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Aberrant miR-3135b and miR-1273g-3p expression in the peripheral blood samples of BRCA1/2 (±) ovarian cancer patients

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ABSTRACT

Ovarian cancer (OC) ranks as the eighth most prevalent malignancy among women globally. The short non-coding RNA molecules, microRNAs (miRNAs) target multiple mRNAs and regulate the gene expression. Here in this study, we aimed to validate miR-3135b and miR-1273g-3p as novel biomarkers for prognostic and diagnostic factor OC. After RNA isolation, we analyzed the miR-3135b and miR-1273g-3p expression in peripheral blood samples derived from 150 OC patients. Subsequently, we compared their expression levels with 100 healthy controls. The differences of miR-3135b and miR-1273g-3p expression were detected using the Quantitative Real Time-PCR (qRT-PCR) technique following miRNA-specific cDNA synthesis pursing miRNA separation. The miR-3135b and miR-1273g-3p were higher in OC patients who tested positive for BRCA1/2 compared to BRCA-negative patients, and healthy cases. The level of miR-3135b demonstrated a roughly 4.82-fold increase in OC patients in comparison to the healthy cases, while miR-1273g-3p expression exhibited a roughly 6.77-fold increase. The receiver operating characteristic (ROC) analysis has demonstrated the potential of miR-3135b and miR-1273g-3p as markers for distinguishing between OC patients and healthy controls. The higher expressions of miR-3135b and miR-1273g-3p could be associated with OC development. Moreover, miR-3135b may have a diagnostic potential and miR-1273g-3p may have both diagnostic and prognostic potential in OC cell differentiation. The string analysis has revealed an association between miR-1273g-3p and the *MDM2* gene, suggesting a potential link to tumor formation through the proteasomal degradation of the *TP53* tumor suppressor gene. Additionally, the analysis indicates an association of miR-1273g-3p with *CHEK1*, a gene involved in checkpoint-mediated cell cycle arrest. String analysis also indicates that miR-3135b is associated with the *MAPK1* gene, causing activation of the oncogenesis cascade. In conclusion, miR-1273g-3p, and miR-3135b exhibit

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significant potential as diagnostic markers. However, further research is needed to comprehensively investigate these miRNAs diagnostic and predictive characteristics in a larger cohort.

1. Introduction

Ovarian cancer (OC) ranks as the eighth most prevalent gynecological malignancy. It is also the fifth highest cause of cancer-related mortality in women. The prevalence of ovarian cancer affects around 313.959 women, with an annual mortality rate of approximately 207.252 [1]. Despite advances in detection and treatment strategies, it continues to be the most severe gynecologic tumor in developed countries. The 5-year overall survival rate for early-stage OC is approximately 90%**,** however, the survival rate in the late-stage disease is 20–40% [2]. The lack of typical symptoms and signs at early stages cause the rapid growth of OC progress from early to advanced stage within one year [3]. Therefore, it is crucial to contribute early identification and diagnosis markers for OC patients.

MicroRNAs (miRNAs) are compact RNA molecules, typically around 22 nucleotides in length, which act as antisense RNA to suppress the post-transcriptional expression of target genes [4]. Dysregulation of miRNAs causes several human diseases, including OC [5]. miRNAs dysregulation contributes to the development of cancer via DNA point mutations, epigenetic mechanisms, chromosomal modifications, adjustments in translation, and alterations in the genetic and epigenetic aspects of both transcriptional and post-transcriptional levels [6]. miR-1273g-3p, 21 nucleotides long, encoded in *SCP2* gene intron [7]. One previous study revealed the potential of miR-1273g-3p in the modulation of hepatic stellate cell behavior by directly affecting *PTEN* [8]. Furthermore, a recent study found that miR1273g3p is upregulated in the A549 lung cancer cell line and enhances cellular migration [9]. However, the status of miR-1273g-3p expression in OC patients has not been elucidated. miR-3135b, a non-coding RNA, which was mapped on chromosome 6p21.32 [10]. The bioinformatics analysis revealed the probable target of the *GOLPH3* for miR-3135b [11] and was reported to be involved in preserving Golgiapparatus structure and enhancing the expression of AKT and mTOR genes. Stimulation of *AKT* and *mTOR* gene expression contributes to the enhancement of cell survival. Furthermore, bioinformatics analysis suggested that miR-3135b might have a role in regulating protein-coding genes to participate in cell survival, chemotherapy resistance, and Golgi functions [10].

BRCA1 and *BRCA2* gene mutations are well-established risk factors for OC [12]. Inherited mutations in these genes can increase the lifetime risk of OC from 20 % (*BRCA2*) to 50 % or higher (*BRCA1*) [13]. In our previous research, we used microarray analysis to discover differences in the miRNA expression profile in monozygotic discordant twins [14]. Out of 2549 miRNAs, we detected 99 miRNAs, including miR-3135b and miR-1273g-3p, that could be used as novel therapeutic targets for diagnosing, treating, and managing epithelial OC, providing new avenues for therapeutic intervention. Here in this study, we analyzed the expression of miR-3135b and miR-1273g-3p in 150 OC patients (n = 120 *BRCA1/2* mutation-negative and n = 30 *BRCA1/2* mutation-positive patients) peripheral blood samples and 100 healthy controls for validation of our previous microarray results. We aimed to determine the potential of miR-3135b and miR-1273g-3p as biomarkers for the early detection of OC. The identification of novel expression patterns of miRNAs in various types of cancer may improve our understanding of their role in cancers including OC.

