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High Level of MIR-142-3p Expressıon in Patıents with Ovarian Carcinoma

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Abstract

Ovarian cancer is responsible from more than 150.000 death annually worldwide. This cancer is detected in the late stage, and is characterised with poor prognosis, therefore most cases result with death. The diagnosis and the treatment of the disease have to be improved for a better quality of life for patients. MicroRNAs are the noncoding RNAs in the length of 19-24 nucleotides which show suppressor effect on target genes. miRNAs are included in the pathology of various diseases including cancer. miRNAs being as the biomarker candidates in diagnosis, and their use in treatment as the inhibitors of the molecules mimicking the miRNA showed that they may be used as the new therapeutic target and agents. We detected with our group in our prior study conducted with disconcordant ovarian cancer twins that many miRNA molecules were different in ovarian cancer compared with the molecules in healthy sibling. The expression level of miR-142-3p that was selected from the miRNAs detected in the previous study was compared, and investigated in a wider ovarian cancer group, and in healthy control group. miR-142-3p expression level was investigated using the real-time PCR method in the present study involving 147 patients, and 100 healthy control group. The differences in the expression levels of miR-142-3p detected in the peripheral blood lymphocytes of ovarian cancer patients, and healthy control were statisticaly evaluated. The expression level of miR-142-3p was detected to have increased 3.11 fold in ovarian cancer patients compared with the levels in healthy controls, and the difference was statistically significant (p:0.00). These results suggest that miR-142-3p that was found significantly increased in the peripheral blood samples of ovarian cancer patients compared with the healthy controls might be used as a sensitive, noninvasive biomarker in the early diagnosis, and treatment and follow up of ovarian cancer.

Keywords: Ovarian Cancer; MiR-142-3p Expression; Biomarker

Abbreviations : ARNTL: Aryl Hydrocarbon Receptor Nuclear Translocator Like; BRCA1: Breast Cancer 1 ; BRCA2: Breast Cancer 2; CDK: Cyclin Dependent Kinase ; cDNA: complementary DNA; Ct: Cycle threshold ; DNA: Deoxyribonucleic acid; EDTA: Ethylenediaminetetraacetic acid; FC: Fold Change; g: Gram; KRAS: Kirsten-rous avian sarcoma; miRNA: microRNA; Ml: Milliliter; mRNA: messenger RNA; ng: nanogram; P21: The cyclin-dependent kinase inhibitor; p53: Tumor protein 53; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; RNA: ribonucleic acid; ROC: Receiver Operator Characteristic; AUC: Area Under the ROC Curve; RPM: revolutions per minute; µL: Microliter; %95 CI: %95 Confidence Interval; NACI: sodium chloride; SD: standart deviation

Introduction

Cancer, which is an important public health problem worldwide is the significant cause of morbidity and mortality in all regions, and all countries worldwide. Ovarian cancer is the third most leading gynecologic cancer among women after cervical, and corpus uteri cancers. Ovarian cancer is the second most leading cancer and has the worst prognosis in the ranking of cancers in accordance with mortality among women [1]. High rate of mortality is associated with the silent development, and difficult imaging of ovarian cancer. 75% of women are detected in the advanced stage owing to the asymptomatic characteristic of the tumor. 5 years survival can be possible in more than 70% of women with early stage disease, however this rate decreases to 30% in women with advanced stage disease [2, 3]. One of the most important prognostic factors for ovarian cancer is the stage at diagnosis. Most patients have the symptoms of abdominal distension, pelvic pain, dyspepsia, urinary tract problems, constipation, nausea and vomiting. These symptoms detected in ovarian cancer correspond with the symptoms of gastrointestinal diseases, and menstrual irregularity which result with the delay in consulting patients to gynecology [3].

There are various current treatment options for ovarian cancer patients which are neoadjuvant chemotherapy, weekly chemotherapy, intraperitoneal chemotherapy, and surgical resection, and each of these treatment methods are currently updated. However, each patient has cellular and molecular diversity in accordance with the cancer subtype. For example, mucinous tumors carry *KRAS* mutations, and high degree serous tumors have *BRCA1*, and *BRCA2* gene mutations[4]. In addition, researchers have recently reported differences in various miRNAs in different cancer types [4]. Many treatments are recently designed considering the individual genetic features in addition to the clinical features of the patients.

MicroRNAs (miRNAs), are the small noncoding RNA molecules with a length of 19-24 nucleotides which have a criticial role in the regulation of gene expression after transcription. Researchers in various studies showed that miRNAs have significant roles in the regulation of critical cellular processes such as in proliferation, differentiation, migration, apoptosis, metabolism, and stress response. miRNAs are known to have roles as the key regulators in the pathogenesis of diseases particularly in cancer [5]. miRNAs are demonstrated as the candidates for prognostic biomarkers, and therapeutic targets in cancer. New therapeutic target, and agents can be developed after identification of miRNAs and target molecules with cancer development and metastasis [6].

There is no effective screening test for identifying asymtpomatic ovarian cancer in the early stage yet [7]. There is a need for disease specific and sensitive biomarkers in the early diagnosis, treatment selection, and monitoring of treatment in ovarian cancer. Our team have detected 99 candidate miRNAS that might be important in the ovarian cancer etiology in their previous study [8]. miR-142-3p which was investigated in a wider ovarian cancer patient group in the present study was selected among these miRNAs, and there is no study which demonstrates the direct association of this miRNA with ovarian cancer in the literature. We investigated and evaluated whether the expression level of miR-142-3p molecule had a noninvasive biological biomarker feature in our study including 150 sporadic and familial ovarian cancer patients with 100 healthy individuals who were matched for age, sex, and ethnicity with the patient group.

