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A Bioinformatics Approach to Male Infertility, MicroRNAs, and Targeted Genes

Erkek İnfertilitesi ile İlgili MikroRNA'lara ve Hedef Genlere Biyoinformatik Yaklaşım

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ÖZ

Amaç: İnfertilite, dünya çapında çiftlerin yaklaşık %12'sini etkileyen bir sağlık sorunudur. İnfertilite oluşumunda erkek kaynaklı sorunların payı yaklaşık %50'dir. Birçok hücresel süreçte rol oynayan mikroRNA (miRNA)'lar, spermatogenez sürecinde de kritik rol üstlenmektedir. Anormal miRNA ifadesinin erkek fertilitesi üzerinde zararlı etkilerinin olduğu gösterilmiştir. İnfertilite ile ilişkili genlerdeki genetik değişikliklerin yanı sıra miRNA'lar gibi gen ekspresyonunu değiştiren epigenetik faktörlerin de infertilite sürecinde kuşkusuz rolü vardır. Bununla birlikte, infertilite ile ilgili genlerle hangi miRNA'ların ilişkili olduğu tam olarak bilinmemektedir. Bu çalışmanın amacı, çeşitli biyoinformatik araçlar kullanılarak infertilite ile ilişkili genlerin düzenlenmesinde rol oynayabilecek miRNA'ları belirlemektir.
Araçlar ve Yöntem: Çalışmada ilk önce Erkek İnfertilitesi Bilgi Bankası (MIK) veri tabanından infertilite ile ilişkili genler seçildi. Seçilen genlerin yolak analizi, bu genlerle ilgili protein-protein etkileşimi (PPI) ve hub proteinler, Elsevier pathway veri tabanı ve Enrichr aracı ile ortaya çıkarıldı. Ardından infertilite ile ilişkili genleri etkileyebilecek miRNA'lar belirlendi. Daha sonra miRNA-hedef gen ilişkisinin erkek infertilitesine etkisi miRPathDB 2.0 veritabanı, StarmiR ve miRNet gibi çeşitli in siliko araçlar kullanılmak suretiyle biyoinformatik olarak ortaya çıkarıldı.
Bulgular: MIK veri tabanından Erkek infertilitesi ile ilişkili 21 gen seçildi ve bu genlerin ifadesini biyoinformatik olarak düzenlemesi en muhtemel 15 miRNA belirlendi. Aynı zamanda seçilen erkek infertilite genleriyle ilişkili olabilecek 10 hub protein tespit edildi.
Sonuç: Biyoinformatik çalışma sonuçlarımız, miR-34a-5p ifade değişiminin *CREM, LAMP3, AGBL5, FOXM1* genleri aracılığıyla ayrıca miR-335-5p'nin *CFAP65, CFTR* ve *GAPDHS* genleri üzerinden erkek infertilitesine neden olabileceğini göstermektedir.

Anahtar Kelimeler: erkek infertilitesi; miR-335-5p; miR-34a-5p

ABSTRACT

Purpose: Infertility affects nearly 12% of couples worldwide, with a male factor being the primary or contributory reason in around 50% of cases. MicroRNAs (miRNAs) are essential post-transcriptional regulators in the spermatogenesis process, and dysregulated miRNAs have been shown to have harmful effects on male fertility. However, it is unclear which miRNAs are associated with infertility-related genes. The aim of this study is, to identify miRNAs that may be involved in the regulation of infertility-related genes using various bioinformatics approaches.
Materials and Methods: The study first selected genes associated with infertility from the Male Infertility Knowledge Base (MIK) database. Pathway analysis of the defined genes, protein-protein interaction (PPI), and hub proteins related to these genes were revealed by the Elsevier pathway collection database and Enrichr tool. Following that, miRNAs that can influence infertility-related genes were determined, and the influence of the miRNA-target gene connection on male infertility was established bioinformatically using various in silico tools such as miRPathDB 2.0 tool, StarmiR, and miRNet.
Results: 21 male infertility associated genes were selected from the MIK database and 15 miRNAs that are most likely to regulate these genes were identified bioinformatically. 10 hub proteins related to defined male infertility genes were analyzed.
Conclusion: Our bioinformatic study results indicate that miR-34a-5p dysregulation may contribute to infertility through *CREM, LAMP3, AGBL5, FOXM1* genes and also miR-335-5p may cause infertility via the *CFAP65, CFTR,* and *GAPDHS* genes.

