, diagnosis and, in addition to targeting to perform their validation, and their distributions in ovarian cancer.

Materials And Methods

Experimental Group, and Patients: The experiment group consisted of 150 ovarian cancer patients selected among patients who presented to Cancer Genetics clinic of Istanbul University, Oncology Institute for *BRCA1/2* testing, and the control group consisted of 100 healthy individuals who were matched for age, sex, and ethnicity with patients and who had no history of cancer in the last three generations in the study. The experimental procedures were maintained using the RNA materials of a total of 127 ovarian cancer patients, and 98 healthy individuals after the quality control of the biologic materials were performed. 103 out of 127 ovarian cancers were only diagnosed with ovarian cancer with no other secondary cancer. 16 patients were diagnosed with both ovarian, and breast cancer, 6 patients were diagnosed with ovarian cancer and other additional cancer, and 2 patients were diagnosed with both ovarian and endometrium cancer.

Declaration: The present study was approved by the Clinical Research Ethics Board of Istanbul Faculty of Medicine in Istanbul University (File No:2018-77), and written consent was granted from all participants. This study has been supported by Istanbul University Scientific Research Projects Unit (Project No: 31200).

Lymphocyte Isolation from complete blood: Approximately 10 mL blood taken into EDTA tube was diluted in a ratio of 1/1 using 0.9% NaCl. 2 mL Ficoll was slowly added, and was centrifugated for 30 min at

400g. After the centrifugation procedure, erythrocyte was differentiated at lower phase, cell pellet in medium phase, and plasma was differentiated in upper phase. The phase just above the erythrocyte phase, and consisting of white color lymphocyte cells gathered as a circle in the plasma phase was taken into a clean tube using a pasteur pipette. The lymphocyte cell phase was centrifugated for 10 min at 400g at room temperature, and the supernatant was removed. 4 mL PBS was added on the remaining pellet, and pipetted, and distributed in cryotubes as to provide 1 mL in each tube, and were centrifugated at room temperature for 5 min at 400g. The supernatant was removed. The precipitated cells were freezed at -80°C for 24 h for step by step freezing for future experimental procedures. 24 h later, they were taken into liquid nitrogen tank for long time storing.

RNA isolation from lymphocyte: For RNA isolation from lymphocyte, the Quick-RNA-TM MiniPrep protocol (Quick-RNA-TM MiniPrep, Zymo Research, U.S.A.) was applied. DNA-RNA Lysis Buffer inside the kit was added on cell pellet which was stored at -80°C or in liquid nitrogen storage tank. First, the cell membranes were bursted with DNA-RNA Lysis Buffer, and the DNA and RNA molecules in the cell were revealed. This mixture was loaded on the filter in the cell in the RNA isolation kit, and was centrifugated for 1 min at +4°C at 12.000g. The DNA molecule was stuck on the filter after centrifugating, the lower phase taken into a clear tube and RNA isolation procedure was maintained because the RNA molecules moved to the lower phase. Ethanol was added into RNA phase that was taken into clean tube, and the new mixture was loaded on the special filter holding the RNA, and was centrifugated for 1 min at +4°C at 12.000g.

Then the 400 µL of RNA Prep Buffer was added on the filter, and then centrifugated for 1 min at +4°C at 12.000g. The filter was transferred into a clean tube, and centrifugated for 1 min at +4°C at 12.000g by adding 700 µL RNA Wash Buffer. 400 µL RNA Wash Buffer was again included in the filter, and centrifugates for 2 min at +4°C at 12.000g.

The RNA molecule was collected by centrifugating for 1 min at 12.000g at +4°C by adding RNase-Free Water. The collected RNA was separated into portions, and stored at -80°C.

Measurement of the purity and concentrations of the RNAs: The high quality undegradated RNA with no inclusion of genomic DNA contamination are required for the success of the cDNA synthesis. The purity, and concentration calculations of the obtained RNAs were performed using the NanoDrop 2000 Spectrophotometer [THERMO SCIENTIFIC] device.

cDNA Synthesis: ID3EAL miRNA qPCR Starter Kit protocol was used (ID3EAL miRNA qPCR Starter Kit, MiRXES, Singapore). Kit protocol consists of two phases as cDNA synthesis, and RT qPCR reaction. The cDNA synthesis is performed for the miRNAs in the first phase, and two different miRNAs were prepared for RT-qPCR assay in the second phase.

All reactions in this phase were performed on ice. The cDNA synthesis procedure was performed in accordance with the kit protocol. The RNA samples of patients were dissolved before cDNA synthesis reaction, and then were vortexed. The mold RNA (approximately 1 µg), 5 µL ID3AL RT Buffer, 1 µL ID3AL RT Primer, and 1 µL ID3AL RT (reverse transkriptase) were added in the tube of cDNA reaction,

respectively. The volume of the obtained mixture was completed to 20 μ L with distilled water. Pipetting was performed using a micropipette, and then vortex was performed. cDNA forming reaction was completed using the BioRad PCR device by incubating at 42°C for 30 min, and at 95°C for 5 min. The obtained cDNAs were diluted in a ratio of 1:10 using sterilised distilled water. The diluted cDNAs were stored at -20°C, and all cDNAs were used within 4 weeks.

Real Time PCR Reaction: 10 μ L ID3AL qPCR Master Mix, 2 μ L ID3AL qPCR assays, 5 μ L diluted cDNA, and 3 μ L distilled water were included into tubes specially manufactured for Mic qPCR as to include a total of 20 μ L volume. Pipetting was performed using a micropipette, and then vortex was performed.

