Identification of a valuable gene network for the diagnosis and treatment of non-obstructive azoospermia: in-silico analyses – experimental research

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Introduction: Non-obstructive azoospermia (NOA) is an etiology of infertility in men. NOA may have various classifications; however, hypogonadotropic hypogonadism can be regarded as a class of NOA associated with genetic factors. Former studies have shown that noncoding RNA (ncRNA) plays an essential role in NOA incidence, but few studies have been performed on the NOA-related ncRNA interaction network. In the current study, genes, NOA-related microRNA (miRNA), and circular RNA (circRNA) were found by bioinformatics methods to offer a new perspective on NOA treatment.

Methods: The gonadotropin-releasing hormone receptor (GnRHR)-related protein–protein interaction (PPI) network was extracted by searching in 'string-database'. GO, KEGG, and Enrichr databases were used to identify pathways, molecular function, and biological processing. Four databases, including TargetScan, mirDIP, miRmap, and miRWalk, were used to extract miRNAs. At last, the circ2GO, circBase, and literature were used to identify circRNAs and their genes.

Results: The current study identified the four proteins associated with the GnRHR signaling; eight shared miRNAs that affect the expression of found proteins and 25 circRNAs and their origin genes that regulate the miRNAs' function.

Conclusion: The two miRNAs, hsa-miR-134-3p and hsa-miR-513C-3p, the three genes, VCAN, NFATC3, and PRDM5, and their associated circRNAs can perform as a valuable gene network in the diagnosis and treatment of NOA pathogenesis.

Keywords: azoospermia, bioinformatics, circular RNA, GNRHR protein, microRNAs

Introduction

Infertility is a prevalent disorder, affecting ~70 million people worldwide. The World Health Organization (WHO) estimates that 9% of couples worldwide have infertility problems, and males cause a 50% incidence of infertility. Furthermore, male infertility may have various types of etiology, such as hormonal and genetic[1].

Congenital hypogonadotropic hypogonadism (CHH), as a classification of central non-obstructive azoospermia (NOA)[2], is characterized by a deficiency of gonadotropin secretion due to the dysfunction of gonadotropin-releasing hormone (GnRH)[3]. The gonadotropin-releasing hormone receptor (GnRHR) gene was one of the first genes to be identified in disease occurrence[4]. GnRHRs are mainly expressed on the surface of pituitary gonadotropin cells, and the beginning of the receptor signaling causes the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH)[5]. Indeed, LH and FSH are involved in spermatogenesis initiation, and impaired LH and FSH secretion is the essential etiology of NOA incidence[6].

With the development of complete genome sequencing, it was discovered that 98% of the human genome is transcribed into noncoding RNAs (ncRNAs)[7]. ncRNAs are classified into different types, such as microRNAs (miRNAs), circular RNAs

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Zabihi et al. Annals of Medicine & Surgery (2023)

Annals of Medicine & Surgery

(circRNAs), long noncoding RNAs (lncRNAs), and small nucleolar RNAs (snoRNAs)[8]. Evidence has shown that ncRNAs are vital regulators of many essential cellular processes such as differentiation, proliferation, transcription, post-transcriptional changes, apoptosis, and cellular metabolism[9]. ncRNAs innately construct correlated genetic networks that affect the expression of various proteins and eventually direct numerous cellular responses. Also, disrupted regulation of ncRNAs may lead to multiple disease incidences[10].

miRNAs are noncoding single-strand RNA molecules that regulate or reduce gene expression. miRNAs are paired to the complementary sequence of target mRNA in the cytoplasm[11]. Furthermore, circRNAs are a product of alternative splicing in cells or tissues, which regulate carefully[12]. Interestingly, disrupted regulation of miRNAs and circRNAs may lead to various disease incidences[13]. The correlation of lncRNA types and men's infertility had been investigated previously, but based on our knowledge, the relationship between circRNAs and miRNAs with GnRHR function in men and NOA needs more investigation[14,15].

In the current in-silico analysis, we identified the GnRHR protein–protein interaction (PPI) network. Then, we recognized the circRNAs and miRNAs that related to the network. Also, we detected the found ncRNA gene expression network. Finally, we provided a new rationale for investigating circRNAs and miRNAs in treating NOA.

Methods

Identification of PPI network

STRING

STRING (https://string-db.org/)[16] collects, scores, and integrates PPI databases and computational predictions of potential functions. In the current investigation, the first stage was carried out to examine the interactions of GnRHR-related proteins utilizing the STRING database. Afterward, rigorous criteria were applied to exclude unreliable or low-confidence interactions, ensuring that our analysis comprises solely biologically significant and high-quality interactions.

Identifying the function of extracted genes and proteins

Enrichr

Enrichr (https://maayanlab.cloud/Enrichr/)[17] is a website for visual analysis that provides variated graphical summaries of the functions of genes. In the current study, this website was utilized to identify extracted genes' functions. The obtained genes were utilized as a query in the present database, and a P-value > 0.05 was considered for the selection of ontologies.

Gene ontology (GO) term enrichment analysis

GO (http://geneontology.org/)[18] is an international standard classification system for gene annotation. In this database, various genes are synchronized to perform biological functions, and the most important biochemical metabolic pathway and signal transduction involved in target genes can also be identified[19]. In the present investigation, this database was utilized to extract the molecular function and biological process related to all five genes. Also, a P-value > 0.05 was considered to find the most validated functions.

HIGHLIGHTS

- The current study identified the four proteins associated with the GnRHR (gonadotropin-releasing hormone receptor) signaling.
- The current study identified the eight shared microRNAs (miRNAs) that affect the expression of found proteins and 25 circular RNAs and their origin genes that regulate the miRNAs' function.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway

KEGG (https://www.genome.jp/kegg/)[20] is an essential comprehensive database for systematically analyzing biological pathways. The main feature of KEGG is the declaration of various biochemical pathways, such as most known metabolic and regulatory pathways. Fisher's test performs the KEGG enrichment analysis based on the supra-geometric distribution to calculate the P-value of each path[21]. In the context of this investigation, the pathways that exhibited a collective involvement of all five genes were chosen, adhering to a P-value threshold of less than 0.05. Through an exhaustive exploration of these pathways, elements unrelated to fertility, spermatogenesis, and sex hormones were excluded from the identified outcomes.

