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Identification of key miRNAs as regulatory biomarkers of gonadotropins leading to infertility in males

Noor A. Oohayyed[1], Mais M. Mohammed[2], Aya M. Al-Rahim[1], Rawaa N. AlChalabi[1], Semaa A. Shaban[3], Ahmed A.J. Suleiman[4]

[1]Al-Nahrain University; Baghdad 64074, Iraq;

[2]Ibn Sina University for Medical and Pharmaceutical Sciences; Baghdad 64074, Iraq;

[3]Tikrit University; Tikrit, Saladin 34001, Iraq;

[4]University of Anbar; Ramadi, Anbar 46006, Iraq

***Corresponding author:** Ahmed A.J. Suleiman, e-mail: ahmed.suleiman@uoanbar.edu.iq*

Abstract

Introduction. Infertility is a highly fatal reproductive system disorder that affects the ability of a couple to reproduce. Over the past decades, a drastic uplift has been recorded in infertility cases among males ranging from 20 to 70 % indicating spermatogenesis impairment.

Aim: to identify key microRNAs (miRNAs) as regulatory biomarkers of gonadotropins involved in dysregulation of fertility-related genes to propose potential therapeutic strategies that would combat the action of oncogenic miRNAs (oncomiRs).

Materials and Methods. Interaction analysis was performed between miRNAs and fertility-related genes namely luteinizing hormone choriogonadotropin receptor (*LHCGR*), gonadotropin-releasing hormone receptor (*GnRHR*), follicle-stimulating hormone receptor (*FSHR*) and cystic fibrosis transmembrane conductance regulator (*CFTR*) to identify key miRNAs as regulatory biomarkers of gonadotropins leading to infertility in males.

Results. A total of 10, 13, 31 and 18 strong and potential binding sites were predicted for miRNAs-LHCGR, miRNAs-GnRHR, miRNAs-FSHR, and miRNAs-CFTR respectively employing miRWalk (comprehensive genetic database including miRNA targets) followed by identification of 6, 18, 55 and 17 significant interactions through RNA22. Subsequently shortlisted miRNAs and messenger RNA (mRNA) regions were subjected to Vfold-Pipeline and RNAComposer individually for 3D structure prediction. Additionally molecular docking was carried out between miRNAs and mRNAs models that discovered potential and stable interactions elucidating miR-6880-FSHR(R2) as a highly stable complex with least binding affinity (-566.3) and high confidence score (0.999).

Conclusion. Hence this study proposes key oncomiRs as a diagnostic biomarker and therapeutic target to bring about a promising treatment strategy against male factor infertility. However wet lab investigations are required for further validations of proposed study.

Keywords: gonadotropin, azoospermia, infertility biomarker, luteinizing hormone choriogonadotropin receptor, LHCGR, gonadotropin-releasing hormone receptor, GnRHR, follicle-stimulating hormone receptor, FSHR

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Идентификация ключевых микроРНК как регуляторных биомаркеров гонадотропинов, приводящих к мужскому бесплодию

Н.А. Охайед[1], М.М. Мохаммед[2], А.М. Аль-Рахим[1], Р.Н. Аль Чалаби[1], С.А. Шабан[3], А.А.Дж. Сулейман[4]

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[1]Университет Аль-Нахрейн; Багдад 64074, Ирак;

[2]Университет медицинских и фармацевтических наук Ибн Сины; Багдад 64074, Ирак;

[3]Университет Тикрита; Тикрит, Саладин 34001, Ирак;

[4]Анбарский университет; Эр-Рамади, Анбар 46006, Ирак

Для контактов: *Ахмед А.Дж. Сулейман, e-mail: ahmed.suleiman@uoanbar.edu.iq*

Резюме

Введение. Бесплодие – это крайне фатальное заболевание репродуктивной системы, которое определяет неспособность пары к воспроизведению потомства. За последние десятилетия зафиксирован резкий рост случаев бесплодия среди мужчин, составляющий от 20 до 70 %, что свидетельствует о нарушении сперматогенеза.