Quantitative Real Time PCR Reaction: The Quantitative Real Time PCR procedure was performed using the Mic qPCR device (Bio Molecular Systems, MIC qPCR Cyclor, Australia). The Quantitative PCR procedure was performed in a cycle of 10 min at 95°C, and then 5 min incubation at 40°C, and 40 sec at 95°C, and 30 sec at 60°C in 40 cycles. The measurement and calculation of the reactions were performed using the Mic qPCR software on the device. The miRNA expression levels investigated using this software were calculated as quantitative values.

The analysis of miRNA molecules augmented by qPCR: The expression levels of both miR-423-5p, and miR-664b-5p were calculated using the CT (Threshold Cycle) values of each sample of patient and controls detected from Mic qPCR device.

Calculations of the Δ CT and $\Delta\Delta$ CT Values: The expression levels were identified by calculations of the Δ CT and $\Delta\Delta$ CT from the CT values.

Δ CT = CT(target gene) - CT(reference gene).

$\Delta\Delta$ CT = Δ CT(target gene) - $\sum \Delta$ CT(reference gene).

$2^{-\Delta\Delta$ CT value was found after calculation of these values. $\Delta\Delta$ CT value was close to 0 and owing to the $2^0=1$ expression, the reference value was accepted as 1. The increase or decrease of the expression levels were detected in accordance with the reference value [12].

Statistical Analysis: The comparison of the miRNA expression levels of patients, and control groups was performed using the IBM Support Statistical Package for the Social Sciences (SPSS) program. We evaluated the Fold&Change (FC) ratios of samples using the "Independent-Samples T-Test" in the specified study groups. The values for Fold&Change ratios of $|FC| \geq 2$, and with p value smaller than <0.05 were taken into consideration. We used the Kaplan-Meier analysis for investigating whether miR-423-5p, and miR-664b-5p expression levels were the variables affecting the survival period. We performed the Receiver Operator Characteristics (ROC) analysis for demonstrating the diagnostic performance of miR-423-5p, and miR-664b-5p molecules as the candidate bioindicators. We performed the "Spearman's correlation analysis" for identifying the possible linear association of numerical measurements of miRNA expression levels and the CA-125 values obtained from the clinical data of the ovarian cancer patients.

Results

We included 150 ovarian cancer patients who presented to Cancer Genetics clinic of Oncology Institute in Istanbul University for *BRCA1/2* investigation, and 100 healthy controls who were matched for age, sex, and ethnicity with patients were included in the study. The experimental procedures were continued using the RNA materials of a total of 127 ovarian cancer patients, and 98 healthy individuals after the quality control of biological material was performed. The mean age of the ovarian cancer patient group was 50 ± 10 (23-84y) years, and the mean age of the control group was 48 ± 11 (23-84y) years.

The normality estimation of the investigated groups was identified using the Kolmogorov-Smirnov test. The normality distributions of the data were calculated for miR-423-5p (Kolmogorov-Smirnov $Z=4,731$; $p<0.05$); and for miR-664b-5p (Kolmogorov-Smirnov $Z=5,619$; $p<0.05$). We used the post-hoc ANOVA test in the resolution of the data as the groups showed normal distribution, and to perform calculation for subgroups, and there were a total of 5 subgroups.

The calculations based on $2^{-\Delta\Delta C_t}$ values using the ANOVA test between the patient and control groups showed that miR-423-5p, and miR-664b-5p expression levels were found statistically significantly higher in patient group compared with the healthy controls ($p:0.000$).

Comparison of the Presence of BRCA mutation and miRNA expression levels of patients: miR-423-5p expression was found to have increased in 52% (13/25), and decreased in 48% (12/25) of patient who were positive for BRCA mutation in our study (**Table 1**). However, miR-423-5p expression was found to have increased in 57.8% (59/102), and was found to have decreased in 42.2% (43/102) of patients who were negative for BRCA mutation. In general, miR-423-5p was found to have similar distribution in positive and negative group for BRCA mutation.

The comparison of the presence of BRCA mutation, and the miR-664b-5p expression showed that miR-664b-5p was detected to have decreased both in most negative, and positive cases. Accordingly, miR-664b-5p expression level was detected to have increased in 36%(9/25) of BRCA mutation positive patients, and to have decreased in 64% (16/25) of the patients. In addition, miR-664b-5p expression level was found to have increased in 39.2% (40/102), and decreased in 60.8% (62/102) in patients with negative BRCA mutation.

Table 1: The Association of the BRCA Mutation Status, and miRNA expression levels in patients with ovarian cancer.

BRCA Mutation Status	miR-423-5p		miR-664b-5p		
	Increased	Decreased	Increased	Decreased	Total
	n(%)	n(%)	n(%)	n(%)	n(%)
Positive	13(52%)	12(48%)	9(36%)	16(64%)	25(19.6%)
Negative	59(57.8%)	43(42.2%)	40(39.2%)	62(60.8%)	102(80.3%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127

Comparison of the miRNA expression levels in the patient and control groups:

The evaluation of miR-423-5p, and miR-664b-5p expressions in all ovarian cancer patient group included in the study showed that miR-423-5p expression was decreased in 43.3% (55/127), and increased in 56.7%(72/127). miR-664b-5p expression was detected to have decreased in 61.5% (78/127), and increased in 38.5%(49/127) out of the total 127 patients (**Table 2**).