Identification of extracted gene-related miRNAs

miRmap

miRmap (https://mirmap.ezlab.org)[22] provides miRNAs target prediction. Indeed, miRNAs suppress the expression of RNAs after transcription. In the present study, we used the website to find the first part of miRNAs. Within these databases, individual gene inquiries were conducted. Subsequently, the resultant miRNAs that emerged were consolidated.

miRWalk

miRWalk (http://mirwalk.umm.uni-heidelberg.de)[23] is an open-access resource that provides an interface of predicted and valid miRNA binding sites for known genes in humans and other animals. The core of miRWalk is a TarPmiR-based stochastic approach software that searches the prediction of the miRNA target site with a complete transcript sequence including 5′-UTR, CDS, and 3′-UTR. This database was used to find the second part of the miRNAs. A collective query involving all five genes was executed within this database. Eventually, a substantial quantity of miRNAs (~10 000) was acquired as a result.

TargetScan

TargetScan (http://www.targetscan.org)[24] predicts the biological targets of miRNAs by searching the 7mer and 8mer protected sites that match the seed site of each miRNA. In mammals, predictions are ranked based on the predicted effectiveness of targeting, which is calculated using the site scores. We also used this website to find the third part of miRNAs. Individually, each gene was queried on this website. Moreover, using this website's data, cases with conserved sequences were chosen as potential targets.

mirDIP

mirDIP (https://ophid.utoronto.ca/mirDIP)[25] offers an integrated score for each unique target miRNAs interaction. These scores are statistically inferred using predictions obtained from individual sources. This website was also used to find the fourth part of miRNAs. Following the comprehensive gene search on the mirDIP website, instances exhibiting class scores categorized as very high and high were specifically chosen[26].

Venn diagram

As the final step in deriving the miRNAs, all the acquired elements were gathered and subsequently visualized using the online Venn diagram tool website. The Venn diagram (https://bioinformatics.psb.ugent.be/webtools/Venn/) allows for calculating overlapping sets of elements. It generates textual results indicating which elements are present in each overlap or unique to specific lists. Users can opt for either symmetric (default) or asymmetric Venn diagrams on this website. Currently, the tool can handle the computation of intersections for 30 lists[27].

Identification of miRNA-related circRNAs

circ2GO

In circ2GO (https://circ2go.dkfz.de)[28], users can explore circRNA profiles, visualize circRNAs expressed from each gene, and examine different transcripts. Additionally, circRNA origin genes and miRNA binding sites for each circRNA can be observed. Moreover, the platform offers a reverse search option to identify circRNAs targeting specific miRNAs. In the present study, we utilized this online resource to showcase desired circRNAs. We inputted the subscribed miRNAs into the website's query, resulting in the extraction of the desired circRNAs. This process included conducting individual searches for each miRNA. Also, specific identified miRNAs were not found in the website's library, which will be addressed in the subsequent analysis phase.

Literature review

To discover different types of circRNAs not found in the circ2GO database, a search of the literature in the PubMed and Scopus databases was performed. Two authors searched from September 2021 to November 2021 separately. Keywords included the desired mir ID and "circRNA" along with using the Boolean operator "AND" 35 articles were included in the study. Subsequently, articles were written before 2000, and animal studies were excluded. The references of each found article were also checked to prevent data loss. Finally, 16 articles were extracted.

Identification of circRNAs origin genes

circBase

The 'circBase' website (http://www.circbase.org/)[29] can provide an integrated dataset of circRNAs and the evidence that supports their expression. circBase also provides scripts for identifying new and known circRNAs in data sequencing. In the current analysis, this website was used to find the origin genes of circRNAs. The identified circRNAs in the preceding sections were investigated on this database, leading to the extraction of associated genes. Subsequently, to pinpoint the most crucial genes, a process of

sharing was undertaken with the gathered elements. Ultimately, the cases acquired and the primal genes were employed to conduct network analysis.

Network formation of extracted genes

GeneMANIA (http://www.genemania.org/)[30] was used to find the genetic interactions, pathways, expression, localization, and protein amplitude of all GnRHR-related genes and their ligands. All genes, including those producing circRNAs and the primal genes, underwent inquiries within these databases, enabling the exploration of their correlations in the context of humans.

Identification of the histological expression of mRNAs

Genotype-tissue expression (GTEx)

The GTEx portal (https://www.gtexportal.org/home)[31] provides free access to data, including gene expression, QTLs, and histological images. We used this website to investigate the extracted genes' location and intensity of expression. According to previous documents, three tissues containing the testicular, pituitary, and hypothalamus were considered[32].

Microarray validation

The Gene Expression Omnibus (GEO) website (http://www.ncbi.nlm.nih.gov/geo/) was used for microarray validation. GEO is an international public resource for microarray and next-generation sequence functional genomic datasets obtained from various researches[33]. In addition, the "NOA" keyword was used as a query for finding proper gene expression profiles (GSE). Then the intended genes (GNRH1, GNA11, GNAS, GNAQ, PRDM5, VICAN, NFATC3, MIR4772, MIR134, MIR579, MIR513A, MIR3606, MIR4766, MIR513C, and MIR5088) were searched in the obtained GSEs data. P-value > 0.05 was considered for gathering valuable data.

Network analysis

Cytoscape (version 3.9.1) software was used for the analysis of the protein–miRNA–circRNAs network. Cytoscape is a powerful open-source software project for integrating biomolecular interaction networks (protein–protein, protein–DNA, and genetic interactions) with high-throughput expression data[34]. This software was employed to identify the most crucial pathway connecting circRNA, miRNA, and genes. Ultimately, the final predicted pathway was derived from this analysis.

Ethical approval

This article does not contain any studies with human or animal subjects performed by any authors and does not require ethical approval and consent.