2. Material and methods

2.1. Selection and description of participants

This research was carried out following the ethical principles outlined in the Declaration of Helsinki [15]. We received approval from the Istanbul University Clinical Research Ethics Committee. Before performing genetic analysis, the patients received genetic counseling, and their informed consent was obtained. In the study, we analyzed peripheral blood samples from 150 OC patients attending our clinic and compared them with 100 healthy cases. The control group was matched in terms of age, gender, and ethnic background, and no cancer history in the family for three generations.

2.2. Leukocyte and RNA isolation

The Ficoll technique was used to separate leukocytes from peripheral blood mononuclear cells. Initially, a 20 mL peripheral blood sample was collected from the patients. First, 3 mL of Ficoll solution (Sigma-Aldrich, Darmstadt, Germany) was added to a centrifuge tube. Then the blood samples were poured into a Ficol-containing tube [16]. Then RNA from the acquired lymphocytes of OC patients and control subjects was isolated using the Quick-RNA Miniprep Kit (Quick-RNA-TM MiniPrep, Zymo Research, USA) according to the kit protocol [17]. The NanoDrop 2000 spectrophotometer (Thermo Scientific) was used to measure the quantity. The purity and quantity of the isolated RNAs were assessed using absorbance measurements at A260/A280 nm wavelengths. Pure/suitable RNA samples were defined as those that had absorbance in the range of 1.6–2.0 OD.

2.3. Reverse transcription and real-time qPCR

An ID3EAL cDNA synthesis system (ID3EAL miRNA qPCR Starter Kit, Singapore) was used to prepare the cDNA template. A real-time PCR instrument (Mic Real-Time PCR System) was used to examine the miRNA expression. Melting curve analysis was used to identify the qPCR products including the target miRNA and reference gene.

Gene expression analysis was assessed using the $2^{-\Delta\Delta CT}$ formula. The average of cycle threshold values was used to calculate triplicate results for each sample. The expression levels of miR-3135b and miR-1273g-3p in the patients were evaluated by comparing them with the levels observed in the control group using the $2^{-\Delta\Delta CT}$ formula.

2.4. Statistics

Using the Statistical Package for the Social Sciences (SPSS) v27.0 software, normality assumptions were tested using the Kolmogorov–Smirnov. As the p-value was less than 0.05, the result indicated that the data did not follow a normal distribution, and the non-parametric Mann-Whitney *U* test was found acceptable for data analysis. In addition, operating characteristic curve analysis (ROC), and logistic regression modeling were used to develop a combination model of miRNAs identified as potential biomarkers, to demonstrate their diagnostic power. The Chi-square test was used to perform pairwise analysis of the medical data. Kaplan-Meier analyses were used to determine the survival rates of patients, considering statistical significance at a p-value below 0.05.

2.5. Bioinformatics analysis for microRNA and target genes

The genes that exhibit significant algorithmic overlap with the sequences of miR-3135b and miR-1273g-3p from the miRTarBase database, respectively, with high scores in the database are listed. The proteins associated with miR-3135b and miR-1273g-3p sequences from the miRTarBase database were examined for their interactions in the String database. The TSV file containing the protein interactions derived from the String database was prepared for use with the Cytoscape bioinformatics software platform. After Cytoscape software platform analysis, the hub genes that had the highest interaction with each other were selected. Hub genes interact extensively with other genes or proteins within a network and are therefore at the center of the network. Identifying hub genes is important for better understanding the network structure and studying biological processes. The research flowchart that is shown in Fig. 1.

3. Results

In the present study, we investigated the miR-3135b and miR-1273g-3p expression in the peripheral blood samples from 150 *BRCA1/2* (±) patients diagnosed with OC and compared them with 100 healthy cases. The mean age of OC patients was 49.6 ± 10.3 years and 46.9 ± 9.1 years for the control group. 120 out of 150 cases were negative for *BRCA1/2* mutation. Also, in 30 of the cancer patients, 14.7 % (22/150) had *BRCA1* mutations and 5.3 % (8/150) had *BRCA2* mutations. 17 of 150 OC patients (11.3 %) had secondary primary cancer. 101 OC patients had metastasis. Table 1 presents the OC patient's clinical characteristics investigated in this study.

In OC patients, miR-3135b and miR-1273g-3p were found to be increased $2^{-\Delta\Delta Ct} = 4.82$ fold and $2^{-\Delta\Delta Ct} = 6.77$ fold, respectively.