The evaluation of the miR-423-5p, and miR-664b-5p expressions in ovarian cancer and ovarian cancer subgroups showed that the number of patients diagnosed with only ovarian cancer with no other primary tumors was 103 out of the total 127 patients diagnosed with ovarian cancer. miR-423-5p expression was increased in 52.4% (54/103), and was decreased in 47.6%(49/103). miR-423-5p expression level was increased in 68.8%(11/16), and was decreased in 31.2% (5/16) in the group diagnosed with both ovarian and breast cancer. miR-423-5p expression was increased in 50% (1/2), and was decreased in 50% (1/2) in patients diagnosed with two different primary cancers as ovarian and endometrium cancer. miR-423-5p expression was found to have increased in all patients 100%(6/6) with a secondary disease with ovarian cancer except breast, and endometrium cancer (**Table 2**).

miR-664b-5p expression was increased in 35% (36/103), and was decreased in 65% (67/103) of 103 patients out of 127 ovarian cancer patients with only diagnosis of ovarian cancer, and no other primary tumors. miR-664b-5p expression level was found to have increased in 43.8% (7/16), and have decreased in 56.2% (9/16) of patient group diagnosed with both ovarian and breast cancers. miR-664b-5p expression was found to have increased in the half, and was decreased in the half of patients who were diagnosed with both ovarian, and endometrium cancers. miR-664b-5p expression was found to have increased in patients with a secondary primary disease with ovarian cancer except breast, and endometrium cancer (**Table 2**).

Table 2: The association of miR-423-5p, and miR-664b-5p expression status with the ovarian cancer, ovarian cancer patients subgroups

Diagnosis	miR-423-5p		miR-664b-5p		Total n(%)
	Increased	Decreased	Increased	Decreased	
	n(%)	n(%)	n(%)	n(%)	
Only Ovarian Cancer	54(52.4%)	49(47.6%)	36(35%)	67(65%)	103(81.1%)
Ovarian Cancer + Breast Cancer	11(68.8%)	5(31.2%)	7(43.8%)	9(56.2%)	16(12.6%)
Ovarian Cancer + Endometrium Cancer	1(50%)	1(50%)	0(0%)	2(100%)	2(1.6%)
Ovarian Cancer + Other Cancer	6(100%)	0(0%)	6(100%)	0(0%)	6(4.7%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127

A statistical significance was detected between the miRAN expressions of both miRNAS in the statistical comparison between the healthy controls, and ovarian cancer patients groups(p:0.000). miR-423-5p expression was found to have increased 2.35 fold in ovarian cancer patients compared with the control group. Some patients with ovarian cancer were also diagnosed with an additional secondary cancer. Accordingly, some patients were diagnosed with ovarian and breast cancer, some patients were diagnosed with ovarian and endometrium cancer, and one group was diagnosed with another secondary cancer in addition to ovarian cancer. The comparison of the ovarian cancer subgroups with the control group showed that miR-423-5p expression level increased 3.65 fold in patients with both ovarian and breast cancer, and increased 4.69 fold in patients with both ovarian cancer and other cancer, however decreased 0.58 fold in patients diagnosed with ovarian and endometrium cancer (**Figure 1**).

The comparison of all ovarian cancer patients with the control group showed that the miR-664b-5p expression increased 2.47. The comparison of ovarian cancer patient subgroups with the control group for miR-664b-5p showed that the levels increased 1.95 fold in both ovarian and breast cancer patients, and increased in 2.95 fold in ovarian and other cancer patients, however the levels decreased 0.27 fold in patients with both ovarian and endometrium cancer (**Figure 2**).

Evaluation of miRNA expression status in accordance with the Family History of the patients: The evaluation of the miR-423-5p Expression Status with the family history of patients showed that (**Table 3**); the expression level decreased in 38.6% (17/44) of ovarian cancer patients with a family history of 2 or more than 2 ovarian and breast cancer, however the expression level increased in 61.4% (27/44). The expression level decreased in 45.8% (38/83), and levels increased in 54.2% (45/83) of ovarian cancer patients with a family history of ovarian and breast cancer less than 2. The expression level decreased in 45.7% (37/81), and increased in 54.3% (44/81) of ovarian cancer patients with a family history of 2 or

more than 2 any type of cancer. The expression level decreased in 39% (18/46), and increased in 61% (28/46) in ovarian cancer patients with a history of less than 2 cancers in the family.

The evaluation of miR-664b-5p expression status in accordance with the family history of patients showed that the expression level decreased in 56.8%(25/44), and increased in 43.2%(19/44) of ovarian cancer patients with a history of 2 or more than 2 ovarian and breast cancers in the family. The expression level decreased in 64%(53/83), and increased in 36%(30/83) of ovarian cancer patients with a family history of less than 2 ovarian and breast cancer. The expression level decreased in 53%(43/81), and increased in 47%(38/81) of ovarian cancer patients with a family history of any type of cancer of 2 or more than 2 in the family. The expression level decreased in 76%(35/46), and increased in 24%(11/46) in ovarian cancer patients with a history of less than 2 cancer in the family.

Table 3: The association of miR-423-5p, and miR-664b-5p expression status with family history

Family History	miR-423-5p		miR-664b-5p		
miRNA Expression Status	Increased	Decreased	Increased	Decreased	Total
History of ovarian and breast cancer in the family	n(%)	n(%)	n(%)	n(%)	n(%)
<2 individuals	45(62.5%)	38(69%)	30(61.2%)	53(68%)	83(65.3%)
>=2 individuals	27(37.5%)	17(31%)	19(38.8%)	25(32%)	44(34.7%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127
History of other cancer in the family					
<2 individuals	28(38.9%)	18(32.7%)	11(22.4%)	35(44.8%)	46(36.2%)
>=2 individuals	44(61.1%)	37(67.3%)	38(77.6%)	43(55.2%)	81(63.8%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127

Comparison of the miRNA expression levels with the clinical features of patients

The clinical and pathologic stage features of patients, and their association with miR-423-5p and miR-664b-5p expression levels, and their distributions are given in Table 5 (**Table 4**). The comparison of the clinical stage and miRNA expression levels of the patients showed that miR-423-5p expression levels were decreased in 43.3%(55/127) of patients, and expression levels increased in 56.7%(72/120) of patients ($p>0.005$). miR-664b-5p expression was found to have decreased in 61.5% (78/127) of the patient, and increased in 38.5%(49/120) ($p>0.005$).