Materıals and Methods

Study Groups: The experimental group consisted of the peripheral blood samples of 150 ovarian cancer patients, and 100 healthy individuals. Patients were selected among the patients who presented to Istanbul University, Oncology Institute, Cancer Genetics Division, and gave consent for the use of their material for scientific purpose. Healthy control group was selected from healthy individuals who were matched for ethnicity, age, and sex with the patients, and nonsmokers with no history of cancer in their last 3 generations. The blood drawn procedure was performed after the informed consent forms were obtained. The study was pursued with the RNA materials of 147 patients, and 100 healthy individuals after the quality control analyses were performed. 122 out of 147 ovarian cancer patients were diagnosed with only ovarian cancer, 17 patients were diagnosed with ovarian and breast cancer, 5 patients were diagnosed with another cancer in addition to ovarian cancer, and 3 patients were diagnosed with endometrium can-

cer and ovarian cancer. The present study was approved by the Clinical Research Ethics Committee of Istanbul Faculty of Medicine (Ethics Board approval: dated 07.05.2019; and no: /2029/624), and was conducted in accordance with the updated version of the Declaration of Helsinki.

Lymphocyte Isolation from Peripheral Blood: The peripheral blood lymphocytes were separated from other components using the Ficoll method. Approximately 10 mL blood was drawn from the donors, and the collected blood samples were transferred into EDTA tube, and were diluted in a ratio of 1/1 using 0.9 % NACI involving isotonic solution. The diluted blood was slowly transferred on the 2 mL Ficoll-Histopaque-1077 (Sigma-Aldrich, USA) using a pipette, and was centrifugated for 30 min in the cooled centrifuge at 1970 RPM. Three different phases were detected as the plasma on the upper phase, lymphocytes in opaque appearance in the middle phase, and the erythrocytes on the lower phase, and the lymphocytes were attentively collected in a clean tube using a pasteur pipette, and centrifugated at 1970 RPM for 10 min. Supernatant was removed, and pellet was resolved by pipettaging with the addition of 4 mL PBS on the pellet. The resolved pellet was distributed in 4 cryotubes as to include 1 mL in each tube. Crytotubes were centrifugated at 1970 RPM for 5 min. The supernatant was removed. The obtained cells were incubated at -80°C for 24 hours. The cells in the cryotubes were transferred into liquid nitrogen storage tank for long time storage after 24 hours.

RNA Isolation: The Quick-RNA MiniPrep Isolation kit (ZYMO RESEARCH) was used for the total RNA isolation of lymphocyte cells obtained from the samples of the patients, and controls. In accordance with the kit protocol; lymphocyte cells were taken out of nitrogen tank, and the samples were studied on ice. Pipetting was performed until the dissolution by addition of 300 µL RNA lysis buffer, and centrifugated at 10.000 x g for 30 seconds. The DNA-RNA in the cell were revealed. The RNA involving supernatant moved to lower phase, and the lower phase was transferred into a new tube, and similar amount of 95-100% ethanol was added on the tube, and the mixture was centrifugated for 30 sec at 10.000 g. Then, the supernatant accumulated at the lower part was removed, and RNA Prep buffer(400 µL) was added on the filter and centrifugated at 12.000g, the residue in the collection tube was removed. Centifugation was performed at 12.0000g by addition of RNA Wash Buffer (700 µL) on the filter, and residue in the collection tube was removed. Centifugation was performed at 12.0000g by addition of 400 µL RNA Wash Buffer for 2 min. Finally, 100 µL DNAse/RNAse-Free Water was included, RNA was isolated by centrifugation at 12.000g for 30 sec after keeping at room temperature for 1 min. The obtained RNAs were controlled, and then were stored at -80°C by distribution in several tubes.

The Quality control of the RNA Samples, and identification of the concentrations: All RNAs were conducted at 160 in 1.5% agarose gel for quality control. The quality control of the RNAs required for cDNA synthesis was performed with the absence of DNA bant on the RNA walk, and with the presence of two major bands representing the 28S/18S rRNAs. The purity, and concentration measurements were performed using the NanoDrop 2000 Spectrophotometer THERMO SCIENTIFIC device after confirmation that RNAs were not degraded, and not included genomic DNA contamination. The purity of the RNA samples were evaluated at A260/A280 nm wavelenght. Highly pure RNA samples which were in the interval of 1.8- 2.2 OD were included in the study.

cDNA Synthesis: Complementary DNA was synthesized using a commercial kit ID3EAL cDNA synthesis system protocol with the miRNA specific reverse transcriptase enzyme. All procedures were performed on ice. RNA samples were dissolved and vortexed for real time PCR reaction. For cDNA reaction, RNA in a volume corresponding to maximum 1 µg template RNA was included into 5 µL ID3EAL RT Buffer, 1 µL ID3EAL RT Primer, 1 µL ID3EAL RT Primer 5s rRNA, 1 µL ID3EAL Reverse Transcriptase enzyme. The volume of the obtained mixture was completed to 20 µL using distilled water. The reaction mixture was vortexed after mixing carefully with pipette. 20 µL mixture was incubated on BioRad PCR device for performing the reverse transcriptase reaction. cDNA synthesis procedure was performed with two stage reactions first incubation at 42°C for 30 min, then with the reaction involving the elimination of enzyme activity at 95°C for 5 min.

Calculation of the Real Time qPCR Reaction, and CT value: Real time quantitative PCR (qRT-PCR) reactions were performed for measuring the miR-142-3p expression level after the total RNA isolation, and cDNA synthesis of the miRNA samples. cDNAs were diluted using a ratio of 1:10(v:v) sterilised DNA/RNAse free distilled water. As to obtain a total volume of 20 µL for real time

qPCR reaction; 5 µL diluted cDNA, 10 µL ID3AL qPCR Master Mix, 2 µL ID3AL qPCR assays, and 3 µL distilled water were added. Pipettaging was performed using micropipette, then were vortexed, and inserted on MIC real time PCR device (MIC qPCR Cycler, Bio Molecular Systems, Australia). The quantitative PCR procedure was performed in 40 cycles including incubation(polimerase activation), at 95°C for 10 min, and then at 40°C for 5 min., then denaturation procedure at 95°C for 10 sec., and at 60°C 30 sec binding and extension procedures. The MIC qPCR Software analysis was performed for the control, and calculation of the expression levels of the performed reactions. The below $2^{-\Delta\Delta Ct}$ formula was used for measuring the miR-142-3p gene expression with this software.

ΔCt, and ΔΔCt values for each patient, and healthy control were measured on Excel program.