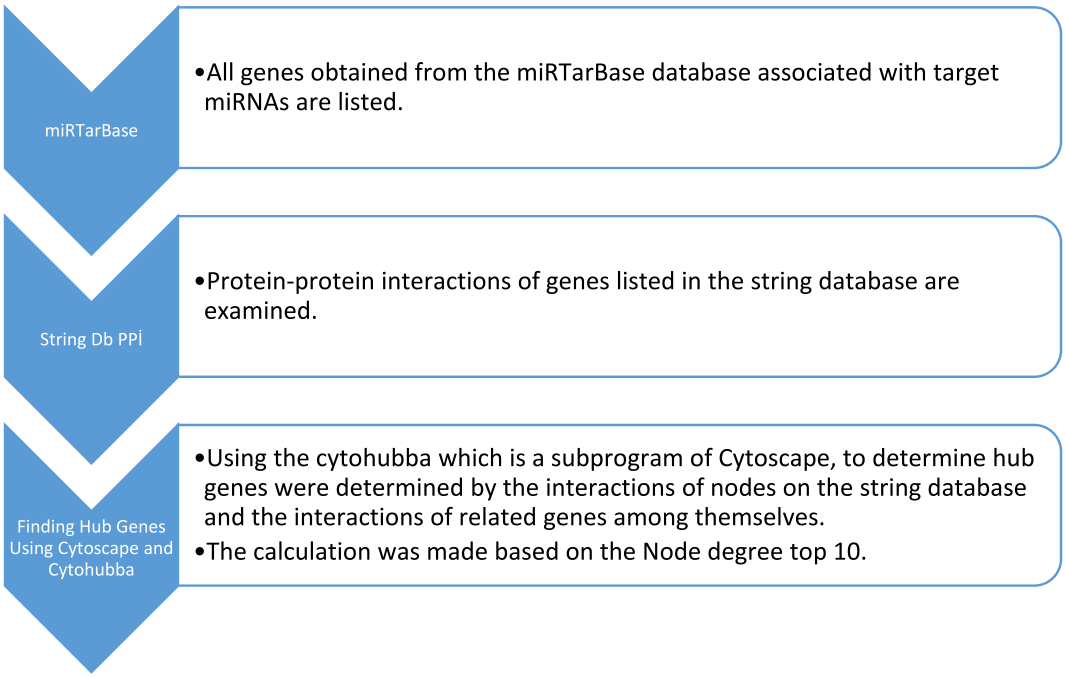

Fig. 1. Research flowchart.

Table 1
The clinical characteristics of ovarian cancer patients investigated in this study.

Clinical Parameters		Frequency(n)	Percentage(%)
The age at diagnosis	49.6 ± 10.3 years		
Patients age	<45y	48	32.0
	>45y	102	68.0
BRCA1 mutation status	Negative	128	83.3
	Positive	22	14.7
BRCA2 mutation status	Negative	142	94.7
	Positive	8	5.3
Secondary Primary Cancer	No	133	88.7
	Yes	17	11.3
Ovarian Metastasis Status	No	49	32.7
	Yes	101	67.3
Histological Grades	1	22	14.7
	2	34	22.7
	3	94	62.7
Histological Subtypes	Serous carcinoma	132	88
	Mucinous carcinoma	6	4.0
	Clear cell carcinoma	10	6,6
	Squamous cell carcinoma	1	0.7
	Endometrioditis	1	0.7
Lymph Nodes Involvement	No	12	8.0
	Yes	138	92.0
Treatment	Only Chemotherapy	5	3.3
	Only Surgery	14	9.3
	Chemotherapy + Surgery	115	76.7
	Chemotherapy + Radiation therapy + Surgery	16	10.7
Smoking	No	110	73.3
	Yes	40	26.7
Final Condition	Death	54	36
	Disease Present	89	59.3
	Unknown	7	4.7

If the FC = 2 or greater means upregulated. The examination based on the $2^{-\Delta\Delta Ct}$ values ($P < 0.05$), the Mann–Whitney *U* test was conducted. For each miRNA, the p-value was (*$P < 0.05$) for miR-1273g-3 and (*$P < 0.05$) for miR-3135b, respectively. The comparison of the expression level differences ($2^{-\Delta\Delta Ct}$) of the OC patients and the healthy controls was shown in Fig. 2.

The association of the expression miR-3135b and miR-1273g-3p and smoking behavior, locally advanced OC, and oral contraception were assessed using the Mann–Whitney *U* test. We identified a statistically significant correlation between the expression levels in miR-3135b level and smoking habits (*P: 0.004), but did not detect a statistically significant association between miR-1273g-3p and smoking behavior ($P > 0.05$). The miR-1273g-3p expression was statistically upregulated in metastatic patients (*P: 0.001), however, there was no correlation found between miR-3135b and metastatic status ($P > 0.05$). The use of oral contraceptives did not demonstrate statistical significance ($P > 0.05$) when compared to the expression of miR-3135b and miR-1273g-3p. The miR-3135b and miR-1273g-3p expression levels of patients younger than 40 years and over 40 years were analyzed independently, and no statistical significance was found in gene expression change with age ($P > 0.05$). Our results identified no significant ($P > 0.05$) miR-3135b and miR-1273g-3p differences between the ages. Following diagnosis, individuals with OC exhibited a median survival duration of 12.11 months (SD ± 0.792 m). Among the 150 patients, 54 (36 %) were deceased, 89 (59.3 %) survived, and the health status of 7 (4.7 %) individuals remained undetermined. The influence of miR-3135b and miR-1273g-3p expression levels on survival was investigated using Kaplan-Meier methods. The expression was not associated with survival rates.

To demonstrate the strength differentiation of miR-3135b and miR-1273g-3p in OC and healthy control, we used the ROC analysis shown in Fig. 3.

The results showed that miR3135b was a more specific biomarker compared with the miR-1273g-3p in this patient group (Table 2). The determined threshold value and the specific miRNAs showed notable statistical importance in diagnosing OC cases.

Additionally, Spearman's correlation analysis was used to analyze potential linear connections between CA-125 values and the levels of miR-3135b and miR-1273g-3p. There was no correlation or significant difference between miR-3135b and miR-1273g-3p expression and CA-125 numerical variables, both at the onset and throughout the therapy course (*$P < 0.05$).