Цель: идентифицировать ключевые микроРНК в качестве регуляторных биомаркеров гонадотропинов, участвующих в нарушении регуляции генов, связанных с фертильностью, для определения потенциальных терапевтических стратегий по противодействию онкогенным микроРНК.

Материалы и методы. Проведен анализ взаимодействия между микроРНК и генами, связанными с фертильностью, такими как ген рецептора лютеинизирующего гормона/хориогонадотропина (англ. luteinizing hormone choriogonadotropin receptor, *LHCGR*), рецептора гонадотропин-рилизинг-гормона (англ. gonadotropin-releasing hormone receptor, *GnRHR*), рецептора фолликулостимулирующего гормона (англ. follicle-stimulating hormone receptor, *FSHR*) и регулятора трансмембранной проводимости при муковисцидозе (англ. cystic fibrosis transmembrane conductance regulator, *CFTR*), для идентификации ключевых микроРНК как регуляторных биомаркеров гонадотропинов, приводящие к мужскому бесплодию.

Результаты. Применение базы данных miRWalk (полная генетическая база данных, включающая в себя мишени для микроРНК) позволило предсказать в общей сложности 10, 13, 31 и 18 сильных и потенциальных сайтов связывания в парах микроРНК-LHCGR, микроРНК-GnRHR, микроРНК-FSHR и микроРНК-CFTR соответственно с последующим выявлением 6, 18, 55 и 17 значимых взаимодействий посредством алгоритма РНК22. Впоследствии включенные в окончательный список микроРНК и области матричной РНК (мРНК) были попарно проанализированы с использованием программ Vfold-Pipeline и RNAComposer для прогнозирования трехмерной структуры. Кроме того, был проведен молекулярный докинг-анализ между моделями микроРНК и мРНК, которые показали потенциальные и стабильные взаимодействия, и выявлен высокостабильный комплекс miR-6880-FSHR(R2) с наименьшей аффинностью связывания (-566,3) и высоким показателем достоверности (0,999).

Заключение. В настоящем исследовании предложены ключевые онко-микроРНК («oncomiRs») в качестве диагностического биомаркера и терапевтической мишени для разработки многообещающей стратегии лечения мужского бесплодия. Однако для дальнейшей проверки результатов данной работы необходимо проведение практических лабораторных исследований.

Ключевые слова: гонадотропин, азооспермия, биомаркер бесплодия, рецептор лютеинизирующего гормона/хориогонадотропина, LHCGR, рецептор гонадотропин-рилизинг-гормона, GnRHR, рецептор фолликулостимулирующего гормона, FSHR

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Highlights

What is already known about this subject?

- This field predicting the most effective gene in infertility.
- Use of bioinformatics tool to elucidate the biomarker related with men infertility.

What are the new findings?

- Predicting new miRNAs effecting gonadotropins.
- Show the infertility hormones regulatory small non coding RNA molecule.

How might it impact on clinical practice in the foreseeable future?

- The new research using the bioinformatics tool to predict the biomarker and treatment strategies through gene regulatory molecules.

Основные моменты

Что уже известно об этой теме?

- Ранее описан ген, являющийся наиболее частой генетической причиной бесплодия.
- Для обнаружения биомаркеров, связанных с мужским бесплодием, используют биоинформатику.

Что нового дает статья?

- Показана возможность определения новых микроРНК, влияющих на гонадотропины.
- Обнаружена малая некодирующая РНК, регулирующая гормоны бесплодия.

Как это может повлиять на клиническую практику в обозримом будущем?

- Для определения биомаркеров и стратегий лечения возможно проведение новых исследований с использованием инструментов биоинформатики в отношении молекул генов-регуляторов.

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Introduction / Введение

Infertility in men (azoospermia) has been considered a major health concern being highly prevalent worldwide indicating abnormalities in sperm parameters. Male infertility refers to impaired reproductive health due to defective semen parameters and lower testosterone levels. According to World Health Organization statistics, about 10–15 % of couples have been reported with infertility however male infertility contributes to half (50.0 %) of the infertility cases [1]. Infertility poses a considerable effect on psychological health and financial condition of patients producing economic burden on health care systems. Moreover, a higher mortality rate was observed among men having infertility factor than fertile men indicating greater risk of cancer among infertile individuals [2].