Table 4: Comparison of the expression status of miR-423-5p, and miR-664b-5p with the clinical and pathologic features

Clinical Characteristics	miR-423-5p		miR-664b-5p		
miRNA Expression Status	Increased	Decreased	Increased	Decreased	Total
Age [mean 50±10 interval (25-84)]	n(%)	n(%)	n(%)	n(%)	n(%)
<40 age	10(13.9%)	6(11%)	6(12.2%)	10(12.8%)	16(12.5%)
>=40 age	62(86.1%)	49(89%)	43(87.8%)	68(87.1%)	111(87.5%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127
Clinical Stage					
Stage 1	12(17%)	10(18%)	6(12.2%)	16(20.5%)	22(17.3%)
Stage 2	8(11.1%)	6(11%)	10(20.4%)	4(5.2%)	14(11%)
Stage 3	48(66.7%)	28(51%)	28(57.2%)	48(61.5%)	76(59.9%)
Stage 4	4(5.5%)	11(20%)	5(10.2%)	10(12.8%)	15(11.8%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127
Pathological Stage					
Stage 1	11(15.3%)	11(20%)	7(14.2%)	15(19.2%)	22(17.3%)
Stage 2	11(15.3%)	8(14.5%)	9(12.5%)	10(12.8%)	19(15%)
Stage 3	50(69.4%)	36(65.5%)	33(67.3%)	53(68%)	86(67.7%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127
Histological Grade					
Grade 1	11(15.3%)	14(25.5%)	7(14.3%)	18(23%)	25(19.7%)
Grade 2	14(19.4%)	10(18.2%)	10(20.4%)	14(18%)	24(18.9%)
Grade 3	47(65.3%)	31(56.3%)	32(65.3%)	46(59%)	78(61.4%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127
Tumor Size					
<2 cm	15(21%)	13(23.6%)	12(24.4%)	16(20.5%)	28(22%)
>=2 cm	57(79%)	42(76.4%)	37(75.6%)	62(79.5%)	99(78%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127
Treatment					
Surgery					
Yes	68(94.4%)	54(98.2%)	46(93.9%)	76(97.4%)	122(96%)

No	4(5.6%)	1(1.8%)	3(6.1%)	2(2.6%)	5(4%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127
Chemotherapy					
Yes	66(91.7%)	51(92.7%)	49(100%)	68(87.1%)	117(92%)
No	6(8.3%)	4(7.3%)	0(0%)	10(12.9%)	10(8%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127
Radiotherapy					
Yes	10(13.9%)	5(9%)	6(12.2%)	9(11.5%)	15(12%)
No	62(86.1%)	50(91%)	43(87.8%)	69(88.5%)	112(88%)
Total	72(56.7%)	55(43.3%)	49(38.5%)	78(61.5%)	127

Survival Analysis

The mean survival time of ovarian cancer patients that we investigated in the study was 103 (SD $\pm$ 5.7 months) months, and we found that 45(35.4%) patients out of 127 patients that we screened between 2010-2019 died, and 78 (61.4%) survived after the diagnosis, and the condition of 4(3.2%) were unknown.

We performed the Kaplan-Meier analysis for investigating the possible variables that might affect the survival time of miR-423-5p, and miR-664b-5p expression levels . We found no correlation between the gene expression levels and survival time ($p>0.005$) (**Table 5, and Figure 3-4**).

Table 5: The association of gene expression levels and survival time (Kaplan-Meier analysis)

miR-423-5p expression	Total Number (n)	n of Events	Censored	
			n	Percentage
Decreased	55	32	23	41.8%
Increased	72	46	26	36.1%
Total	127	77	49	38.6%
miR-664b-5p expression				
Decreased	78	44	34	43.6%
Increased	49	34	15	30.6%
Total	127	78	49	38.6%

ROC Analysis

We performed the Receiver Operator Characteristics (ROC) analysis for demonstrating the differentiation strenght of ovarian cancer patient, and healthy control group and also the diagnostic performance of miR-423-5p, and miR-664b-5p molecules as the candidate bioindicators (**Figure 5**). **Table 6**, and **Figure 5** demonstrate the identified ROC-AUC values for each miRNA, and 95% CI (confidence interval). These results showed that the diagnostic strenght of miR-664b-5p miRNA variables were statistically significant in the diagnosis of ovarian cancer patients ($p < 0.005$), however the diagnostic strenght of miR-423-5p was found statistically insignificant ($p > 0.005$).

Table 6: ROC-AUC values of miRNAs in differentiation of ovarian cancer patients, and healthy groups

miRNAs	ROC-AUC	95% CI	p value
miR-423-5p	0.531	0.465 - 0.606	.429
miR-664b-5p	0.374	0.301 - 0.448	.001

The analysis of the change of CA-125 value in ovarian cancer patients

We performed the "Spearman's correlation analysis" for identifying the possible linear association between the numerical measurements of miRNA expression levels and the Ca-125 values obtained from

the clinical data of the ovarian cancer patients. We found no correlation and statistical significance between the expression levels of 423-5p, and miR-664b-5p with the Ca-125 numerical variables in the beginning of treatment, and during treatment ($p>0.05$) (**Table 7**).