Keywords: male infertility; miR-335-5p; miR-34a-5p

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INTRODUCTION

Infertility is defined by the World Health Organization (WHO) as the failure to conceive following at least one year of regular, sexual contact without protection.[1] According to the Global Burden of Disease survey, the prevalence of infertility increased by 0.370% in women and 0.291% in the male population in recent years.[2] Male infertility is caused by a variety of reasons, including congenital or idiopathic conditions that disrupt spermatogenesis. Many health issues may impact male fertility, emphasizing the importance of a complete patient screening to determine treatable or reversible lifestyle variables or medical diseases. The utilization of assisted reproductive techniques has substantially improved infertile individuals' willingness to have biological children.[3] Spermatogenesis is a complex, multi-step process. Various defects in the spermatogenesis process can result in male infertility, male reproductive system illnesses, and even malignancy.[4] Infertile men's spermatogenic dysfunction can be caused by many factors, and the precise molecular pathways remain unknown. Although sperm evaluation remains the gold standard for detecting and managing male infertility, more advanced diagnostic methods are required to assess sperm quality and function.[3]

Numerous genes have been associated with male infertility in the literature. For example in the last thirty years, many studies have revealed the effect of *CFTR* mutations on sperm function. Studies have shown that poor sperm quality or low fertilization rate in IVF increases due to *CFTR* gene mutations.[5] *FOXM1* is a gene that is closely associated with infertility and encodes a very important transcription factor for the G2/M phase transition. It has been shown that the balance between *FOXM1* gene and *NANOS3* gene or *FOXM1-PUM1* expression levels may be associated with testicular cancer.[6] *GAPDHS* is a sperm-specific glycolytic enzyme involved in the production of the energy required during spermatogenesis and sperm motility.[7] *OAZ3* is a testicular-specific gene that has been reported to have a major impact on male fertility.[8]

MicroRNAs (miRNAs) are known to be involved as key players in many biological processes, including spermatogenesis. The first publication on altered miRNA expression in nonobstructive azoospermia (NOA) participants revealed that 154 miRNAs were differently downregulated and 19 elevated in NOA patients compared to fertile males.[9] MiR34b/c and miR-449 deletion in mice has been demonstrated to disrupt both meiosis and sperm maturation, leading to oligoasthenoteratozoospermia (OAT).[10] In the study of Salas-Huetos et al., it was demonstrated that miR-34b-3p plays a role in the regulation of male meiosis via the E2F-pRB pathway, and miR-132-3p is associated with sperm differentiation and cell cycle progression via *MYC*.[11]

Although the effect of genetic mutations in various genes on infertility is better understood, the molecular mechanisms of miRNA-induced infertility are still unclear. It is necessary to first determine which miRNAs are involved in the regulation of infertility-related genes. The goal of our study is to identify miRNAs that may have a role in the regulation of infertility-related genes utilizing various bioinformatics approaches. For this purpose, in the study, first of all, genes related to infertility were determined. Pathway analysis of these genes, PPI interaction and hub proteins that may be related to these genes were determined. Afterward, miRNAs that can regulate infertility-related genes were identified and the effect of miRNA-target gene relationship on male infertility was demonstrated bioinformatically.