Infertility is caused by several risk factors that are categorized as acquired, idiopathic and congenital [3]. Acquired factors comprise exogenous factors (chemotherapy, radiation, heat, medications), severe diseases (renal failure, liver cirrhosis), acquired hypogonadotropic hypogonadism and varicocele which is a most common acquired factor of infertility with incidence rate of 40 % [4]. Moreover idiopathic factors incorporate obesity, psychological stress, smoking cigarettes, excessive intake of drugs, advanced paternal age, dietary practices and environmental exposure to toxins. Whereas, congenital factors (genetic factors) include chromosomal abnormalities, resulting in impaired testicular function and microdeletions in Y chromosome leading to spermatogenic abnormalities [5].

About 15 to 30 % of male infertility has been reported as a consequence of genetic factors including Y chromosome microdeletions and single gene defects [6]. Y chromosome is a male determining chromosome since most of its genes are specific to male fertility such as *SRA* (serum resistance associated) gene coupled with *DAZ, RBMY, BDY* (well known male fertility factors) regulate development of male genitalia. However microdeletions of fertility-related genes give rise to male-factor infertility anomalies [7]. Whereas single gene disorders occur as a result of mutations in a single gene for instance cystic fibrosis disorder (lung disease) is caused by recessive mutation in cystic fibrosis transmembrane conductance regulator (*CFTR*) that is responsible for congenital absence of the vas deferens (if inherited from both parents) leading to male infertility [8].

Hormones are the signaling molecules that regulate physiology and behavior of mammals sending signals into the bloodstream and tissues. The hypothalamic-pituitary-gonadal (HPG) axis (male reproductive hormone axis) is involved in regulation of male reproductive system through three major components namely hypothalamus, pituitary gland and gonads [9]. Furthermore, the gonadotropin-releasing hormone (GnRH) released by the hypothalamus binds to the gonadotropin-releasing hormone receptor (GnRHR), which is expressed on the gonadotrope cell surface that produces gonadotropins – follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [10]. Gonadotropins are glycoprotein hormones involved in the regulation of the reproductive system and production of gametes and sex steroids hence essential for normal growth, sexual development and reproduction. However abnormal functioning of gonadotropins causes hypogonadism that occurs when sex glands do not produce enough hormones for normal functioning of the reproductive system [11]. Additionally malfunctioning hypothalamus and pituitary glands produce hypogonadotropic hypogonadism. It refers to the condition in which the hypothalamus and pituitary gland do not produce hormones including FSH, LH and GnRH which are essential for the stimulation of sex glands [12]. Therefore, it is a prerequisite to identify the key microRNAs (miRNAs) involved in the downregulation of essential gonadotropins.

MicroRNAs (consisting of 18 to 22 nucleotides) are known as non-coding RNAs involved in the post transcriptional regulation of gene expression through either mRNA (matrix RNA) degradation or translational repression [13]. MicroRNAs have been emerged as disease biomarkers due to their influence on gene expression as tumor suppressors or oncogenic miRNAs (oncomiRs). Generally oncomiRs (upregulated miRNAs) show elevated expression in cancer whereas tumor suppressor miRNAs are underexpressed therefore regulation of miRNAs for therapeutic purposes is a concern of ongoing research. In breast cancer, high expression of oncomiRs majorly miR-532, miR-19b and miR-20b has been observed indicating underexpression of tumor suppressor genes considerably *RERG*, *PTPRG* and *PTEN* [14]. Moreover, overexpression of miR142-3p has been reported in testicular germ cell tumors suggesting that miR142-3p act as an oncomiR inducing the tumorigenesis through downregulation of key target PTPN23 (tumor suppressor gene) [15]. Another study has reported the causative role of oncomiRs in non-obstructive azoospermia elucidating that overexpression of miR-100 and let-7b downregulates the expression of estrogen-alpha (ERα) among infertile patients when compared with fertile men [16]. Considering the potential role of oncomiRs in male infertility, significant oncomiRs that are involved in the downregulation of fertility-regulating gonadotropins – GnRHR, FSHR, CFTR and luteinizing hormone choriogonadotropin receptor (LHCGR) needs to be investigated as disease biomarkers.