Results

Identification of PPI network

We extracted the GnRHR-related 'PPI network' by searching in the 'string database' (Fig. 1A). In addition, to select the most critical interaction module, we considered the interactions, which included 'from curated databases' and 'experimentally determined' modes. At last, the intended module was detected, which provides GNRH1, GNAQ, GNA11, and GNAS nodes (Fig. 1B).

Zabihi et al. Annals of Medicine & Surgery (2023)

Annals of Medicine & Surgery
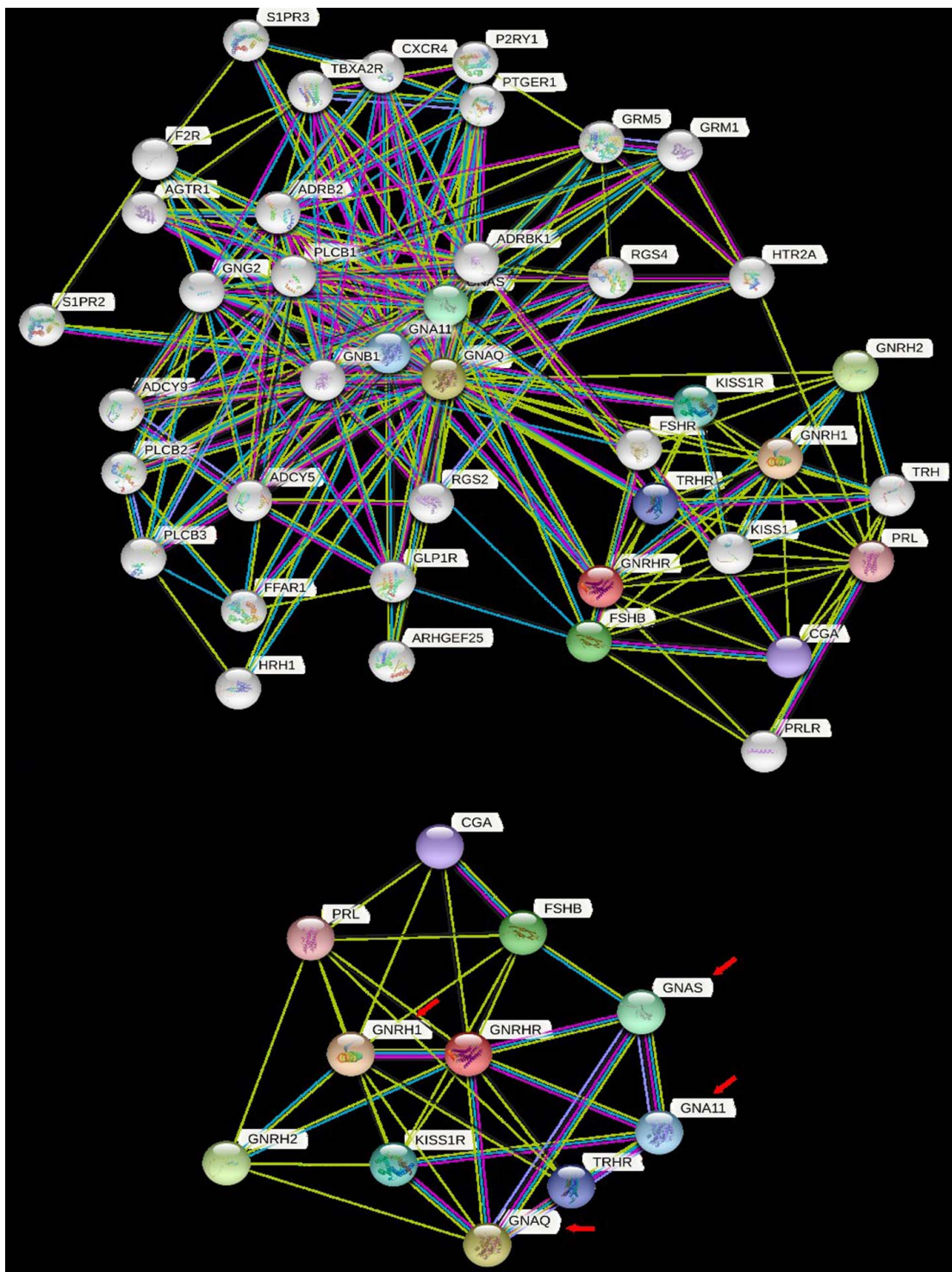
Figure 1. GnRHR PPI network's major nodes include GNRH1, GNA11, GNAS, and GNAQ.

Function of identified proteins and genes

We detected involved pathways by searching in KEGG and Enrichr databases. Furthermore, we identified molecular function and biological processing using the GO website (Table 1). To select the valuable data in 'molecular function' and 'biological processes' information, we considered a *P*-value <0.05 in the

dataset. At last, we used a negative logarithm of determined data for better visual perception.

miRNAs identification

miRNAs were extracted from four databases, including TargetScan, mirDIP, miRmap, and miRWalk. Then for sharing

Table 1

KEGG signaling pathway.