MATERIALS and METHODS

Identifying Genes Associated with Infertility

The Male Infertility Knowledge Base (MIK) (http://mik.bicnirrh.res.in/index.html) database was used to identify infertility-related genes. During the gene selection process from the MIK, genes with a PubMed Identifier (PMID) evidence number of 6 or more in high-throughput datasets were included in the investigation. MIK is a useful data repository recently developed to support the elucidation of the complex genetic etiology of male infertility. In this database, information about 17 000 gene-related pathways, gene ontology, and gene and sequence-based analysis tools are integrated and presented to the user. The Molecular Signatures Database (MSigDB) (https://www.gsea-msigdb.org/gsea/msigdb) tool and Orphanet (https://www.orpha.net/consor/cgi-bin/index.php)

databases were used to confirm whether the identified genes are relevant to infertility. MSigDB is a repository of many thousands of annotated gene sets. It is organized into two catalogs: Human and Mouse.

Pathway Analysis of Selected Infertility-Associated Genes and Hub PPI Analysis

The Elsevier pathway database was used for detection pathway analysis and PPI analysis constructed using Enrichr (https://maayanlab.cloud/Enrichr/) tool.

Identification Of Mirnas That Match Infertility-Related Genes

MiRNAs matching selected infertility-related genes were identified using miRNet tool (https://www.mirnet.ca/). Potential sequence matching between miRNAs and genes was revealed using STarMir tool (https://sfold.wadsworth.org/cgi-bin/index.pl).

Pathway Analysis of Selected Potential miRNAs Associated with Infertility

Pathway analysis of defined miRNAs was performed via the miRPathDB 2.0 tool (https://mpd.bioinf.uni-sb.de/overview.html).

Comparison of Selected Potential mRNAs with High-Throughput Studies and Identification of More Meaningful Mirnas By Literature Search

Selected potential infertility-related miRNAs were compared with the results of 3 important High-throughput studies to identify candidate miRNAs that may be more closely connected with infertility (Table 1).

Statistical Analysis

Functional enrichment evaluations were carried out using free platforms. The statistical cut-off for enrichment analysis in tools was set at P-value<0.05 by default.

RESULTS

Selected Infertility-Associated Genes

21 infertility associated genes were selected from the MIK database. Confirmation analysis via MSigDB database revealed that most of these genes could be related to spermatogenesis (P=0.0003659). The Orphanet database has also validated the link between these genes and infertility (Table 2).

Table 1. Comparison of miRNAs matching selected infertility 21 genes with the findings of three high-throughput studies published in the literature.

miRNAs	P-value	Fold Change	Regulation	Reference/Study
hsa-miR-16-5p	0.00023	10.7	Down	[12] (study-1)
hsa-miR-210-3p	0.0008	2.3	Up	[12] (study-1)
hsa-miR-146a-5p	0.002	2.1	Up	[12] (study-1)
hsa-miR-129-2-3p	0.02	2.2	Up	[12] (study-1)
hsa-let-7b-5p	0.009	2.7	Up	[12] (study-1)
miR-26b-5p	0.0025	5.7	Up	[12] (study-1)
miR-335-5p	0.004	3.8	Down	[12] (study-1)
miR-374a-5p	0.007	2.9	Up	[12] (study-1)
hsa-let-7a-5p	0.005	2.7	Up	[12] (study-1)
hsa-miR-34a-5p	0.009	4.7	Up	[13] (study-2)
miR-335-5p	0.005	3.9	Down	[13] (study-2)
hsa-miR-1-3p	0.02	1.9	Up	[14] (study-3)

Pathway Analysis and Defining Hub Proteins

Based on the Elsevier pathway collection database, selected 21 genes were found to be associated with male infertility (p=0.00004424). These genes are also associated with diseases such as transcytosis, cystic fibrosis, prostate cancer, as well as infertility (Figure 1). The hub proteins of the selected genes are demonstrated in Figure 2. Of these genes, *APP* and *CDK1* have been associated with infertility in the literature (Table 3).

Table 2. The selected 21 genes and their association with different diseases (Orphanet Augmented 2021). (Adj. p-value: Adjusted p-value, Odds R.: Odds Ratio, C.Score: Combined Score).