GnRHR is a receptor protein located on the cell surface of pituitary gonadotropes. It has been involved in the transduction of signals from GnRH (released by hypothalamus) to synthesize and stimulate the secretion of FSH and LH [17]. However FSHR is a follicle-stimulating hormone receptor mediated by G protein that activates downstream signal transduction pathways such as PI3K-AKT and ERK1/ERK2 leading to cellular response

[18]. Whereas LHCGR is a G protein-coupled receptor for LH and chorionic gonadotropin and contributes to activation of signals that affect cell development and function [19]. CFTR is involved in regulation of ion channels by maintaining salt and water level on surfaces of the organ therefore such ion channels are essential for the proper functioning of organs such as lungs and pancreas [20]. Additionally mutated CFTR has been associated with male infertility suggesting mutations in the *CFTR* gene accounts for 78 % of infertility cases with congenital bilateral absence of the vas deferens [21].

Nevertheless, this study proposes key oncomiRs as regulatory biomarkers that may be involved in male infertility by dysregulating the essential fertility-related genes. These miRNAs may suggest potential therapeutic targets for combating the oncomiRs. Moreover, the crucial interactions predicted between miRNA-mRNA complexes and their strong and stable conformations might suggest their diagnostic and prognostic significance in biomedical research. Additionally, the early diagnosis and prognosis will not only alleviate infertility determinants but will also ensure general health and well-being. Hence, infertility oncomiRs exerts potential role in acting as diagnostic, prognostic, and eventually therapeutic biomolecules for future perspectives.

Aim: to identify key miRNAs as regulatory biomarkers of gonadotropins involved in dysregulation of fertility-related genes to propose potential therapeutic strategies that would combat the action of oncomiRs.

Materials and Methods / Материалы и методы

Overview / Краткое описание

In this study, binding sites of miRNAs were predicted on fertility-regulating genes, namely *LHCGR, GnRHR, FSHR* and *CFTR*. The interactions between miRNAs and aforementioned genes were analyzed with respect to binding potential in order to identify the key miRNAs involved in downregulation of fertility-regulating genes (**Fig. 1**).

Binding site prediction of miRNAs on fertility-regulating genes / Прогнозирование сайта связывания микроРНК на генах, регулирующих фертильность

The binding sites of miRNAs were predicted on fertility-related key genes *LHCGR, GnRHR, FSHR* and *CFTR* employing miRWalk (http://mirwalk.umm.uni-heidelberg.de). MiRWalk provides key insights of miRNA-target interactions based on machine learning predictions and experimental validations. It enables the user to predict miRNA-target interactions by either single gene or miRNA search option or through an advanced option called target mining [22]. Single gene search option was utilized to predict binding sites of miRNAs on fertility-regulating genes individually. Binding predictions