The Identification of the genes and proteins with which miR-3135b and miR-1273g-3p interact by string analysis and identification of hub genes: Experimentally validated miR-3135b and miR-1273g-3p targets were obtained from miRTarBase database [16]. MicroRNA target interactions are based on manual curation. First, it was obtained from the miRTarBase database that 4857 genes related to miR-3135. Then, 313 genes that could be the target of miR-3135b were identified in a string analysis, and 38 showed strong interactions with each other. The genes, which had an interaction value greater than 0.95, were analyzed (Fig. 4). The same procedures were performed for miR-1273g-3p. It was obtained from the miRTarBase database that 2579 genes related to miR-1273g-3p. Then 365 genes that could be miR-1273g-3p targets were identified in a string analysis, and 39 showed strong interactions with each other. The interaction value of genes, which had an interaction value greater than 0.95 were analyzed (Fig. 6). The interactions of the proteins, which are the products of genes associated with the miR-3135b and miR-1273g-3p sequences from the

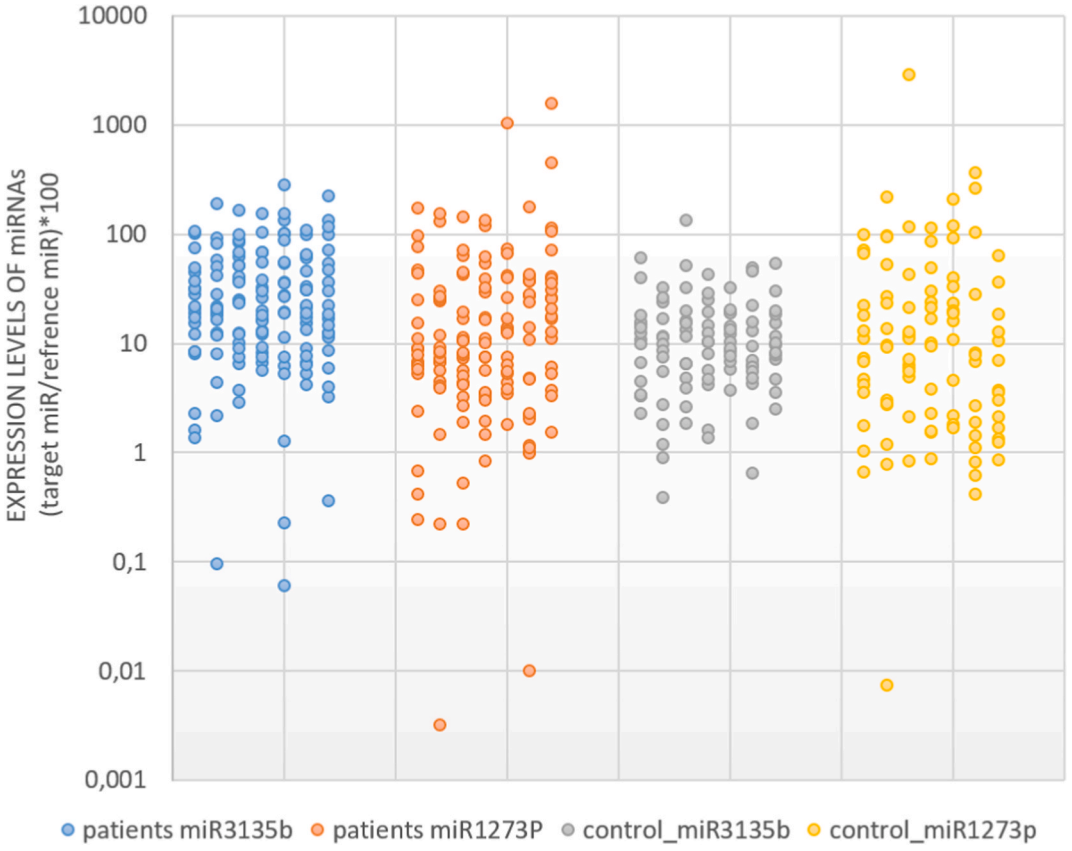

Fig. 2. The miR-3135b and miR-1273g-3p expression profiles of OC compared to healthy controls.

miRTarBase database, with each other in the String database were examined. The TSV file containing the protein connections derived from the String database was then made ready to be run on the Cytoscape bioinformatics software platform. After Cytoscape software platform analysis, which interacted with miR-3135b and each other, the top ten hub genes were selected (Fig. 5) and which interacted with miR-1273g-3p and each other, the top ten hub genes were selected (Fig. 7).

3.1. Expression analysis of miR-3135b and mir-1273g-3p with BRCA1/2 mutation status

miR-3135b expression was found to have increased in 44 % (14/30) of the BRCA1/2 patients and 50 % (15/30) for miR-1273g-3p in BRCA1/2 positive patients. Whereas, 42 % (14/30) of BRCA1/2 negative OC patients showed an increase in miR-1273g-3p and 37 % (12/30) for miR-3135b.

4. Discussion

In the early stages of OC, there are no apparent symptoms, and over than 60 % of patients with OC have abdominal or distant metastases [17]. Expression profiling methods have advanced greatly in recent years, allowing a better understanding of the clinical consequences of cancer, as well as prospective applications in diagnosis, treatment, and drug development. A pelvic exam, a trans-vaginal ultrasound, and CA-125 are the typical methods used for diagnosing OC, however, the sensitivity and specificity of these tests are not adequate for early detection [18]. In clinical practice, due to a lack of specific and sensitive biomarkers, OC cases are diagnosed at advanced stages [19]. Therefore, the exploration of relevant biomarkers is crucial for the management of OC. The circulating miRNAs in various body fluids are highly expressed in stable forms [20] indicating their appropriateness for identifying cancer at an early stage [21]. Abnormal miRNA expression has been observed in numerous cancers. Different studies highlighted the abnormal expression of miRNA in OC is associated with cell migration and invasion [19]. Here, we examined the expression levels of miR-3135b and miR-1273g-3p for *detection* and prognosis biomarkers for OC cases. The results indicate the upregulation of oncogenic miRNAs blocks the tumor-suppressing mRNAs leading to tumor formation. Consequently, in this study, the miR-1273g-3p expression level was higher in an advanced stage. The results suggest that miR-3135b may have diagnostic value, and miR-1273g-3p may have both diagnostic and prognostic value, as previously indicated in our study [14]. In our analysis, we investigated the correlation between level CA-125 and the miR-3135b, miR-1273g-3p and detected a statistically significant association. Furthermore, we detected major