Disease	P-value	Adj. p-value	Odds R.	C.Score
Non-syndromic male infertility	1.042e-7	0.00002615	56.97	915.8
Situs inversus totalis	0.000004	0.0005	44.5	546.8
Primary ciliary dyskinesia	0.00001	0.0009	35.3	403.3
Retinal macular dystrophy type 2	0.004	0.11	21.1	112.0
Orofaciodigital syndrome type 3	0.004	0.11	21.1	112.0

Table 3. Hub proteins of the selected 21 infertility-associated genes (https://maayanlab.cloud/Enrichr/). (Adj. p-value: Adjusted p-value, Odds R.: Odds Ratio, C.Score: Combined Score).

Gene Name	P-value	Adj P-value	Odds R.	C.Score
APP	0.02734	0.3023	8.48	30.52
CREBBP	0.04739	0.3023	6.23	19.00
PRKCB	0.04841	0.3023	6.15	18.63
PRKG1	0.11970	0.3023	8.27	17.56
CDK1	0.03045	0.3023	4.91	17.14

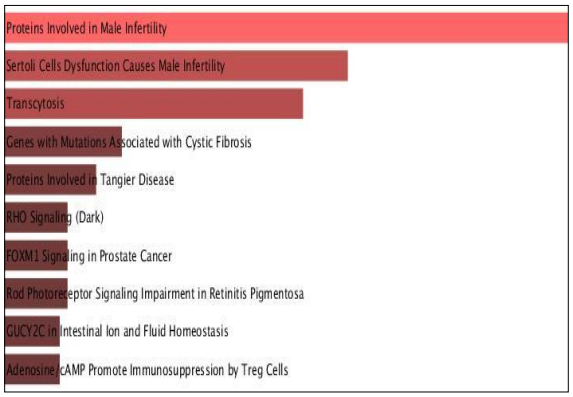

Figure 1. Diseases that are most likely to be linked to the selected 21 genes according to the Elsevier pathway collection database (p=0.00004424). (Obtained using https://maayanlab.cloud/Enrichr/ database).

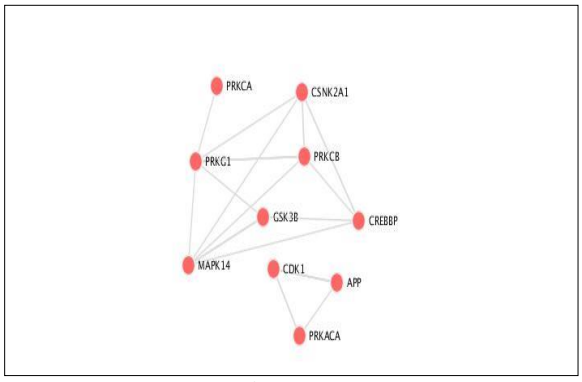

Figure 2. The hub proteins of the selected 21 genes.

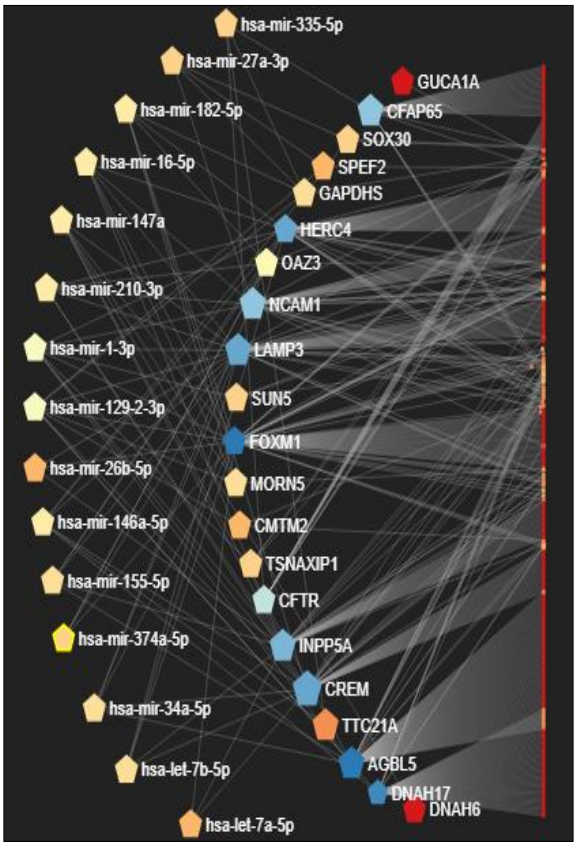

Figure 3. miRNAs capable of targeting 21 selected genes.