Table 7: The correlation statistics of miRNA expression levels and CA-125 variables

p values	Initial CA-125	Final CA-125
miR-423-5p	0.278	0.742
miR-664b-5p	0.313	0.362

Discussion

Ovarian cancer is the 7th most common cancer among women worldwide, and is the 3rd most common cancer in women following the breast and servix cancer, and the incidence is 10-15 in 100.000. Ovarian cancer is the 5th most fatal cancer among women worldwide [13]. The importance of molecular biological indciators associated with treatment associated molecular targetted treatments, and their monitoring has been increasing with the increase of information on the ovarian tumor etiology. Various small molecule inhibitors and monoclonal antibodies targetting multiple cancer have been used in the clinic since the last decade. There is a need for more efficient and specific biologic indicators in treatment period, and in monitoring the effects of therapeutics, and in the early diagnosis of ovarian cancer [14]. The miRNAs identified in the recent years are reported as the new and promising molecules in the etiology of ovarian cancer[1]. The biologic indicator potential of miRNA molecules in ovarian cancer was measured with the comparison of CA-125 tumor marker which is commonly used in ovarian cancer in clinic, and miRNAs were reported to as a more stronger biomarker in the early diagnosis of ovarian cancer [15]. The increase or decrease of the expression of miRNA molecules have the potential to be used as an agent in cancer treatment or as a biologic marker [16-18]. In summary, the miRNA expression profiles in the tissue and blood may be used in the diagnosis and treatment of ovarian cancer.

The expression levels of two miRNAs as miR-423-5p, and miR-664b-5p which were detected to be important for ovarian cancer etiology in familial and sporadic ovarian cancer patients from the previous submitted results were selected from the high risk ovarian cancer families in our study, were investigated [19]. We aimed to investigate the place of these miRNAs in the etiology, diagnosis of ovarian cancer, and to perform the validation and identify their distribution in ovarian cancer. We used the peripheral blood lymphocytes of a total of 127 ovarian cancer patients, and 98 healthy controls. The expressions of these miRNAs were found to have significantly increased in the peripheral blood lymphocytes of ovarian cancer patients compared with the levels in the samples of the healthy controls in our study ($p:0.000$). In ovarian cancer patient subgroups, the miRNA expressions were found significantly increased in only ovarian cancer, ovarian and breast cancer, and ovarian and other secondary tumor patients compared with the

levels in the control group, however, the expression in ovarian and endometrium cancer patients were found to have decreased compared with the levels in the control group.

There are only 2 different studies in the literature suggesting that miR-423-5p was a diagnostic biomarker. miR-423-5p expression was reported to be associated with metastasis and progression of ovarian cancer in tumor tissue and plasma of the ovarian cancer patients in these studies [20, 21]. In addition, miR-423-5p was reported to inhibit the proliferation of colon cancer cells by stimulating the apoptosis in colon and gastric cancers [22], and was reported to be effective in the gastric cancer cell proliferation and invasion by inhibiting a tumor suppressing molecule- TFF1[23]. Wan et al. showed that the overexpression of miR-423-5p decreased the apigen sensitivity of glioma stem cells [24], and facilitated the glioma cell proliferation, angiogenesis, and invasion in in vitro [25]. The evaluation of these studies showed that in cancers with overexpression of miR-423-5p the apoptosis mechanisms induced by Apigenin was severely destroyed. miR-423-5p was reported to be frequently overexpressed in glioma stem cells, and significantly increased apigenin related apoptosis developed in the use of miR-423-5p inhibitors [26]. The overexpression of miR-423-5p was reported to increase the colonisation, mobility, migration, and invasion capacity of LAD cells (Human mast cell greed LAD1, and LAD2) [27], however, in rat studies was reported to induce the tumorigenesis, and brain metastasis[28]. These results suggest that miR-423-5p was essential in metastasis, and miRNA-423-5p functioned as a tumor suppressor in ovarian cancer, and might be used as a potential negative biologic indicator. The results of our study showed that miR-423-5p might be used as a biologic indicator, and might show the metastatic potential. Considering that approximately 90% of cancer related deaths are associated with metastasis, miR-423-5p might be used as a biologic indicator in predicting the early metastasis, and might inhibit the metastasis when used as an anti-therapeutic agent. This is the first study describing the results associated with the evaluation of lymphocytes in peripheral blood of miR423-5p expression compared with healthy controls although miR-423-5p expression was increased in ovarian cancer patients compared with the controls, and these results were consistent with the studies conducted with very limited number of patients associated with ovarian cancer in the literature.

There is only one study which investigated the miR-664b-5p in the literature. That study includes the data associated with breast cancer rather than ovarian cancer. miR-664b-5p was shown to function as a tumor suppressor, and had a significant role in chemotherapy sensitivity by targeting CCNE2 in *BRCA1* mutation triple negative breast cancer patients in that study [11]. A decrease was detected in miR-664b-5p expression in this unique study in the literature investigated the *BRCA1* mutation triple negative breast cancers. In general, miR-664-5p expression was found to have increased in the ovarian cancer patient group compared with the healthy controls in our study investigating the miR-664b-5p expression in the peripheral blood lymphocytes of ovarian cancer patients. However, the evaluation of miR-664b-5p expression level with the presence of BRCA gene mutation showed that the levels were decreased in 64% of the group with having BRCA mutation. In the preliminary study of our group, the detection of *BRCA1* gene mutation carrier of ovarian cancer patients who had decreased miR-664b-5p expression level corresponds with the only study performed with breast cancer patients in the literature, and with the BRCA mutation patient subgroup in our study, and miR-664-5p expression was found to have decreased in 64%

of patients in this group. However different than other studies, a decrease in miR-644-5p expression was detected in 60.8% of ovarian cancer patient who were negative for BRCA mutation. These results suggested that the decrease in the miR-664-5p expression was independent from the presence of BRCA mutation.

We also found both miR-423-5p, and miR-664b-5p expression levels in patients diagnosed with both ovarian and endometrium cancer. Although the number of such patients was small, it will be appropriate to investigate these miRNAs in endometrium cancer patients in the future studies.

Conclusion

The present study showed that both two miRNA molecules might be used as a potential non-invasive biologic marker for ovarian carcinoma. Future studies will be valuable to emphasize and show the benefit of measurement of these miRNA molecules in peripheral blood before treatment and during treatment with specific intervals and also on primary tumor tissue in time of diagnosis of the patients with ovarian cancer.