ΔCT= CT (target gene) – CT (reference gene)

$\Delta\Delta CT = \Delta CT \text{ (patient)} - \Delta CT \text{ (healthy control)}_{mean}$

$2^{-\Delta\Delta CT} = 2^{-[(Ct \text{ target} - Ct \text{ ref}) \text{ patient} - (Ct \text{ target} - Ct \text{ ref}) \text{ healthy control}]}$

The fold change was calculated by taking the ratio of $2^{-\Delta\Delta Ct}$ of patient samples to healthy $2^{-\Delta\Delta Ct}$ (fold change=fc), and the gene expression ratio was identified. The reference value of gene expression of healthy control group ($\Delta\Delta Ct = 0 \rightarrow 2^0 = 1$) was taken as 1.

Statistical Analysis: Statistical Analysis was performed using the IBM Support SPSS 21.0 program, and STRING analysis. The fold ratio of the expression levels of groups were identified (Fold-Change). The values with a Fold-Change ratio of $|FC| \geq 2$, and $p<0.05$ were evaluated. The association of the gene expression levels of the patients and healthy control groups, and association of gene expression levels and clinical data were compared using the Mann-Whitney U test. The diagnostic performance of the candidate biologic biomarker miR-142-3p was demonstrated using the receiver operator characteristics curve (ROC) analysis. The significance of the differences between the obtained results from the indicated groups was investigated using the Student's t test (Independent-Samples T-Test). Pair comparison of clinical data was performed using the Chi-square test. The survival analysis of patients was investigated using the Kaplan-Meier test.

Results

150 ovarian cancer patients who presented to the Istanbul University, Oncology Institute, Cancer Genetics polyclinic, and 100 healthy individuals who were matched for ethnicity, sex, and age with the patients constituted the scope of the study. 3 patients with inadequate RNA quality were excluded from the study. The statistical results were evaluated over 147 patients (n:147), and 100 healthy controls (n:100). The mean age of the ovarian cancer patients was 50±10 years (23-84y), and the mean age of the healthy control group was 48±11 years (23-84y).

The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) v21 program. Normality distribution test was performed for conducting the analysis. Normality distribution was identified using the Kolmogorov Smirnov test. Accordingly, the value of $p<0.05$ was accepted significant, and the distribution was accepted as not-normal. The Mann Whitney U test was performed for the comparison of the means of independent data sequences showing non-normal distribution. The evaluation based on the $2^{-\Delta\Delta Ct}$ values on Mann Whitney U test showed that the values in the patient group was found statistically significant compared with the levels in the healthy control group (p:0,00) (Table 1). miR-142-3p was found to have increased 3.11 fold in the patient group compared with the level in the healthy group (Figure 1).

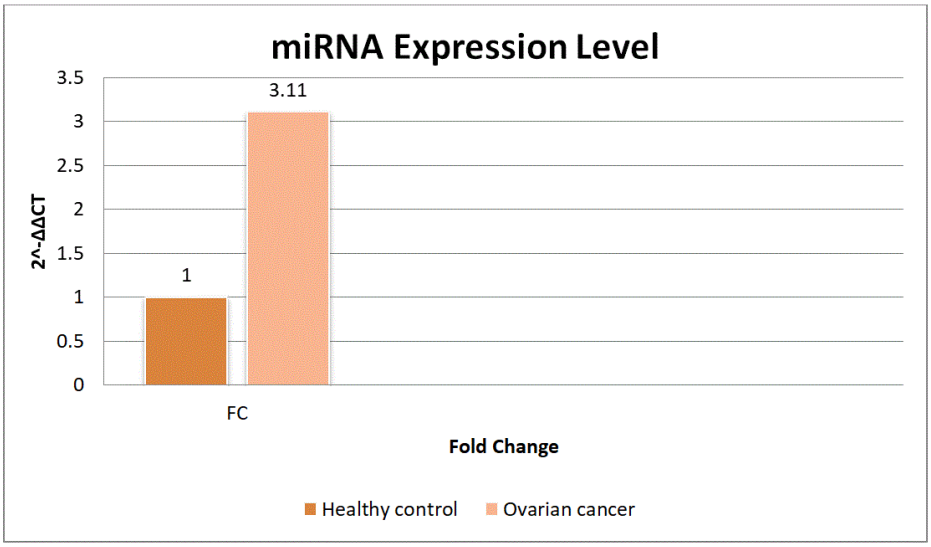

Figure 1: Comparison of miR-142-3p expression level in patient, and healthy control group

	miR-1423p
Mann-Whitney U	5221.000
Wilcoxon W	10271.000
Z	-3.863
p value	**.000**

Table 1: Statistical value of miR-142-3p gene expression level in patient, and healthy control groups

Evaluation of miR-142-3p Expression Level İn Accordance With The Diagnosis: The expression level of miR-142-3p was increased in 53% (53/100) of the healthy control group, and was decreased in 47% (47/100) of the healthy control group. The expression level of miR-142-3p was increased in 75.4% (92/122), and was decreased in 24.6% (30/122) of the ovarian cancer patients. The expression level of miR-142-3p was increased in 64.7% (11/17), and was decreased in 35.3% (6/17) of patients diagnosed with two different primary tumors as ovarian and breast cancer. The expression level of miR-142-3p was increased in 33.3% (1/3), and was decreased in 66.7% (2/3) in patients with ovarian and endometrium cancers. The expression level of miR-142-3p was increased in 80% (4/5), and was decreased in 20% (1/5) of patients with a secondary cancer except breast and endometrium cancers (Table 2). The Pearson's Chi-Square analysis showed that there was a significant association between the diagnosis and miR-142-3p expression ($p<0.05$).

Diagnosis	**miR-142-3p Expression**		**Total n (%)**
	Decreased n (%)	**Increased n (%)**	
Healthy Controls	47 (47%)	53 (53%)	100 (40.5%)
Only Ovarian Carcinoma	30 (24.6%)	92 (75.4%)	122 (49.4%)
Ovarian Ca + Breast cancer	6 (35.3%)	11 (64.7%)	17 (6.9%)
Ovarian Ca + Endometrium cancer	2 (66.7%)	1 (33.3%)	3 (1.2%)
Ovarian Ca + Other type of cancer	1 (20%)	4 (80%)	5 (2%)
Total	86 (34.8%)	161 (65.2%)	247 (100.0%)

Table 2: Distribution of miR-142-3p expression level in patient, and healthy controls

The association of miR-142-3p expression level, and clinical data: The expression level of miR-142-3p was increased in 70.8% (17/24), and was decreased in 29.2% (7/24) of ovarian cancer patients aged below 40 years. The expression level of miR-142-3p was increased in 74% (91/123), and was decreased in 26% (32/123) of patients aged 40 years and above 40 years.