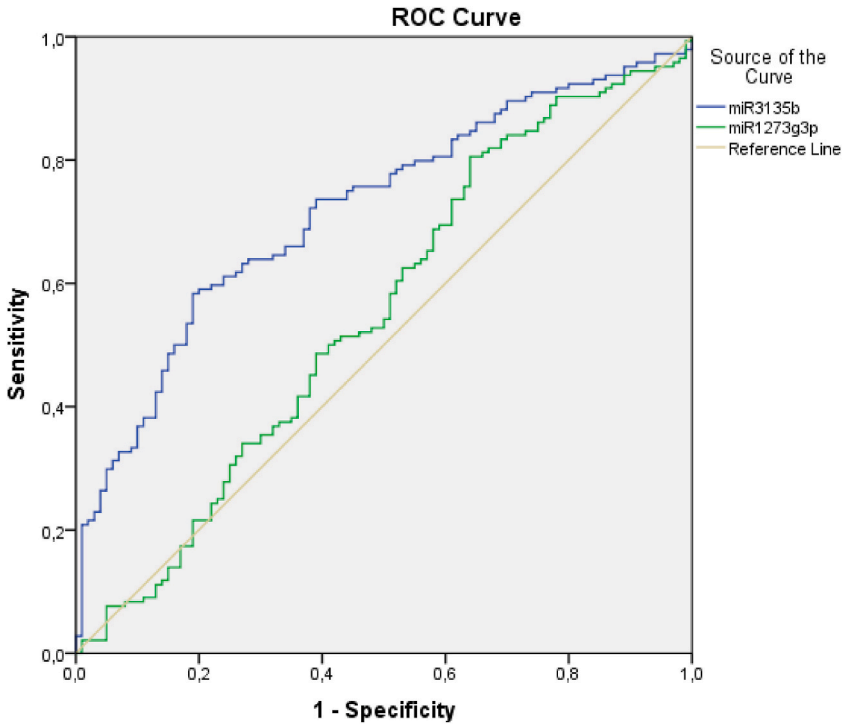

Fig. 3. ROC Curve Analysis of miR-3135b and miR-1273g-3p ovarian cancer patinets and healthy cases.

Table 2
The ROC curve analysis of miR-3135b and miR-1273g-3p distinguish ovarian cancer from healthy controls for possibility of biomarker.

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95 % Confidence Interval		Sensitivity (%)	Specificity (%)	Cut-off value
				Lower Bound	Upper Bound			
miR3135b	.719	.033	*.000*	.656	.783	72.2	62	1.35
miR1273g3p	.552	.038	.168	.477	.627	58.3	49	0.96

[a] Under the nonparametric assumption.
[b] Null hypothesis: true area = 0.5.

differences in metastasis patterns among patients with higher miR-1273g-3p levels. miR-1273g-3p was reported to act as a key regulator for multiple biological targets. For example, the decrease in miR-1273g-3p level compared to tumor-derived cells was reported in OC stem cells, suggests its potential role in the development of serous ovarian cancer. Cancer stem cells were also reported to play a role in developing resistance to chemotherapy and radiotherapy [22]. The potential role of miR-1273g-3p in contributing to resistance in ovarian cancer patients warrants further investigation. According to the literature, the miR-1273g-3p regulates four key genes linked to the regulation of OC. These genes are MMP-2, MMP-9, TNF-α, and Alpha-1 collagen. *TNF-α* expression is regulated by miR-1273g-3p [23]. *TNF-α* is typically overexpressed in OC [24], and is linked to a higher tumor grade [25]. DNA hypomethylation *TNF-α* in OC cell lines was also reported. *TNF-a* has demonstrated the ability to impede the COL1A1 promoter [26]. *COL1A1* facilitates the target genes that regulate proliferation and migration in OC. In addition, *COL1A1* affects chemoresistance through a hypermethylation mechanism. DNA hypermethylation *COL1A1* and epigenetic gene silencing have been linked to resistance to platinum in OC [27,28]. These studies showed that *TNF-α and COL1A1* genes are associated with OC. In our study, we observed an up-regulating miR-1273g-3p expression in OC patients. The string analysis suggests that the target gene of miR-1273g-3p, MDM2, encodes a nuclear-localized E3 ubiquitin ligase. This protein promotes tumor formation by targeting the *TP53* tumor suppressor genes [29]. *TP53* acts as a tumor suppressor gene and plays a critical role in apoptosis and cell cycle regulation, senescence, and autophagy to prevent angiogenesis and metastasis [30] and *TP53* mutations were detected in 96 % of all serious cancers. miR-1273g-3p gene is also associated with *CHEK1* according to the String Database. The *CHEK1* gene produces a member of the Ser/Thr protein proteins kinase family [31], and is essential for inducing cell cycle arrest in response to DNA damage through checkpoint mechanisms.