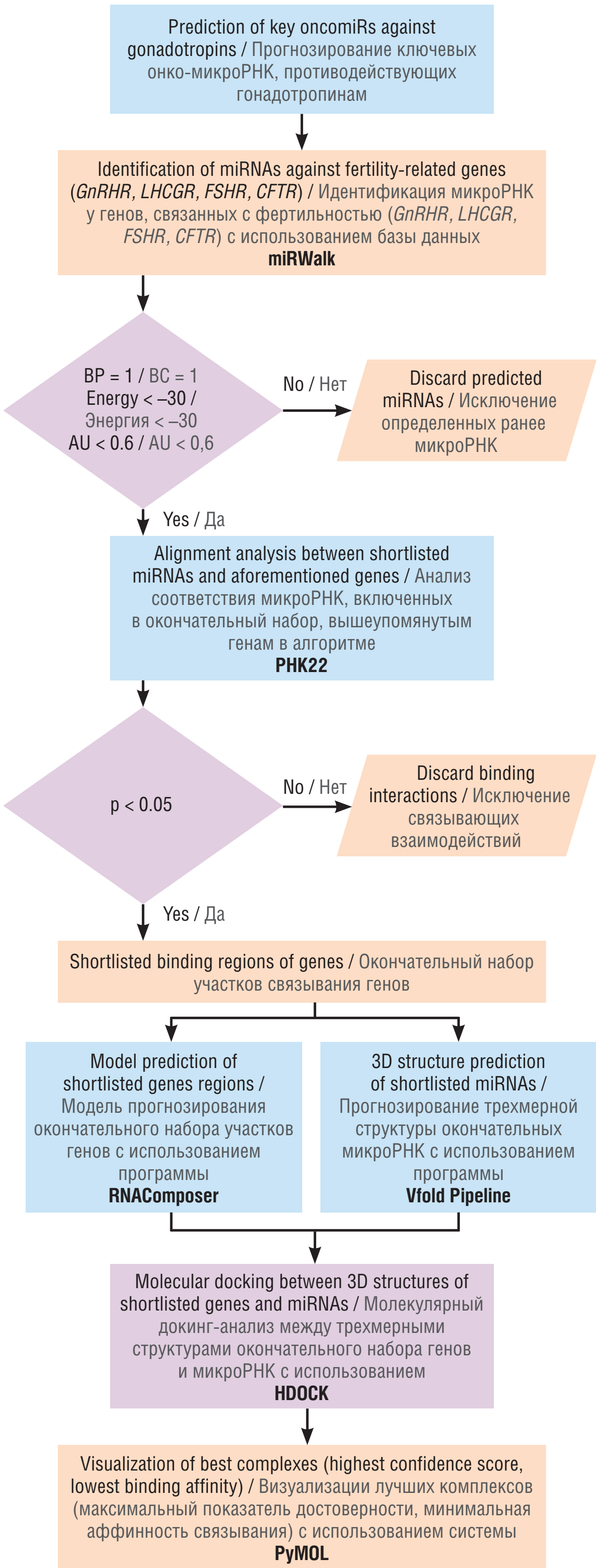

Figure 1. Overall workflow of miRNA-mRNA interaction analysis.

Note: BP – binding probability; AU – Adenine Uracil content.

Рисунок 1. Общий процесс анализа взаимодействия микроРНК-мРНК.

Примечание: *ВС – вероятность связывания; AU – содержание аденин-урацила.*

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were shortlisted on the basis of binding energy < –30 kcal/mol, binding probability = 1 and Adenine Uracil (AU) content < 0.6 [23]. Least binding energy represents highest stability while highest binding probability denotes strong binding plausibility. However AU content refers to AU rich elements of gene located at 30 nucleotides upstream and downstream of the predicted binding site. Additionally, AU rich elements are well known destabilizing elements, therefore least AU rich elements estimate highly stable structure [24].

Alignment analysis between potential miRNAs and fertility-related genes / Анализ соответствия потенциальных микроРНК генам, связанным с фертильностью

Predicted shortlisted microRNAs were aligned against fertility-related genes through RNA22 (https://cm.jefferson.edu/rna22/Interactive/) for the identification of significant binding interactions between miRNAs and their respective genes [25]. RNA22 is based on supervised machine learning method that employs combined approach to predict significant interaction i.e. pattern based approach (patterns of known miRNAs are used to predict putative target regions) and estimation of folding energy [26]. Shortlisted microRNAs were aligned against fertility-related genes (*LHCGR, GNRHR, FSHR* and *CFTR*) individually employing default parameters such as sensitivity = 63 %, specificity = 6 %, seed size = 7, minimum number of paired-up bases in heteroduplex = 12, maximum folding energy = –12 kcal/mol. For target gene sequence, NCBI (National Center for Biotechnology Information) Gene database (https://www.mirbase.org) was accessed to retrieve gene sequences, *LHCGR* (NM_000233.4), *GnRHR* (NM_000406.3), *FSHR* (NM_000145.4) and *CFTR* (NM_000492.4) [27]. The individual gene sequences were fed to RNA22 along with their corresponding shortlisted miRNA sequences retrieved through miRBase database (https://www.mirbase.org) [28]. Subsequently resultant interactions of miRNAs and genes were shortlisted on the basis of least p-value i.e. p-value < 0.05. Furthermore, gene regions depicting maximum and significant binding interactions with shortlisted miRNAs were extracted from genes and plotted in terms of binding sites and folding energy using the scatterplot function of seaborn (v0.11.2) library in python.