The miR-142-3p gene expression level was separately investigated using the Mann-Whitney U test, and no statistical significance was detected between the miRNA and other matched clinical data ($p>0.05$).

The expression level of miR-142-3p was increased in 66.7% (30/45) and was decreased in 33.3% (15/45) of patients with no metastasis. The expression level of miR-142-3p was increased in 76.5% (78/102), and was decreased in 23.5% (24/102) of the patients with metastasis (Table 3).

The expression level of miR-142-3p was increased in 75% (6/8), and was decreased in 25% (2/8) of Stage I patients in accordance with the clinical staging. The expression level of miR-142-3p was increased in 64.3% (9/14), and was decreased in 35.7% (5/14) of stage II patients. The expression level of miR-142-3p was increased in 78% (64/82), and was decreased in 22% (18/82) of Stage III patients. The expression level of miR-142-3p was increased in 67.4% (29/43), and was decreased in 32.6% (14/43) of Stage IV patients (Table 3).

The expression level of miR-142-3p was increased in 75% (6/8), and was decreased in 25% (2/8) of stage I patients in accordance with the pathologic staging. The expression level of miR-142-3p was increased in 61.5% (8/13), and was decreased in 38.5% (5/13) of Stage II patients. The expression level of miR-142-3p was increased in 77.2% (71/92), and was decreased in 22.8% (21/92) of Stage IV patients. The expression level of miR-142-3p was increased in 67.6% (23/34), and was decreased in 32.4% (11/34) in Stage IV patients (Table 3).

The expression level of miR-142-3p was increased in 80% (4/5), and was decreased in 20% (1/5) of Grade 1 patients in accordance with the histological staging. The expression level of miR-142-3p was increased in 73.5% (25/34), and was decreased in 26.5% (9/34) of Grade 2 patients. The expression level of miR-142-3p was increased in 73.1% (79/108), and was decreased in 26.9% (29/108) of Grade 3 patients (Table 3).

The expression level of miR-142-3p was increased in 65.6% (21/32), and was decreased in 34.4% (11/32) of patients with tumor size smaller than 2 cm in accordance with the tumor size evaluation. The expression level of miR-142-3p was increased in 75.7% (21/32), and was decreased in 24.3% (11/32) of patients with tumor size of 2 cm or larger than 2 cm (Table 3).

The expression level of miR-142-3p was increased in 60% (3/5), and was decreased in 40% (2/5) of patients who did not undergo surgery in accordance with the surgery condition. The expression level of miR-142-3p was increased in 73.9% (105/142), and was decreased in 26.1% (37/142) of patients who underwent surgery (Table 3).

In accordance with the final condition, expression level of miR-142-3p was increased in 67.4% (31/46), and was decreased in 32.6% (15/46) of deceased patients. Expression level of miR-142-3p was increased in 76% (76/100), and was decreased in 24% (24/100) of survivor patients (Table 3).

Expression level of miR-142-3p was increased in 80.4% (41/51), and was decreased in 19.6% (10/51) of patients with cancer diagnosis in less than 2 individuals in the family in accordance with the total number of cancer in the family. Expression level of miR-142-3p was increased in 69.8% (67/96), and was decreased in 30.2% (29/96) of patients with cancer diagnosis in 2 or in higher than 2 individuals in the family (Table 3).

Expression level of miR-142-3p was increased in 75.5% (71/94), and was decreased in 24.5% (23/94) of patients with ovarian or breast cancer diagnosis in less than 2 individuals in the family in accordance with the number of ovarian and breast cancer in the

family. Expression level of miR-142-3p was increased in 69.8% (37/53), and was decreased in 30.2% (16/53) of patients with ovarian or breast cancer diagnosis in 2 or more than 2 individuals in the family (Table 3).

Expression level of miR-142-3p was increased in 71% (22/31), and was decreased in 29% (9/31) of patients with no pregnancy history. Expression level of miR-142-3p was increased in 74.1% (86/116), and was decreased in 25.9% (30/116) of patients with pregnancy history (Table 3).

Expression level of miR-142-3p was increased in 70.6% (48/68), and was decreased in 29.4% (20/68) of premenopause patients. Expression level of miR-142-3p was increased in 75.9% (60/79), and was decreased in 24.1% (19/79) of postmenopause patients (Table 3).

Expression level of miR-142-3p was increased in 71.6% (73/102), and was decreased in 28.4% (29/102) of patients who did not use oral contraceptives. Expression level of miR-142-3p was increased in 77.8% (35/45), and was decreased in 22.2% (10/45) of patients who used oral contraceptives (Table 3).

Expression level of miR-142-3p was increased in 73.6% (103/140), and was decreased in 26.4% (37/140) of patients who did not receive infertility treatment. Expression level of miR-142-3p was increased in 71.4 % (5/7), and was decreased in 28.6% (2/7) of patients who had previously received infertility treatment (Table 3).