Potential Infertility-Related miRNAs

There were 474 edges of 319 miRNAs associated with 21 genes identified in the initial analysis in MIK. We then reduced it to 86 miRNAs and 241 edges by selecting "miRNA nodes only" as "degree cut off: 1.0" in the analysis. We then selected 15 miRNAs by confirming with starmiR and miRWalk (Figure 3).

Pathway Analysis Results of the Selected Potential Infertility-Related miRNAs

Figure 4. represents the pathways of the 15 chosen miRNAs. Almost all of these miRNAs, which play a range of biological roles, have been related to cancer.

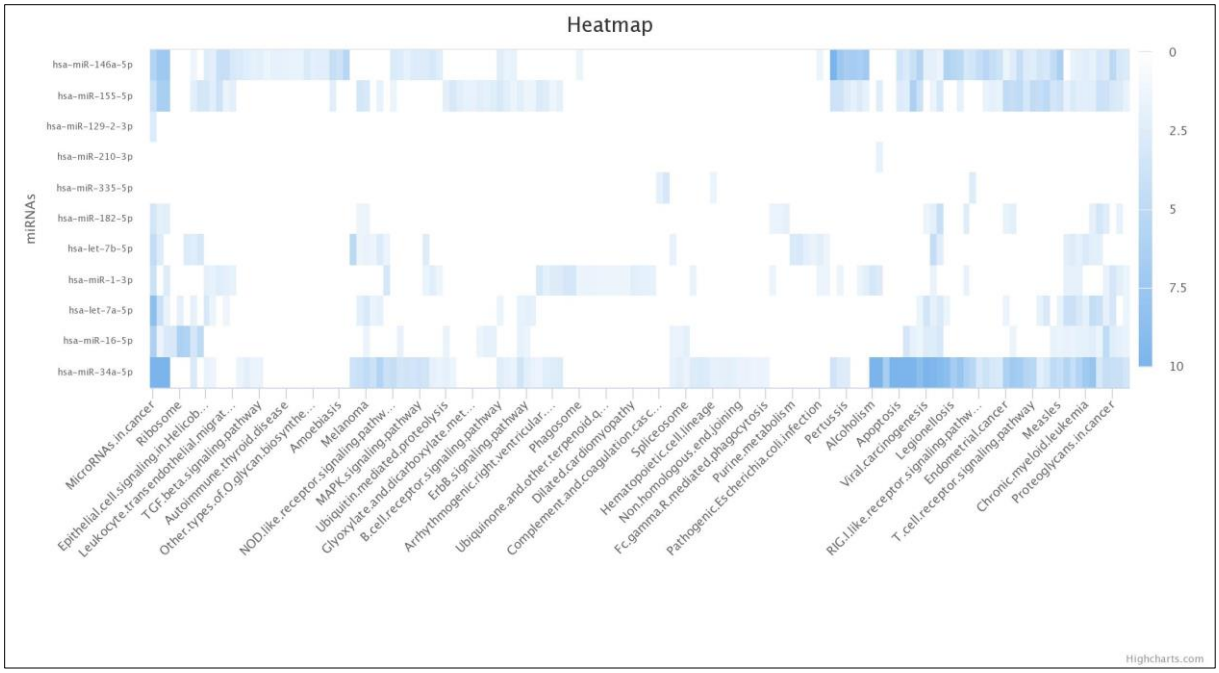

Figure 4. The relationship between 15 chosen miRNAs and diseases.