3D structure prediction of fertility-related genes / Трехмерное прогнозирование структуры генов, связанных с фертильностью

Modeling of fertility-related genes was carried out through RNAComposer (v1.0) (https://rnacomposer.cs.put.poznan.pl) [29]. RNAComposer uses sequence information and secondary structure topology of genes for their 3D structure prediction employing a dedicated database consisting of 3D RNA fragments obtained through RNA FRABASE. RNAComposer generates the model by comparing 3D RNA fragments with secondary structure topology of the queried gene. It offers two modes of gene modeling i.e. interactive mode (for single gene modeling at once) and batch mode (for modeling of 10 genes at a time) [28]. Interactive mode was utilized for modeling of fertility-related genes (parameters such as input sequence up to 500 nt, CONTRAfold as a secondary structure prediction method).

Modeling of shortlisted miRNAs / Моделирование окончательного набора микроРНК

Shortlisted microRNAs (with respect to maximum binding interactions with shortlisted regions of genes) were subjected to modeling through Vfold Pipeline (http://rna.physics.missouri.edu/vfoldPipeline/) [30]. Vfold Pipeline is a statistical approach of modeling that offers 2D and subsequently 3D structure prediction of miRNAs along RNA folding thermodynamics stabilities employing sequence information [30]. Shortlisted microRNA sequences (individually) were fed to Vfold Pipeline using default parameters. It generated a 3D structure file (pdb format) that was used for further analysis.

Molecular docking between shortlisted miRNA models and fertility-related gene models / Молекулярный докинг-анализ между моделями окончательного набора микроРНК и моделями генов, связанных с фертильностью

MicroRNA models were docked against gene models using HDOCK (v2021) (http://hdock.phys.hust.edu.cn/) [31]. It predicts the interactions between receptor and ligand molecule through a hybrid algorithm that offers template-free and template based docking. It takes either amino acid sequence or protein structures as an input and generates interaction results in the form interactive visualizations showing stable conformations with least binding affinity [32]. Individual 3D structures of shortlisted microRNAs and genes were submitted to HDOCK as ligands and receptors respectively. Highly stable conformation (showing least binding affinity and highest confidence score) of miRNA-mRNA docked model was shortlisted out of 10 confirmations for each complex. Furthermore, shortlisted miRNA-mRNA docked complexes were visualized through PyMOL (v2.5.4) (https://pymol.org/2/). PyMOL is a molecular visualization system based on the python programming language that is employed for 3D visualizations of macromolecules including nucleic acids and proteins [33].

Results / Результаты

Prediction of miRNA interactions against fertility-related genes / Прогнозирование взаимодействий микроРНК с генами, связанными с фертильностью

The miRWalk database was employed to identify microRNAs and predict stable and strong interactions

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between predicted microRNAs and fertility-related genes, considerably *LHCGR*, *GnRHR*, *FSHR* and *CFTR*. It was observed that a total of 10, 13, 31 and 18 microRNAs showed strong and stable interactions (binding probability = 1, binding energy < –30 kcal/mol, AU content < 0.6) against aforementioned genes respectively. The interactions of predicted microRNAs against each fertility-related gene are provided in **Tables 1–4** and scatterplot function of seaborn.

Table 1. Predicted interactions between luteinizing hormone choriogonadotropin receptor (*LHCGR*) and miRNAs through miRWalk.