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PS recruited and referred patients to the study. BK performed the experiments and help writing the manuscript. ŞBT and DAÖ performed statistical analysis. All experiments were required in the laboratory of HY. All the chemicals and KITs required for the experimental processes of the study was provided by HY. HY also wrote the manuscript, contributed towards the interpretation of data, writing of the manuscript and increased the scientific value of writing adding intellectual information. AAG and MAS recruited healthy cases to the study and also collected the data and drawing pedigrees and fill up demographic information forms and prepared all documents for the samples. ÖŞE and SKE performed ROC analysis. BÇ criticized the manuscript according to literature intellectually.

Competing Interests of Authors

The authors declare that they have no competing interests.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of Istanbul Medical Faculty in Istanbul University (approval no. 30.03.2018/121108). Written informed consent was provided by the parents/guardians of the participants included in the study.

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Figures

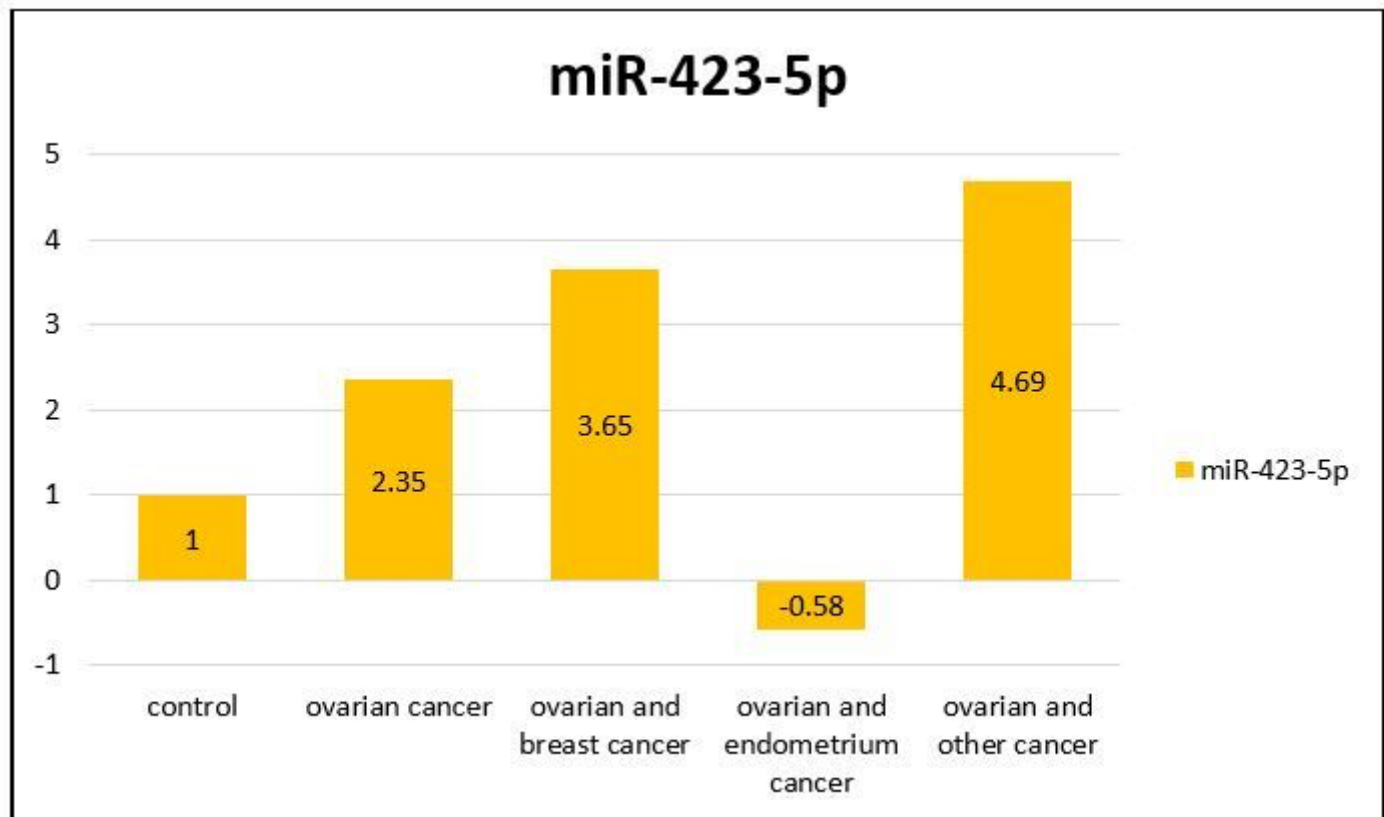


Figure 1

The distribution of miR-423-5p expression level in the ovarian cancer, and ovarian cancer patients subgroups.