Age	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
<40 y	7 (29.2%)	17 (70.8%)	24 (16.3%)
≥40y	32 (26%)	91 (74%)	123 (83.7%)
Clinical Stage	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
Stage 1-2	7 (32%)	15 (68%)	22 (15%)
Stage 3-4	32 (25.6%)	93 (74.4%)	125 (85%)
Pathological Grade	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
Grade 1	2 (25%)	6 (75%)	8 (5.4%)
Grade 2	5 (38.5%)	8 (61.5%)	13 (8.8%)
Grade 3	21 (22.8%)	71 (77.2%)	92 (62.6%)
Grade 4	11 (32.4%)	23 (67.6%)	34 (23.1%)
Histological Grade	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
Grade 1	1 (20%)	4 (80%)	5 (3.4%)
Grade 2	9 (26.5%)	25 (73.5%)	34 (23.1%)
Grade 3	29 (26.9%)	79 (73.1%)	108 (73.5%)
Tumor Size	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
<2 cm	11 (34.4%)	21 (65.6%)	32 (21.8%)
>=2 cm	28 (24.3%)	87 (75.7%)	115 (78.2%)
Surgery	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
No	2 (40%)	3 (60%)	5 (3.4%)

Yes	37 (26.1%)	105 (73.9%)	142 (96.6%)
Pregnancy	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
No	9 (29%)	22 (71%)	31 (21.1%)
Yes	30 (25.9%)	86 (74.1%)	116 (78.9%)
Menopause	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
Premenopause	20 (29.4%)	48 (70.6%)	68 (46.3%)
Postmenopause	19 (24.1%)	60 (75.9%)	79 (53.7%)
Oral Contraceptive	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
No	29 (28.4%)	73 (71.6%)	102 (69.4%)
Yes	10 (22.2%)	35 (77.8%)	45 (30.6%)
Infertility Treatment	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
No	37(26.4%)	103(73.6%)	140 (95.2%)
Yes	2(28.6%)	5(71.4%)	7 (4.8%)
Metastasis	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
No	15(33.3%)	30 (66.7%)	45 (30.6%)
Yes	24(23.5%)	78 (76.5%)	102 (69.4%)
Ovarian & Breast cancer histories in the family	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
<2	23 (24.5%)	71 (75.5%)	94 (63.9%)
≥2	16 (30%)	37 (70%)	53 (36.1%)
Total cancer history in the family	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
<2	10(19.6%)	41 (80.4%)	51 (34.7%)
≥2	29(30.2%)	67 (69.8%)	96 (65.3%)
Total	39 (26.5%)	108 (73.5%)	147 (100%)
Last status	**miR-142-3p Expression**		**Total n(%)**
	Decreased n(%)	**Increased n(%)**	
Ex	15(32.6%)	31(67.3%)	46 (31.5%)
Alive	24(24%)	76(76%)	100 (68.5%)
Total	39 (26.7%)	107 (73.3%)	146 (100.0%)

Table 3: Comparison of the expression level of miR-142-3p with the clinical features of patients

Expression level of miR-142-3p was increased in 84.6% (11/13), and was decreased in 15.4% (2/13) of serous type ovarian cancer patients in accordance with the ovarian histological subtype. Expression level of miR-142-3p was increased in 66.7% (4/6), and was decreased in 33.35% (2/6) of mucinous type ovarian cancer patients. Expression level of miR-142-3p was increased in 100% (1/1) of papillary, endometriode type, and squamous cell ovarian cancer patients. Expression level of miR-142-3p was increased in 69% (20/29), and was decreased in 31% (9/29) of ovarian patients with adenocarcinoma histology. Expression level of miR-142-3p was increased in 69.1% (38/55), and was decreased in 30.9% (17/55) of serous adenocarcinoma type ovarian patients. Expression level of miR-142-3p was increased in 75.8% (25/33), and was decreased in 24.2% (8/33) of serous papillary adenocarcinoma type ovarian

patients. Expression level of miR-142-3p was increased in 87.5% (7/9), and was decreased in 12.5% (1/8) of clear cell carcinoma type ovarian patients (Table 4).

Histological Subtype	miR-142-3p Expression		Total
	Decreased	Increased	
Serous	2 (15.4%)	11 (84.6%)	13 (8.8%)
Mucinous	2 (33.3%)	4 (66.7%)	6 (4.1%)
Papillary	0 (0%)	1 (100%)	1 (0.7%)
Squamous cell carcinoma	0 (0%)	1 (100%)	1 (0.7%)
Adenocarcinoma	9 (31%)	20 (69%)	29 (19.7%)
Serous adenocarcinoma	17 (30.9%)	38 (69.1%)	55 (37.4%)
Serous papillary adenocarcinoma	8 (24.2%)	25 (75.8%)	33 (22.4%)
Clear cell carcinoma	1 (12.5%)	7 (87.5%)	8 (5.4%)
Endometrioid	0 (0%)	1 (100%)	1 (0.7%)
Total	39 (26.5%)	108 (73.5%)	147 (100.0%)

Table 4: Distribution of miR-142-3p expression levels in accordance with the histological subtypes.

ROC Analysis

Receiver operating characteristic (ROC) curve shows the sensitivity, and specificity of the diagnostic tests. We performed the ROC analysis for demonstrating the differentiating strength of ovarian cancer patients, and healthy control group (Figure 2).

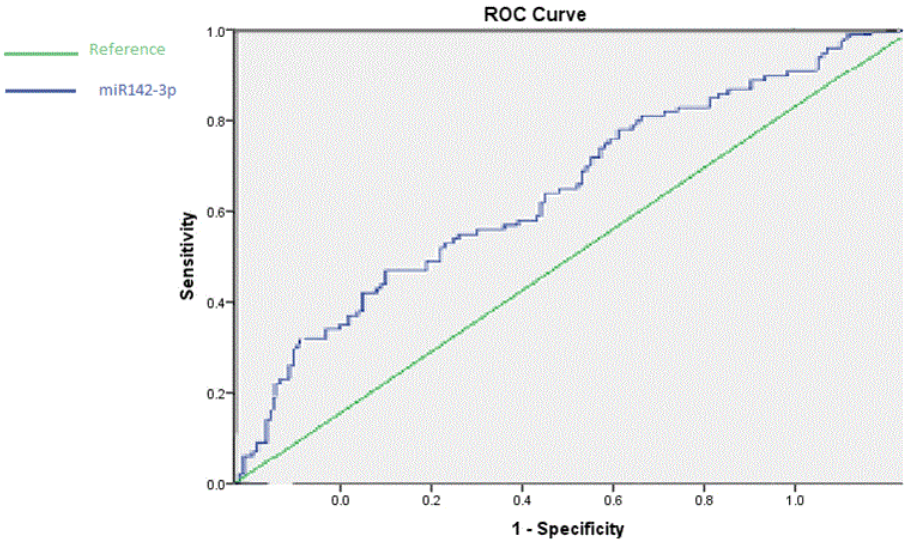

Figure 2: ROC curve analysis of the miR-142-3p expression level

In the ROC, the diagnostic strenght of the variable miR-142-3p miRNA was found statistically significant in the diagnosis of ovarian cancer patients ($p<0.005$). ROC-AUC values and 95% CI (confidence interval) results for miR-142-3p için are shown in Table 5 (Table5).