No.	Term	Overlap	P	Adjusted P	Odds ratio	Combined score	Genes
1.	GnRH signaling pathway	May-93	1.95E − 12	1.29E − 10	99 535	2 683 821	GNA11;GNAQ;GNRHR;GNAS; GNRH1
2.	GnRH secretion	Mar-64	3.11E − 07	5.38E − 06	490.1803	7344.501	GNA11;GNAQ;GNRH1
3.	Cortisol synthesis and secretion	Mar-65	3.26E − 07	5.38E − 06	482.25	7202.927	GNA11;GNAQ;GNAS
4.	Insulin secretion	Mar-86	7.63E − 07	9.00E − 06	359.8554	5069.011	GNA11;GNAQ;GNAS
5.	Gap junction	Mar-88	8.18E − 07	9.00E − 06	351.3529	4924.779	GNA11;GNAQ;GNAS
6.	Aldosterone synthesis and secretion	Mar-98	1.13E − 06	9.37E − 06	314.2105	4301.834	GNA11;GNAQ;GNAS
7.	Parathyroid hormone synthesis, secretion, and action	3/106	1.44E − 06	9.48E − 06	289.6893	3897.412	GNA11;GNAQ;GNAS
8.	Growth hormone synthesis, secretion, and action	3/119	2.04E − 06	1.22E − 05	257.056	3368.603	GNA11;GNAQ;GNAS
9.	Cushing syndrome	3/155	4.51E − 06	2.29E − 05	195.8191	2410.223	GNA11;GNAQ;GNAS
10.	Calcium signaling pathway	3/240	1.68E − 05	7.38E − 05	125.0506	1375.09	GNA11;GNAQ;GNAS
11.	Endocrine and other factor-regulated calcium reabsorption	Feb-53	6.85E − 05	2.83E − 04	260.7059	2499.636	GNAQ;GNAS
12.	Thyroid hormone synthesis	Feb-75	1.38E − 04	4.91E − 04	181.9361	1617.436	GNAQ;GNAS
13.	Pathways in cancer	3/531	1.79E − 04	5.90E − 04	55.30398	477.2243	GNA11;GNAQ;GNAS
14.	Circadian entrainment	Feb-97	2.31E − 04	6.73E − 04	139.6491	1169.537	GNAQ;GNAS
15.	Inflammatory mediator regulation of TRP channels	Feb-98	2.35E − 04	6.73E − 04	138.1875	1154.461	GNAQ;GNAS
16.	Serotonergic synapse	2/113	3.13E − 04	7.25E − 04	119.4234	963.6995	GNAQ;GNAS
17.	Cholinergic synapse	2/113	3.13E − 04	7.25E − 04	119.4234	963.6995	GNA11;GNAQ
18.	Glutamatergic synapse	2/114	3.18E − 04	7.25E − 04	118.3512	952.9641	GNAQ;GNAS
19.	Platelet activation	2/124	3.77E − 04	8.29E − 04	108.5956	856.1814	GNAQ;GNAS
20.	Dopaminergic synapse	2/132	4.27E − 04	9.08E − 04	101.8718	790.4633	GNAQ;GNAS
21.	Estrogen signaling pathway	2/137	4.60E − 04	9.48E − 04	98.07407	753.7245	GNAQ;GNAS
22.	Oxytocin signaling pathway	2/154	5.80E − 04	0.0011262	87.0307	648.5708	GNAQ;GNAS
23.	Long-term potentiation	Jan-67	0.01664	0.0244048	75.48864	309.1989	GNAQ
24.	Sphingolipid signaling pathway	1/119	0.029401	0.0388091	42.11229	148.5188	GNAQ
25.	Relaxin signaling pathway	1/129	0.03184	0.0412042	38.80273	133.7547	GNAS
26.	Apelin signaling pathway	1/137	0.033787	0.0420745	36.50551	123.6688	GNAQ
27.	Phospholipase D signaling pathway	1/148	0.03646	0.0429705	33.7551	111.7815	GNAS

gained miRNAs, the 'calculate and draw custom Venn diagrams' website was used. The ultimate miRNAs included hsa-miR-4772-3p, hsa-miR-134-3p, hsa-miR-579-3p, hsa-miR-513a-3p, hsa-miR-3606-3p, hsa-miR-4766-5p, hsa-miR-513C-3p, and hsa-miR-5088 (Fig. 2).

Identification of miRNA-related circRNAs

We collected circRNAs by searching previous documents and the circ2GO database (Table 2). Then we used the Bioinformatics & Evolutionary Genomics website (https://bioinformatics.psb. ugent.be/webtools/Venn/) for sharing gained circRNAs (Fig. 3).

Identification of circRNAs origin genes

We used the circBase database to collect the circRNA origin genes (Table 3). Then, we investigated the obtained genes to form a network with the primary genes (GnRHR, GNRH1, GNAQ, GNA11, GNAS) on the GeneMANIA website (Fig. 4).

Bulk tissue gene expression of extracted genes

We analyzed bulk tissue gene expression (TPM) of each circRNA gene in the testis, pituitary, and hypothalamus tissues (Table 3). Due to the repetition of genes, three genes, VCAN, PRDM5, and NFATC3, were considered for tissue expression.

Microarray validation

The fold change (upregulation or downregulation) of intent genes in NOA is summarized in Table 4. These data indicated that the GNAS might be considered the most upregulated gene, and GNAQ could be the most downregulated. Notably, the lowest P-value was considered for certainty among the common gene data.

Network analysis

Biomolecular network analysis was performed. Two parameters, betweenness centrality and the number of interactions, were considered (Fig. 5). Results indicated that two miRNAs, hsa-miR-513a-3p and hsa-miR-134-3p, can be regarded as critical nodes.

Discussion

In recent years, growing evidence suggests the impact of ncRNAs, specially circRNA and miRNA, on GnRHR dysfunction and azoospermia incidences[31–33]. The current study identified the four proteins associated with the GnRHR signaling; eight shared miRNAs that affect the expression of found proteins, and 25 circRNAs and their origin genes that regulate the miRNAs' function.