Wu et al. indicated that miR-3135b is associated with vital biological processes and functions [32]. In another study, the expression profile of miR-3135b from the database GEO -151 was evaluated using the online GCBI program, and was reported that miR-3135b was differentially expressed in granulosa cells of patients with polycystic ovarian syndrome (PCOS). Gap junction is important for oocyte

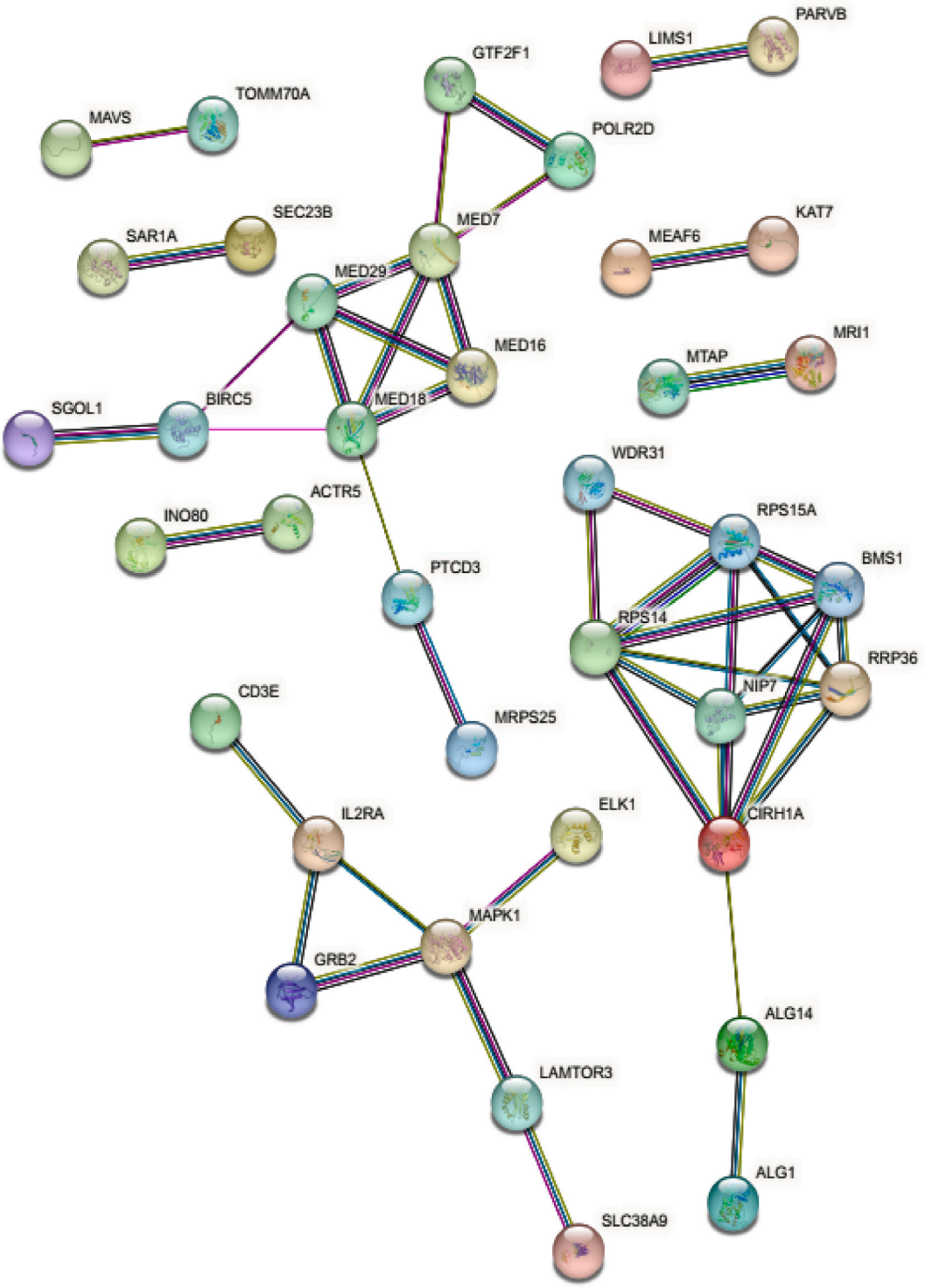

Fig. 4. miR-3135b gene-protein interaction network study. The STRING database was used to generate gene interactions. Genes are represented by network nodes, whereas gene-protein interactions are represented by network rounds.

growth and maturation, and dysfunction of GCs causes abnormal follicle maturation [33]. Dicer, a ribonuclease III enzyme involved in the synthesis of mature functional miRNAs, was found both in oocyte and follicle granulosa cells. Lei et al. stated that the inactivation of Dicer1 in follicle GCs resulted in enlarged primordial follicles, accelerated early follicle recruitment, and more dysfunctional follicles [34]. Consequently, the loss of miRNA in GCs resulted in aberrant oogenesis and a barrier to follicle development. Furthermore, Wang