miRNA	ROC-AUC	95% CI	p value
miR-142-3p	0.355	0.285- 0.425	0.000

Table 5: The ROC-AUC value and reliability interval of miR-142-3p in the differentiation of ovarican cancer patients, and healthy group. a

Survival Analysis

The mean survival time of the ovarian cancer patients was 80 (SD ±49 m) months in our study, 47 out of 147 patients screened between 2010-2019 have died, and 100(68%) patients survived after diagnosis. We performed the Kaplan-Meier analysis for investigating whether miR-142-3p expression level was a variable affecting the survival time. We found no correlation between the gene expression levels, and survival time (p>0.005) (Table 6, and ve Figure 3).

	Chi-Square	df	p value
Log Rank (Mantel-Cox)	.799	1	.371
Breslow (Generalized Wilcoxon)	1.959	1	.162
Tarone-Ware	1.579	1	.209

Table 6: Evaluation of the correlation of the survival time and miR-142-3p expression level.

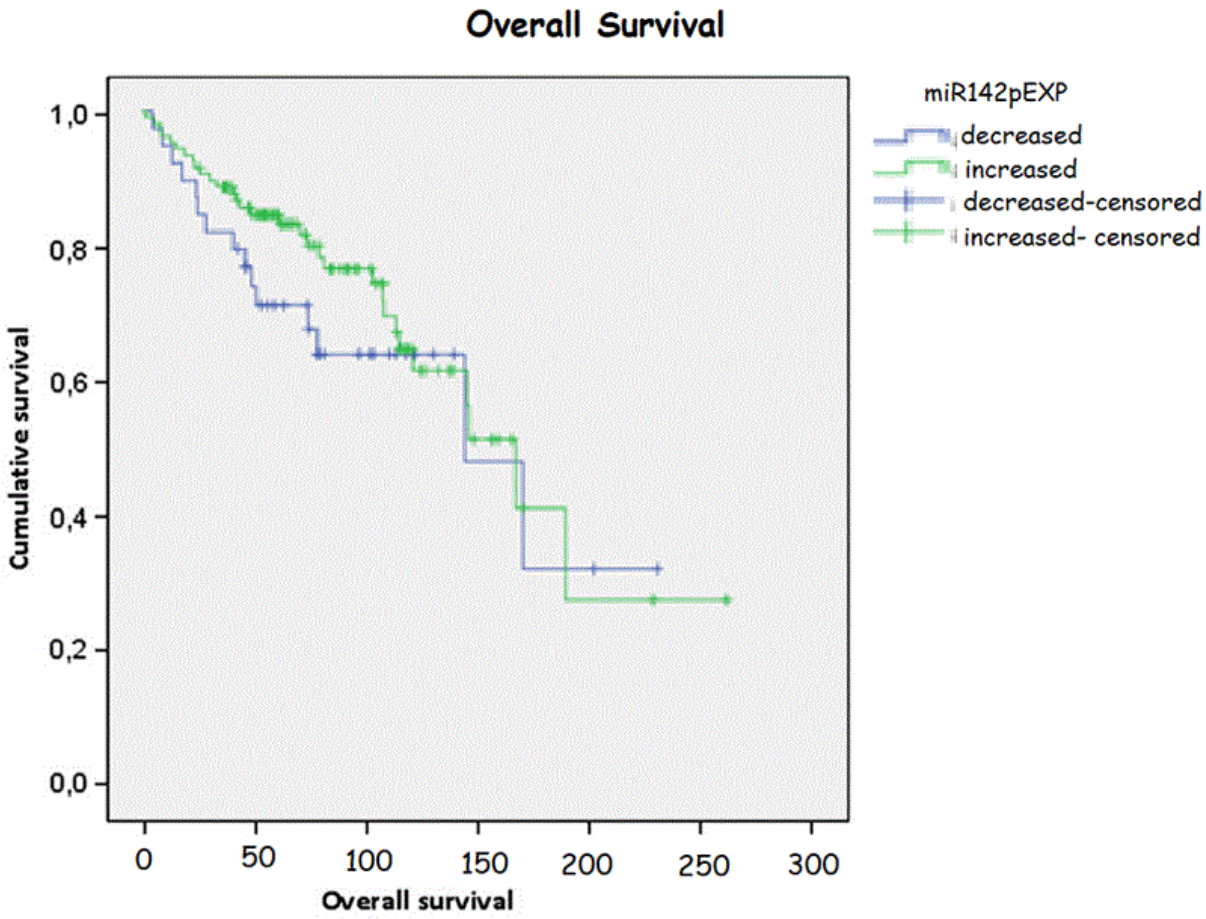

Figure 3: Survival analysis of the ovarian cancer patients in accordance with the miR-142-3p expression.

String Analysis

The target genes of miR-142-3p were screened in miRTarBase, and TargetScan databases. A total of 376 target genes were identified for hsa-miR-142-3p. These target genes were also compared with the data of the other databases. The number of target genes were decreased to 151 genes after this comparison. A group of gene sequence was identified. The gene groups were evaluated using the STRING database for identifying the interaction of this gene sequences with the proteins (Figure 4). The STRING analysis results were found statistically significant (p:0.00000238) (Figure 4).

In accordance with the STRING analysis result, the most important target genes associated with miR-142-3p were identified as *MYH9, ARNTL, CCNT2, IRAK1, TAB2, TIRAP, WASL, GNAQ, and RAC1*. There are studies in the literature demonstrating the association of these genes with the tumor. Researchers in a study reported that the function of *MYH9* gene was completely suppressed by the overexpression of miR-142-3p [9]. The lower expression of miR-142-3p in gastric cancer was reported to result with carcinogenesis with the increase of *CCNT2* expression [10]. *GNAQ, WASL, and RAC1* genes were shown to cause tumor by suppression with the higher expression of miR-142-3p in another study [11]. One of the other important proteins is the *ARNTL* which is encoded by a critical circadian transcription factor of *BMAL1* gene. *ARNTL* gene is known to target more than 150 regions in human genome, and the suppression of *ARNTL* gene was detected to have inactivated the p53 gene by modulating the CDK inhibitor of p21 in most cancers [12] [13].