In the current study, the PPI network shows that GnRHR (PPI) network included GNRH1, GNAQ, GNA11, and GNAS, which appear to be involved in the NOA incidence. According to the findings, all four proteins are involved in G-protein-dependent
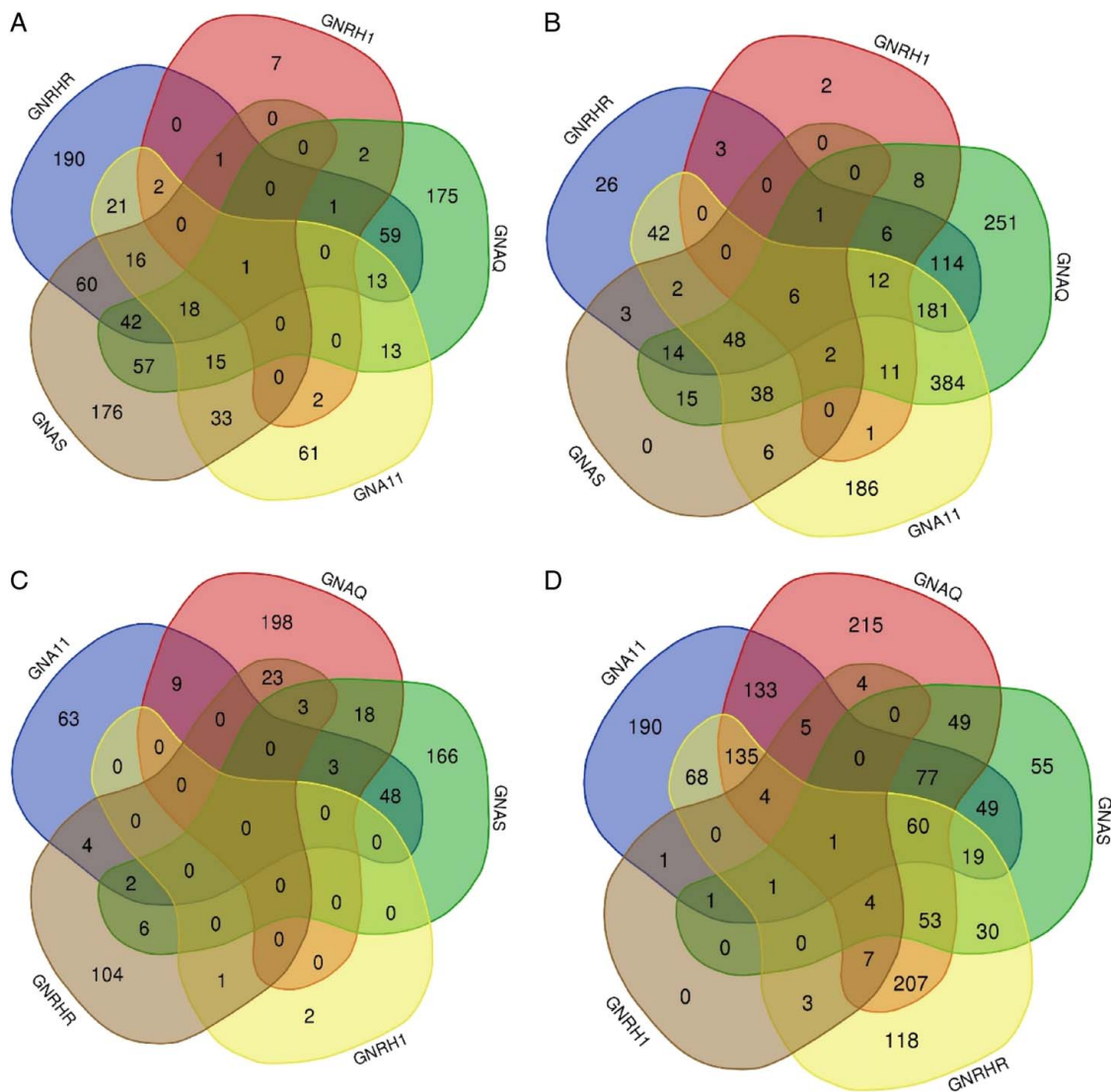
Zabihi et al. Annals of Medicine & Surgery (2023)

Annals of Medicine & Surgery

Figure 2. Venn diagram of found miRNAs from four databases. (A) miRWalk, (B) miRmap, (C) mirDIP, and (D) TargetScan.

and GnRH signaling. In addition, GNA11, GNAQ, and GNRH1 are also involved in GnRH secretion. Furthermore, GNAQ and GNAS also involved in sex hormone signalings, such as estrogen and oxytocin. In a case study, Sagi et al.[52] have shown that a mutation in the pituitary GNRH1 gene played a role in developing azoospermia, and in microarray data, GNRH1 has significant downregulation. Li et al.[53] also indicated that GNAQ protein, expressed in testicular tissues, may be involved in spermatozoon development. Additionally, microarray validation indicated that GNAS is the most downregulated gene in NOA. Furthermore, a study in China showed that RGS22 gene disorder disrupts the expression of GNA11 protein and can be regarded as an azoospermia risk factor; however, this study was performed specifically on testicular tissues. The expression of GNA11 protein in the pituitary was not examined[54]. Also, a case report in France revealed that GNAS gene mutations in testicular tissues could induce Leydig tumors and lead to NOA[55]. Futhermore, by microarray validation, GNAS is the most upregulated gene in NOA. Interestingly, GNAS has also been implicated in

depression[56], and this topic may indicate the bilateral influence of azoospermia and depression on each other[57]. However, despite the significant expression of these proteins in the pituitary gland, according to the authors' data search, the relationship between the expression disturbance of these proteins in the pituitary gland and the incidence of NOA is still not well understood, and it requires more experimental research to investigate.

Eight miRNAs were identified, which include hsa-miR-4772-3p, hsa-miR-134-3p, hsa-miR-579-3p, hsa-miR-513a-3p, hsa-miR-3606-3p, hsa-miR-4766-5p, hsa-miR-513C-3p, and hsa-miR-5088. Likewise, the network analysis showed that the two miRNAs hsa-miR-513a-3p and hsa-miR-134-3p, in addition to interacting with all GnRHR network proteins, also interact with the highest amount of circRNA and can regulate many proteins expression, which involved in NOA. Notably, by microarray validation, hsa-miR-134-3p has significant upregulation among NOA cases. In Spain, Ramirez[58] relieved that hsa-miR-134-3p, hsa-miR-513a-3p, and hsa-miR-513C-3p may be associated with

Table 2

Extracted circRNAs.