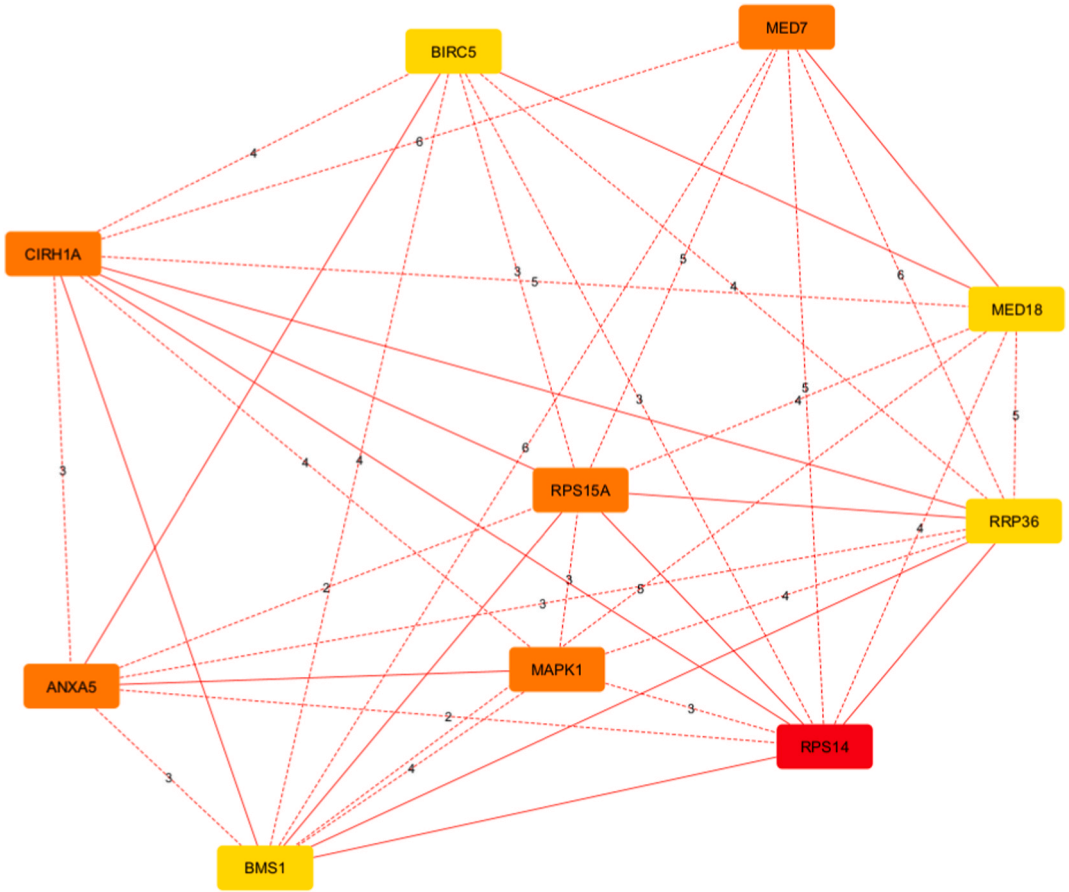

Fig. 5. miR-3135b gene interaction network containing Hub genes. The genes were detected using the 'cytoHubba' plugin through mixed character calculation. As the dot color intensifies, the importance of hub genes rises.

et al. used qRT-PCR methods to confirm the differentially expressed miRNAs and the expression of miR-3135b was found to have significantly increased in PCOS GCs [35]. However, the function and molecular mechanism of the recently discovered miR-3135b is unknown and has not been reported in OC patients. In this study, higher expression of miR-3135b was detected in OC patients. The string analysis revealed that miR-3135b is related to the *MAPK1* gene, coding mitogen-activated protein kinase protein. MAPK1 is also reported to be stimulated by chemotherapeutic medicines and growth factors. Hence, the proliferation, apoptosis, and survival in OC in response to these external stimuli such as reproductive hormones in epithelial surfaces of ovarian may be significantly influenced by the MAPK signaling system [36].

BRCA1/BRCA2 mutations confer risk for OC. Several studies have shown that *BRCA1/BRCA2* mutation-carriers women had a greatly increased risk of OC [12,37–39]. We also observed elevated expression of miR-3135b and miR-1273g-3p among OC patients who were carrying BRCA1/2 mutations. Additionally, the combination of miR-3135b and miR-1273g-3p may serve as a valuable marker for the detection of ovarian cancer patients. We discovered that miR-3135b and miR-1273g-3p were associated with BRCA1/2 positive OC patients in our previous study [14]. This study also revealed increased miR-3135b and miR-1273g-3p expression among OC patients contrary to healthy controls. The inclusion of cancer patients from a single hospital in the study could potentially introduce bias and restrict the applicability of the findings to a broader population. These novel biomarkers show promise as diagnostic indicators, but further research is necessary to assess the diagnostic and predictive qualities of miR-1273g-3p and mir-3135b in a more extensive sample of OC patients.

Early diagnosis increases curative treatment of a variety of malignancies. The expression profile of miRNA at different stages, grades, treatment resistance status, and other diagnostic and therapeutic targets in OC remains to be described. The aberrant miRNA expression profile could potentially serve for the early detection of OC. Once miRNAs and their functional targets are discovered, their clinical significance can further be explored. Since conventional diagnostic approaches have been unsuccessful in the early diagnosis of cancer, miRNA profiling in peripheral blood samples might help to overcome a critical obstacle in OC.

Data availability statement

Data will be made available on request.