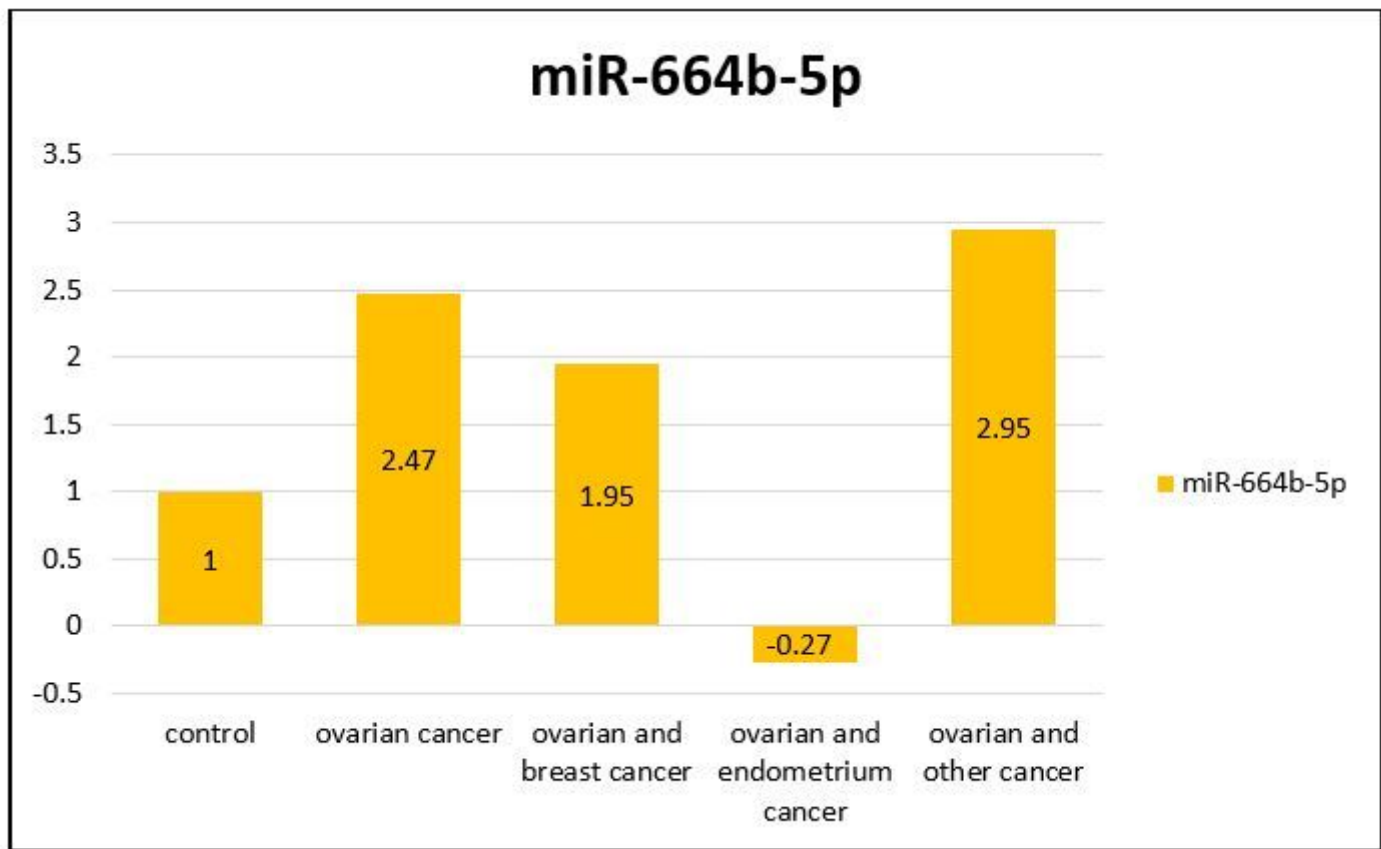


Figure 2

Distribution of miR-664b-5p expression level in ovarian cancer patients, and in the subgroups of ovarian cancer.