No.	miRNAs	circRNA	References
1	hsa-miR-134-3p	hsa_circ_0129854	[26,35–39]
		hsa_circ_0129855	
		hsa_circ_0000711	
		hsa_circ_0125189	
		hsa_circ_0125188	
		hsa_circ_0125187	
		hsa_circ_0125186	
		hsa_circ_0001855	
		hsa_circ_0004904	
		hsa_circ_0003692	
		hsa_circ_0031787	
		hsa_circ_0086241	
2	hsa-miR-513C-3p	hsa_circ_0129854	[26,37,40–46]
		hsa_circ_0129855	
		hsa_circ_0000711	
		hsa_circ_0125189	
		hsa_circ_0125188	
		hsa_circ_0125187	
		hsa_circ_0125186	
		hsa_circ_0078299	
		hsa_circ_0077426	
		hsa_circ_0060975	
		hsa_circ_0004458	
		hsa-circ-0071127	
		hsa_circ_0001013	
		hsa_circ_0007334	
		hsa_circ_0061395	
		hsa_circ_0000972	
3	hsa-miR-5088	hsa_circ_0014213	[47]
4	hsa-miR-5003	hsa_circ_0002131	[48–51]
		hsa_circ_0082689	
		hsa_circ_0082688	

Table 3

circRNA origin genes.

No.	Gene name	circRNA	Primary miRNA	Secondary miRNA
1.	VCAN	hsa_circ_0129854	hsa-miR-134-3p	hsa-miR-513C-3p
2.	VCAN	hsa_circ_0129855	hsa-miR-134-3p	hsa-miR-513C-3p
3.	NFATC3	hsa_circ_0000711	hsa-miR-134-3p	hsa-miR-513C-3p
4.	PRDM5	hsa_circ_0125189	hsa-miR-134-3p	hsa-miR-513C-3p
5.	PRDM5	hsa_circ_0125188	hsa-miR-134-3p	hsa-miR-513C-3p
6.	PRDM5	hsa_circ_0125187	hsa-miR-134-3p	hsa-miR-513C-3p
7.	PRDM5	hsa_circ_0125186	hsa-miR-134-3p	hsa-miR-513C-3p
8.	RNF38	hsa_circ_0001855	hsa-miR-134-3p	
9.	POLE2	hsa_circ_0004904	hsa-miR-134-3p	
10.	FNDC3B	hsa_circ_0003692	hsa-miR-134-3p	
11.	POLE2	hsa_circ_0031787	hsa-miR-134-3p	
12.	RFX3	hsa_circ_0086241	hsa-miR-134-3p	
13.	AKAP12	hsa_ circ_ 0078299	hsa-miR-513C-3p	
14.	USP45	hsa_ circ _0077426	hsa-miR-513C-3p	
15.	PMEPA1	hsa_circ_0060975	hsa-miR-513C-3p	
16.	PSD3	hsa_circ_0004458	hsa-miR-513C-3p	
17.	NR3C2	hsa-circ-0071127	hsa-miR-513C-3p	
18.	KIAA1841	hsa_circ_0001013	hsa-miR-513C-3p	
19.	MBOAT2	hsa_circ_0007334	hsa-miR-513C-3p	
20.	BACH1	hsa_circ_0061395	hsa-miR-513C-3p	
21.	MBOAT2	hsa_circ_0000972	hsa-miR-513C-3p	
22.	SPRR1B	hsa_circ_0014213	hsa-miR-5088	
23.	BNIP3L	hsa_circ_0002131	hsa-miR-5003	
24.	PARP12	hsa_circ_0082689	hsa-miR-5003	
25.	PARP12	hsa_circ_0082688	hsa-miR-5003	

Figure 3. Sharing found circRNAs. The common circRNAs between hsa-miR-134-3p and hsa-miR-513C-3p, containing seven cases which include: hsa circ_0129854, hsa_circ_0125187, hsa_circ_0125189, hsa_circ_0000711, hsa_circ_0125188, hsa_circ_0125186, and hsa_circ_0129855.

testicular atrophy in Klinefelter syndrome. Also, Meunier et al.[59] discovered that hsa-miR-579-3p could be associated with spermatogenesis. In 2018, Barceló et al.[60] also found that the expression of hsa-miR-513a-3p was altered in patients with azoospermia. Jia et al.[61] also indicated that the upregulation of hsa-miR-5088 is also associated with cryptorchidism as the critical factor of NOA. According to the suppressive role of miRNAs in the expression of proteins, upregulation of these ncRNAs will disrupt the expression of essential proteins[62] and might be related to NOA incidence. Although a significant number of NOA-related miRNAs have been discovered, according to the authors' findings, the association of hsa-miR-4772-3p, hsa-miR-3606-3p, and hsa-miR-4766-5p with spermatogenesis, male fertility, and NOA is not discovered. However, these miRNAs can be considered a proposed network in future studies.