Comparison of the selected potential miRNAs and High-throughput studies

miRNAs targeting infertility-related genes in the current study were compared with three high-throughput studies' results (study-1:[12], study-2:[13], study-3:[14]) (Table 1). In the first of these studies, which included nine AZS men, microarray analysis revealed that 50 miRNAs were overexpressed and 27 miRNAs were down-regulated in AZS spermatozoa compared to the control group.[12] In the second study which was performed on spermatozoa samples obtained from 10 AZS individuals, 736 miRNAs were evaluated and it was reported that 26 miRNAs were up-regulated and 6 miRNAs were down-regulated.[13] In the third profiling study comparing thirty-nine individuals with idiopathic AZS and 35 healthy fertile individuals, 18 miRNAs with altered expression were detected.[14] Of the 15 miRNAs evaluated in our study, 9 miRNAs overlapped with study-1. miR-335-5p was shown to be downregulated in both study-1 and study-2. MiR-34a-5p was detected only in study 2, whereas miR-1-3p was found only in study-3.

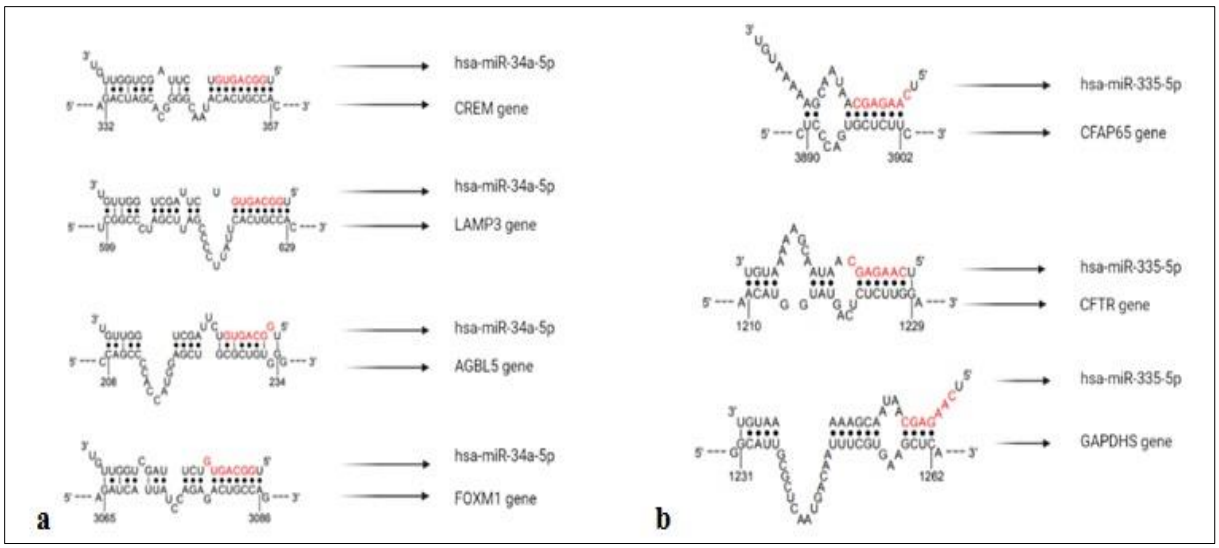

Figure 5. a) Matching between miR-34a-5p and potential target genes' sequences. **b)** Matching between miR-335-5p and potential target genes' sequences.

DISCUSSION

The relationship between the *CREM* gene, which plays a vital role in spermatogenesis, and infertility has been known for many years.[15,16] Nevertheless, evidence to establish the link between the *CREM* gene and miRNAs are limited.