These data show that miR-142-3p has both oncogeneic and tumor suppressor role. The results of these study suggest that miR-142-39 was included in the cancer mechanisms with oncogenic pathways.

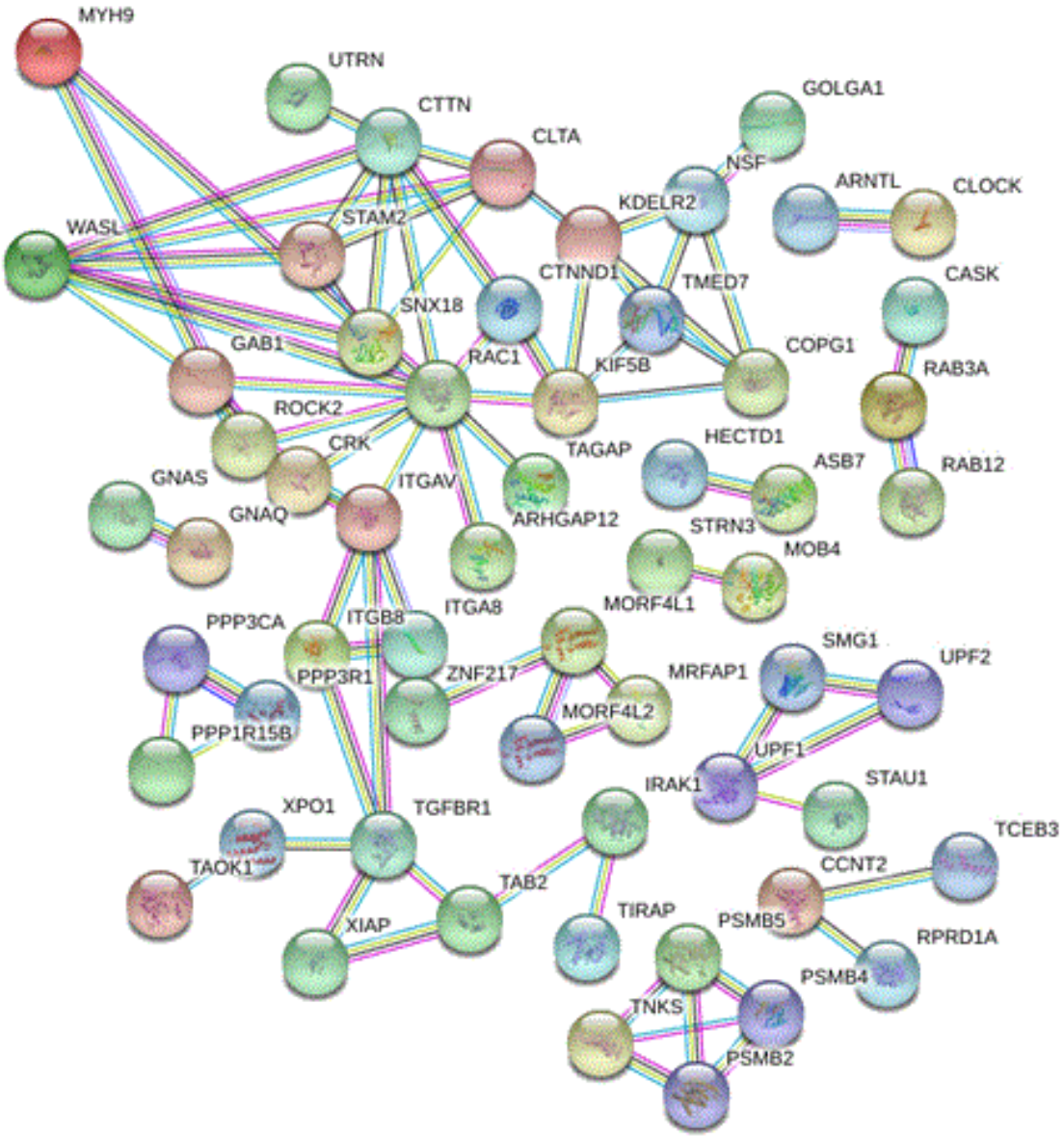

Figure 4: STRING analysis demonstrating the protein interaction of miR-142-3p.

Discussion

Ovarian cancer is a common gynecologic cancer, and treatment is difficult. The Globocan data showed that 295.414 cases were detected, and 184.799 women died worldwide in 2018. The Turkish Statistical Institute data of 2018 in Turkey, 3.729 new cancer cases were detected, and 2.191 women died of ovarian cancer [14]. The worldwide prevalence of ovarian cancer is 6.6 in 100.000, however ovarian cancer is the second leading cancer following the servical cancer in ranking of fatal gynecologic cancer with a mortality rate of 3.9 among women. The prevalence in Turkey is 7.5 in 100.000, and the prevalence rate in developed countries particularly in Central and East Europe was reported as 9 in 100.000 [15, 16]. The genetic, environmental, and hormonal factors effective in cancer development are used in the classification of the subgroups of ovarian cancer [17, 18]. The reason for not being able to clarify the pathogenesis of ovarian cancer was described with the heterogeneity in the samples taken from different regions of cancer tissue. The mechanisms forming this heterogeneity are the epigenetic, and genetic mechanisms. Epigenetic mechanisms also include the regulator feature for other microRNAs in addition to methylation, and modifications. miRNAs which are small noncoding RNA parts functioning as regulators in various diseases by destruction of the binding mRNA, are known to have oncogene and tumor supressor role in cancer. miRNAs are regarded as the sensitive and non-invasive biormarker candidate in ovarian cancer diagnosis [8, 19]. miRNAs were suggested to be possibly used as indicative factors in identifying the disease prognosis, metastasis tendency, and in chemotherapy response in addition to their possible biomarker role in the literature studies [20]. Researchers also suggested that the treatment efficacy could be improved with the use of miRNA mimics, and antagonists [21]. There is no available biomarkers in the early diagnosis of ovarian cancer which increases the risk of late diagnosis of the patients, and eliminates the treatment chance. Therefore, better understanding the ovarian biology will improve the success in diagnosis, prognosis, and treatment of ovarian cancer.