By bioinformatic analysis, seven circRNAs that interacted with at least two miRNAs were identified, including hsa_circ_0129854, hsa_circ_0129855, hsa_circ_0000711, hsa_circ_0125189, hsa_circ_0125188, hsa_circ_0125187, and hsa_circ_0125186, which can be suggested as a circRNAs network. Furthermore, VCAN, NFATC3, and PRDM5 genes had the highest interaction among the circRNAs origin genes, with the highest expression in hypothalamic, testicular, and pituitary tissues. Moreover, VCAN and PRDM5 have significant fold changes in microarray data. Rai et al.[63] reported that VCAN gene expression is associated with the development and function of hypothalamic neurons, especially oligodendrocytes. Also, based on past evidence, dysfunction of oligodendrocyte cells can play a significant role in NOA incidence[64]. Therefore, it can be expected that the expression disruption of this gene and its related circRNAs can play a role in the development of NOA. In addition, Hadziselimovic et al.[65] found that PRDM5 expression was
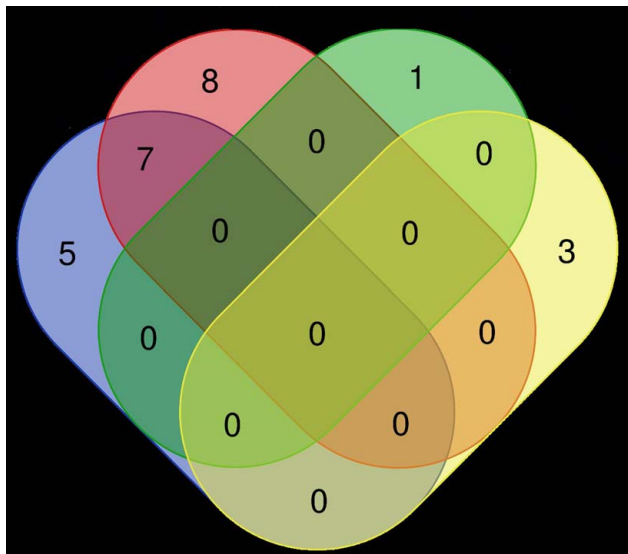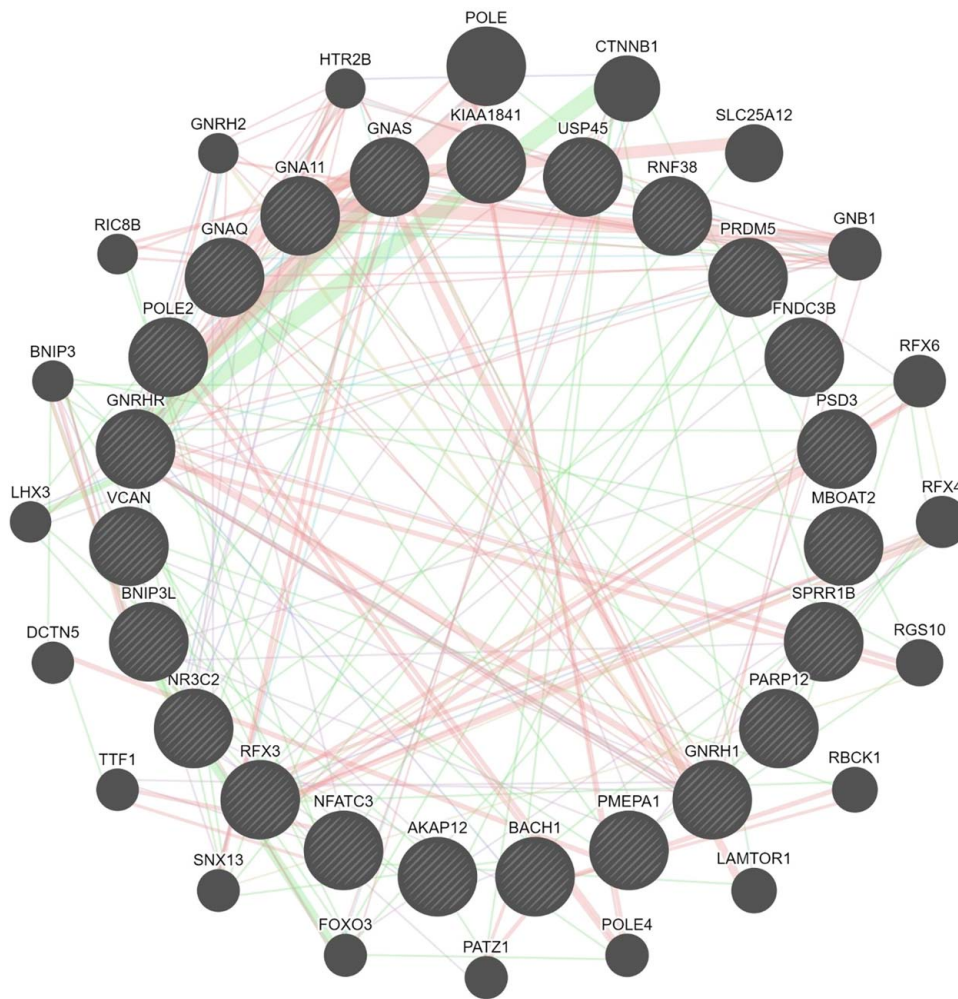
Zabihi et al. Annals of Medicine & Surgery (2023)

Annals of Medicine & Surgery

Figure 4. Gene network between circRNAs origin genes and GnRHR, GNRH1, GNAQ, GNA11, GNAS genes illustrated by Genemania website.

significantly reduced in patients with cryptorchidism compared with healthy cases. In China, Lu *et al*.[35] found that the PRDM5 gene and related-mRNA are associated with sperm motility. Zhu *et al*.[36] have shown that the NFATC3 gene is highly expressed in testicular tissues and plays a vital role in sperm motor development. In summary, according to the bioinformatics study, it can be shown that the found circRNAs can function as miRNA sponges, which are also involved in the regulation of genes involved in NOA. However, experimental studies are required to confirm our prediction.

Limitations

The current study was a bioinformatics analysis based on online data and datasets, which has the following limitations:

- Although the paper gives a variety of gene and RNA sets, the authors did not validate the data experimentally.
- While the in-vivo mechanisms of regulating gene expression may involve various elements, the research concentrates on the circRNAs–miRNA–mRNA axis.
- Considering the difference between the animal azoospermia model and human azoospermia pathophysiology, the present results may not be applicable in animal studies.

- The current study was conducted on one classification of NOA.

Recommendations for future research

Based on the gathered data, a future study could be performed in the following fields:
- Validating current gene sets in azoospermia pathology experimentally.

Table 4

Microarray validation.