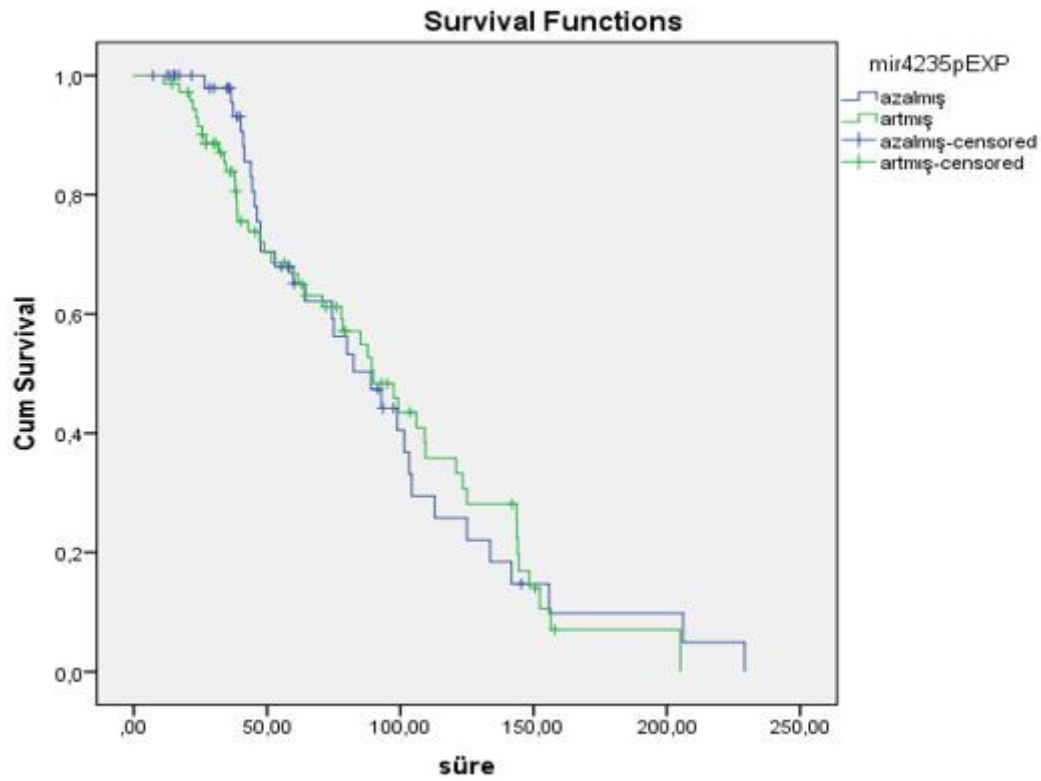


Figure 3

The association of miR-423-5p gene expression level, and survival time

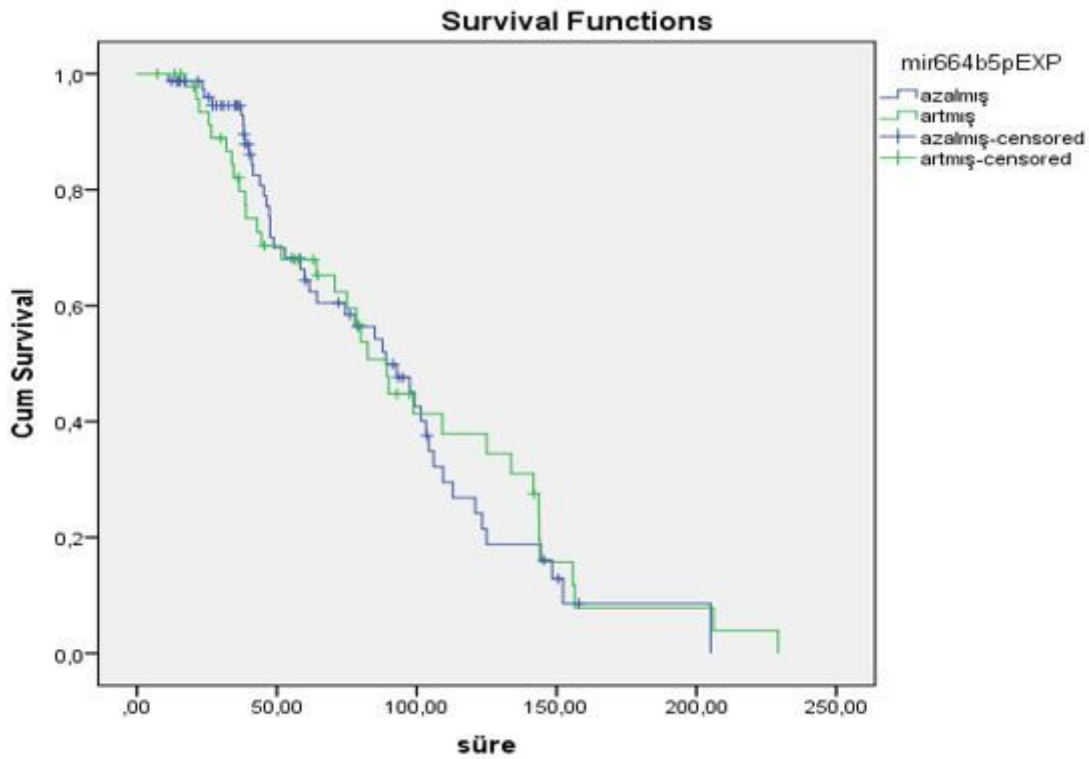


Figure 4

The association of miR-664b-5p gene expression level and survival time

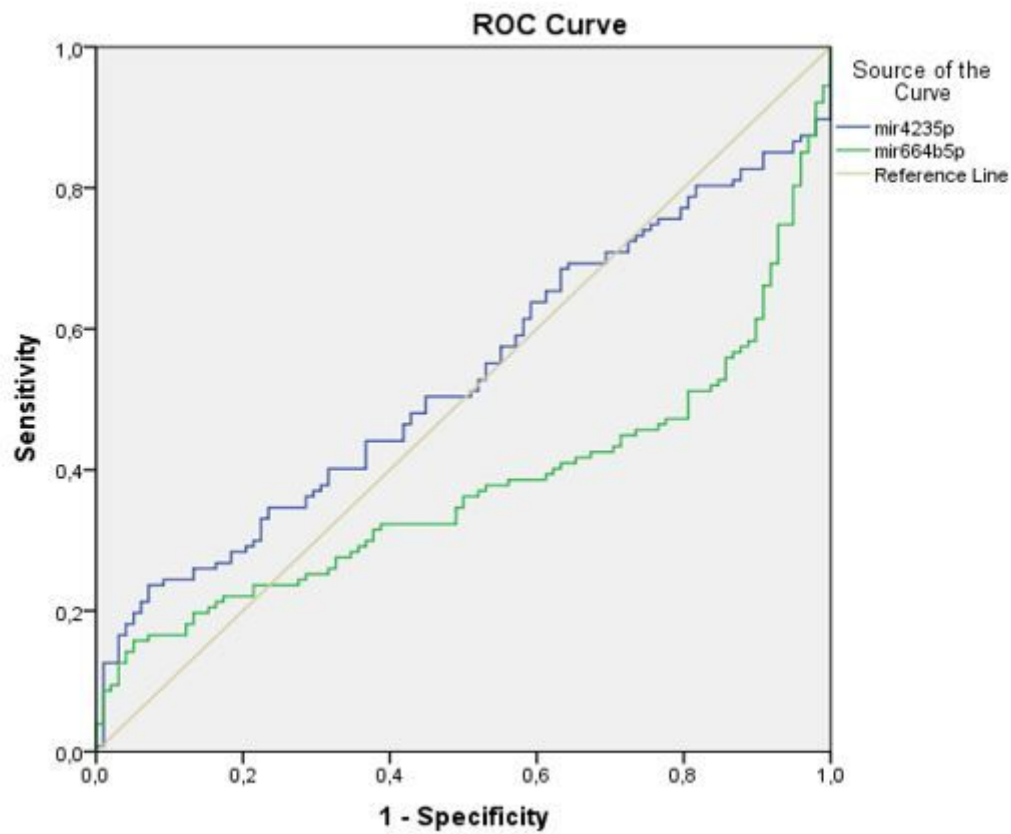


Figure 5

The diagram of ROC-AUC values of miRNAs in differentiation of ovarian cancer patients, and control group