There are vaious studies in the literature investigating the association of ovarian cancer, and miRNA from different perspectives primarily for cancerisation, prognosis, and invasion [19, 22-25]. Researchers in the first study reported that the expression level of miRNA was different in normal, and cancer tissues, and this difference could differentiate both tissues [26]. miR-200a, miR-141, miR-200c, and miR-200b were reported to have been overexpressed, however, miR-199a, miR-140, miR-145, and miR-125b1 were reported to have lower expression, and there was an association between the miRNA expression levels and the histologic type, lymphovascular invasion, and metastasis in the same [26]. In another study researchers showed that miR-126 had tumor suppressor effect by inhibiting the overexpression of PAK4 detected in cancer cells in SKOV3 cell line of the ovarian cancer cell lines [27]. Yamamoto et al. showed that the lower expression of miR-1 /133a gene cluster in endometrium cancer resulted with the overexpression of *PDE7A* gene. *PDE7A* gene overexpression was shown to result with metastasis, and metastasis could be inhibited by regulating the miR-1 /133a miRNA expression level [28]. miR-200, and miR-429 miRNA molecules were reported as effective in ovarian cancer cell lines in the studies investigating the metastasis and prevention of recurrence or early detection [29]. Some miRNAs such as let-7b [30], let-7f [30], miR-200 family were reported to be associated with tumor growth, metastasis ability, tumors malignant and benign difference, tumor angiogenesis, and prognosis in another study in ovarian cancer [31, 32]. Although miR-142-3p was investigated in different cancer types in the literature, there was no study revealing the association with ovarian cancer. miR-142-3p, which was investigated in different cancer groups except in ovarian cancer, was reported to have tumor suppressor effect in some cancers, and have oncogeneic effect in some cancers [10, 33]. Higher miR-142-3p expression level was reported to have an oncogeneic role in prostate cancer [34], colorectal cancers [35][28], in leukemia[36], and esophagial [37] cancer. Researchers reported that miR-142-3p behaved like an oncogene in renal cell carcinoma (RCC) , and when inhibited, significantly supressed the cell migration, and proliferation, and induced the cell apoptosis [38]. There were some studies in the literature supporting that lower miR-142-3p level created a tumor supressor effect [39] [40]. miR-142-3p which is known to have a regulatory role in the differentiation of normal myeloid cells was found to have performed lower expression in acute myeloid leukemia [39]. miR-142-3p was reported to cause poor prognosis in cervix cancer [40], increased the invasiness in breast cancer [41], and was effective by triggering the CDK4 in colorectal cancer [42]. miR-142-3p was shown to have been highly expressed in the peripheral blood samples of monozygotic twin siblings who were discordant for ovarian cancer [8]. This study is valuable for being the first study in the literature which shows the association of miR-142-3p, and ovarian cancer. miR-142-3p which is one

of the molecules detected in the preliminary study [8] limited to two individuals was investigated with the comparison of wider ovarian cancer patient groups and with healthy controls, and its efficacy in ovarian cancer was identified. The present study was the first study demonstrating the expression difference in the peripheral blood of ovarian cancer patients compared with the healthy controls, and the potential of being a biologic marker in early diagnosis of ovarian cancer was detected. These results showed that miR-142-3p expression level was increased 3 fold in ovarian cancer patients compared with the levels in healthy controls, and was statistically statistically significant (p:0.00). We suggest that this microRNA showing high expression promoted the oncogenesis of ovarian cells by behaving such an oncogene. miR-142-3p expression level, and the clinical data, diagnosis, and family histories were analysed in details. miR-142-3p miRNA expression was found statistically significant in the analyses performed in subgroups consisting of individuals with only ovarian cancer diagnosis, with breast cancer in addition to ovarian cancer, with endometrial cancer in addition to ovarian cancer, and with another type of cancer in addition to ovarian cancer ($p<0.05$). miR-142-3p expression level was detected higher in almost all the subgroup with the diagnosis of only ovarian cancer diagnosis in the evaluations. The study showed that there was an association with the presence of family members with cancer history in the family with the miR-142-3p expression level, and that was shown to be statistically significant ($p<0.05$). These data suggest that there might be a genetic association between the miRNA level in the family, and the cancer transition. Contrary to these results, no association was detected between the presence of metastasis, stage, age, oral contraceptive use, and other clinical data with the miR-142-3p expression level ($p>0.05$). In addition, miR-142-3p molecule was shown to differentiate the ovarian cancer patients compared with the healthy controls in the peripheral blood search in the ROC analysis conducted for identifying the diagnostic strength of miR-142-3p ($p<0.05$).

We found in the STRING analysis that the genes associated with miR-142-3p functioned as both an oncogene, and a tumor suppressor. However, no data suggesting the miR-142-3p and ovarian cancer association was detected in these studies. Therefore, our study is the first study in the literature which first showed the association of miR-142-3p with the ovarian cancer. The correlation of higher miR-142-3p expression level and the the prevalence of cancer cases in the family showed that miR-142-3p might have a role in increasing the familial cancer history associated with some unclarified genetic pathways, and this must be investigated. The weakness of our study was that miR-142-3p expression level was not investigated in the peripheral blood of the the tissues of ovarian cancer patients, and individuals with benign ovarian diseases.

In summary, our results confirm that miR-142-3p molecule has a noninvasive biomarker feature in the diagnosis, treatment, and follow up of ovarian cancer. However, this molecule must be evaluated in wider groups, and in the peripheral blood samples of individuals with benign ovarian diseases.

Declaratıons

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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. YG performed the experiments and help writing the manuscript. SBT and DAO 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. XJ, GU and BK 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. OSE and SK performed STRING analysis. BC 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 XXXX Medical Faculty in XXXX University (approval no. 2019524). Written informed consent was provided by the parents/guardians of the participants included in the study.

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