| GSE	Gene	Log fold change	P	Category
GSE145467	GNAS	2.2469981	1.35E − 06	Protein
GSE145467	PRDM5	1.1262543	8.72E − 06	circRNA
GSE45885	MIR134	0.5559249	2.58E − 03	miRNA
GSE9210	VCAN	0.247348	0.0000192	circRNA
GSE145467	GNRHR	− 0.3285583	3.17E − 02	Protein
GSE45885	GNA11	− 0.6453602	1.79E − 04	Protein
GSE145467	GNRH1	− 1.4709544	2.95E − 03	Protein
GSE145467	GNAQ	− 1.5048151	2.97E − 07	Protein
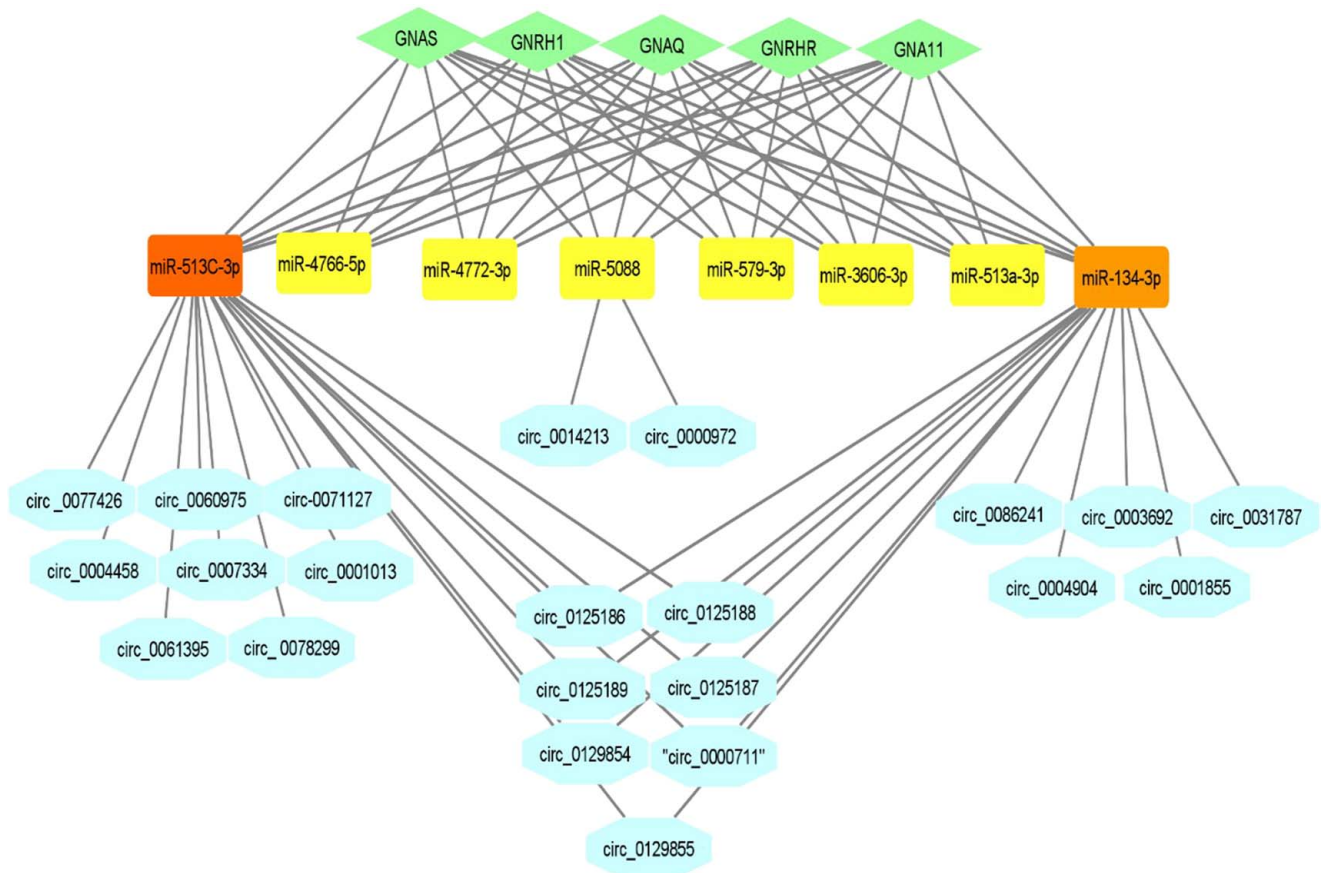
Figure 5. Protein–miRNA–circ RNA interaction illustrated by Cytoscape 3.9.1.

- Identification of other gene-regulating elements, such as lncRNAs, that play a role in azoospermia pathophysiology.
- Investigating the effects of achieved genes and RNA expression changes on the prognosis of azoospermia.
- Development of cohort studies to understand the impact of the obtained gene mutations on the incidence of azoospermia.
- Development of animal models of azoospermia using found genes.

Clinical implications for health managers and policymakers

The present data can be considered for developing biomarkers for the prevention, diagnosis, and prognosis of azoospermia. Also, the obtained gene data can be used to create gene therapy drugs. Moreover, the received data can be applied to identify infertile individuals and improve fertility among various populations.

Conclusion

According to the findings, two miRNAs, hsa-miR-134-3p and hsa-miR-513C-3p, may have the most critical role in regulating the expression of the GnRHR genes network. Furthermore, circRNAs, hsa_circ_0129854, hsa_circ_0129855, hsa_circ_0000711, hsa circ_0125189, hsa_circ_0125188, hsa_circ_0125187, and hsa circ_0125186, can also be considered as regulators of the miRNAs and consequently regulators of the entire GnRHR expression network. In addition, the three genes VCAN, NFATC3, and PRDM5 and their associated circRNAs can serve as a valuable gene network in producing the circRNAs. Finally, these ncRNAs and genes may play an essential role in NOA and may provide new ideas for the diagnosis, prognosis, and therapeutic targeting of NOA.

Ethical approval

This article does not contain any studies with human or animal subjects performed by any authors and does not require ethical approval and consent.

Consent

This article does not contain any studies with human or animal subjects performed by any authors and does not require ethical approval and consent.

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Zabihi et al. Annals of Medicine & Surgery (2023)

Annals of Medicine & Surgery

Author contribution

Study concept and design: M.R.Z., N.N., S.K., and M.A.; data acquisition: M.R.Z. and M.A.; data interpretation: M.R.Z. and M.A.; drafting the manuscript: M.R.Z., N.N., S.K., and M.A.; revision of the manuscript: M.R.Z., N.N., S.K., and M.A.; the final version of the manuscript is approved: M.R.Z., N.N., S.K., and M.A.

Conflicts of interest disclosure

None.

Research registration unique identifying number (UIN)

We could not register our manuscript in the Research Registry UIN: www.researchregistry.com due to internet access restrictions and international sanctions. We live in Iran. We hardly even meet the basic needs of our daily life. We do not receive any funding for our research, and we cannot pay for our research. Please excuse us from registering this manuscript in the Research Registry UIN: www.researchregistry.com.

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Data availability statement

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

Provenance and peer review

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