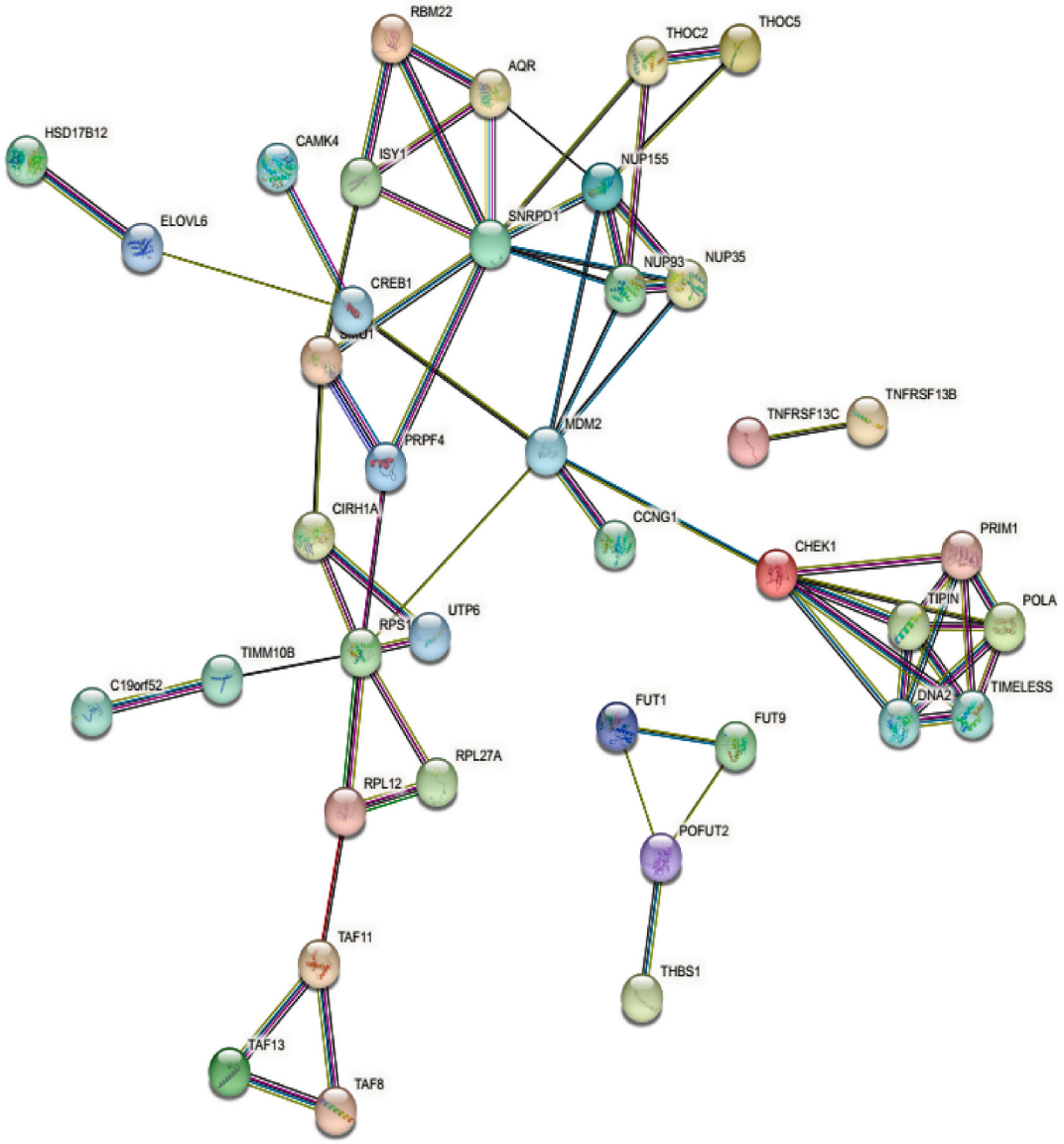

Fig. 6. miR-1273g-3p gene-protein interaction network study. The STRING database was used to generate gene interactions. Genes are represented by network nodes, whereas gene-protein interactions are represented by network rounds.

CRediT authorship contribution statement

Seref Bugra Tuncer: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Betul Celik:** Writing – review & editing, Writing – original draft, Formal analysis. **Seda Kılıc Erciyas:** Formal analysis, Data curation. **Ozge Sukruoglu Erdogan:** Visualization, Data curation. **Ozge Pasin:** Formal analysis. **Mukaddes Avsar:** Methodology, Data curation. **Busra Kurt Gultaslar:** Methodology, Data curation. **Arash Adamnejad Ghafour:** Writing – original draft, Data curation. **Gamze Uyaroglu:** Methodology, Data curation. **Demet Akdeniz Odemis:** Methodology, Formal analysis. **Hulya Yazıcı:** Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

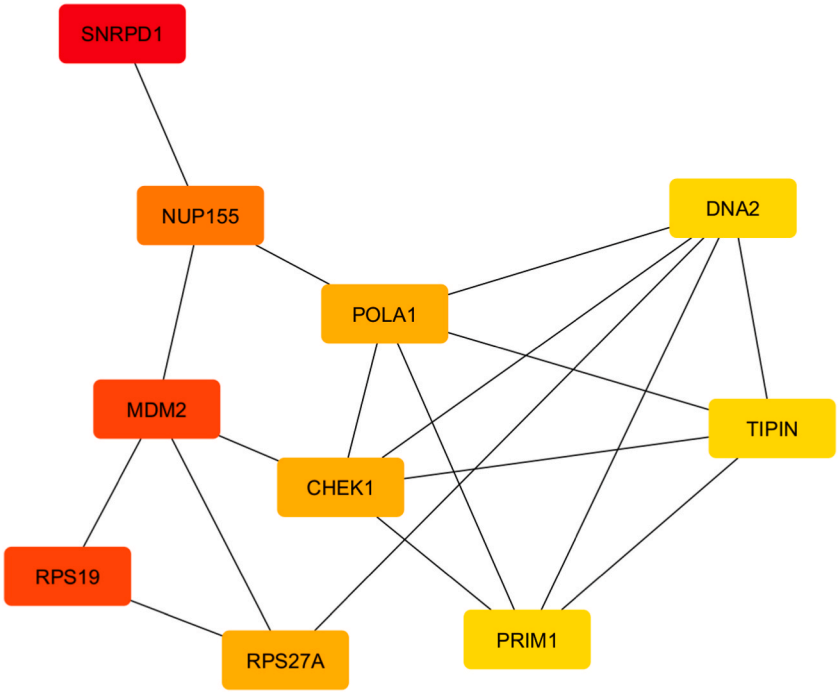

Fig. 7. miR-1273g-3p gene interaction network containing Hub genes. The 'cytoHubba' plugin pinpointed these genes through a mixed-character computation method. The importance of hub genes escalates with the rising intensity of the dot color.

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