Few high-throughput miRNA expression investigations on spermatozoa samples from AZS men have been performed.[12-14] If the results of three of these limited studies (study-1, study-2, study-3) are compared, only the hsa miR-3609 overlaps (Table 1). The partial inconsistency in the results of these three miRNA expression studies could be due to various reasons. For instance low RNA amount, different measurement methods, molecular mechanism complexity of AZS, etc. However, it is noteworthy that 9 of the 15 selected miRNAs in our study overlapped with those in study-1. Many of the 15 miRNAs have been associated with infertility in the literature. For instance, miR-16-5p is one of these miRNAs. In the miRNA microarray study of Liu et al., miRNA expressions in normal semen of 86 healthy men and abnormal semen samples of 86 sterile men were compared. In the study results, it was found that the expression of 7 miRNAs increased, and the expression of 6 miRNAs, including miR-16-5p, decreased (confirmed by qRT-PCR).[17] miR-16-5p may be an important miRNA for male infertility, our study results support the literature findings because our study results suggest that miR-16-5p has the potential to regulate the expression of infertility-related genes such as *OAZ3*, *NCAM1*, *LAM3*, *FOXM1*.

It has been revealed that miR-210-3p may play a role in sperm cell apoptosis by activating caspase-3.[18] According to current study results, miR-210-3p may play a role in infertility by regulating the expression of infertility-related genes such as *OAZ3*, *HERC4* and *INPP5A*. Another prominent miRNA according to the study is miR-155-5p. This miRNA is an oncogenic miRNA (oncomiR) and it has been associated with many cancers[19]. At the same time, it has been proposed that miR-155-5p, which is reported to be closely related to infertility, may be a candidate biomarker for infertility.[20]

miR-34a-5p is one of the important tumor suppressor miRNAs (TsmiR) associated with many cancers.[21] Studies show that miR-34a-5p may also play a critical role in infertility. Momeni et al. showed that miR-34a-5p is downregulated in sperm samples of infertile men. It was emphasized that the abnormal decrease in miR-34a-5p expression may be associated with hypermethylation in the promoter region of miR-34a-5p.[22] As seen in Figure 5a, miR-34a-5p dysregulation may contribute to infertility through *CREM*, *LAMP3*, *AGBL5*, *FOXM1* genes.

Glycolysis can provide energy for sperm cell motility via anaerobic respiration. MiRNAs are known to engage in the glycolytic process by regulating target genes. Reduced expression of let-7b-5p has been demonstrated to reduce glycolysis in asthenozoospermia through inhibiting *AURKB*.[23] The relationship between let-7b-5p and infertility has also been shown in different studies. For example, in the study of Abhari et al., let-7b-5p was demonstrated to be significantly decreased in the sperm of oligospermia men compared to the sperm of fertile men.[24] The present study results show that let-7b-5p may play a role in the infertility process through genes such as *CREM* and *FOXM1*.

It has been reported that miR-335-5p, which was downregulated in both study-1 and study-2, may be closely related to infertility.[25] miR-335-5p may be associated with infertility by regulating the expression of *CFAP65*, *CFTR*, *GAPDHS* genes (Figure 5b).

The most relevant gene in the PPI analysis of the selected genes was found to be *APP* (Table 3). *APP* is a gene with cellular functions such as cell motility, adhesion, apoptosis and intercellular signaling and is associated with male infertility.[26] *CDK1*, another gene that was significant in PPI analysis, has also been reported to be required for male fertility by playing a role in male meiotic progression.[27]

According to the results of miRNA pathway analysis, it is striking that the selected miRNAs are more associated with cancer (Figure 4). The fact that a large part of miRNA research is on cancer diseases may explain this situation. However, the number of research focusing on the relationship between infertility and miRNAs is limited. With new research on the relationship between infertility and

miRNAs, it will be revealed that many more miRNAs are closely related to infertility than are currently known. The infertility-related miRNAs and genes identified by bioinformatics approaches in our work should be supported by in vitro and in vivo techniques. As a result, the function of the miRNAs- targeted genes connections in the mechanism of male infertility can be revealed, and the complicated mechanism of male infertility may be partially elucidated.

Conflict of Interest

The authors declare that there is not any conflict of interest regarding the publication of this manuscript.

Ethics Committee Permission

Because our study data is gathered from open sources that are freely available in the public domain, no ethical approval is required.

Authors' Contributions

Concept/Design: MK. Data Collection and/or Processing: MK. Data analysis and interpretation: MK. Literature Search: MK. Drafting manuscript: MK. Critical revision of manuscript: MK.

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