Таблица 1. Прогнозируемые взаимодействия между геном рецептора лютеинизирующего гормона/хориогонадотропина (*LHCGR*) и микроРНК с использованием базы данных miRWalk.

miRNA id Идентификатор микроРНК	miRNA sequence Последовательность микроРНК
miR-339-3p	UGAGCGCCUCGACGACAGAGCCG
miR-3147	GGUUGGGCAGUGAGGAGGGUGUGA
miR-3677-3p	CUCGUGGGCUCUGGCCACGGCC
miR-3714	GAAGGCAGCAGUGCUCCCCUGU
miR-3135b	GGCUGGAGCGAGUGCAGUGGUG
miR-4689	UUGAGGAGACAUGGUGGGGGCC
miR-4739	AAGGGAGGAGGAGCGGAGGGGCCCU
miR-4745-5p	UGAGUGGGGCUCCCGGGACGGCG
miR-4758-5p	GUGAGUGGGAGCCGGUGGGGCUG
miR-6777-5p	ACGGGGAGUCAGGCAGUGGUGGA

Table 2. Predicted interactions between gonadotropin-releasing hormone receptor (*GnRHR*) and miRNAs through miRWalk.

Таблица 2. Прогнозируемые взаимодействия между геном рецептора гонадотропин-рилизинг гормона (*GnRHR*) и микроРНК с использованием базы данных miRWalk.

miRNA id Идентификатор микроРНК	miRNA sequence Последовательность микроРНК
miR-638	AGGGAUCGCGGGCGGGUGGCGGCCU
miR-1538	CGGCCCGGGCUGCUGCUGUUCCU
miR-3620-5p	GUGGGCUGGGCUGGGCUGGGCC
miR-4640-5p	UGGGCCAGGGAGCAGCUGGUGGG
miR-4685-5p	CCCAGGGCUUGGAGUGGGGCAAGGUU
miR-4749-5p	UGCGGGGACAGGCCAGGGCAUC
miR-5001-3p	UUCUGCCUCUGUCCAGGUCCUU
miR-5787	GGGCUGGGGCGCGGGGAGGU
miR-6089	GGAGGCCGGGGUGGGGCGGGGCGG
miR-6741-5p	GUGGGUGCUGGUGGGAGCCGUG
miR-6752-5p	GGGGGGUGUGGAGCCAGGGGGC
miR-6857-5p	UUGGGGAUUGGGUCAGGCCAGU
miR-8069	GGAUGGUUGGGGGCGGUCGGCGU

Alignment analysis between shortlisted miRNAs and fertility-related genes / Анализ соответствия окончательного набора микроРНК генам, связанным с фертильностью

Significant alignments between shortlisted miRNAs and fertility-related genes have been obtained through RNA22. A total of 6 significant alignments were observed between shortlisted miRNAs and shortlisted regions of *LHCGR* gene while RNA22 predicted 18 significant

Table 3. Predicted interactions between follicle-stimulating hormone receptor (*FSHR*) and miRNAs through miRWalk.

Таблица 3. Прогнозируемые взаимодействия между геном рецептора фолликулостимулирующего гормона (*FSHR*) и микроРНК с использованием базы данных miRWalk.

miRNA id Идентификатор микроРНК	Duplex Дуплекс
miR-211-3p	GCAGGGACAGCAAAGGGGUGC
miR-296-3p	GAGGGUUGGGUGGAGGCUCUCC
miR-762	GGGGCUGGGGCCGGGGCCGAGC
miR-939-5p	UGGGGAGCUGAGGCUCUGGGGGUG
miR-1233-5p	AGUGGGAGGCCAGGGCACGGCA
miR-1207-5p	UGGCAGGGAGGCUGGGAGGGG
miR-3192-5p	UCUGGGAGGUUGUAGCAGUGGAA
miR-4463	GAGACUGGGGUGGGGCC
miR-4632-5p	GAGGGCAGCGUGGGUGUGGCGGA
miR-4651	CGGGGUGGGUGAGGUCGGGC
miR-4656	UGGGCUGAGGGCAGGAGGCCUGU
miR-1343-5p	UGGGGAGCGGCCCCCGGGUGGG
miR-4739	AAGGGAGGAGGAGCGGAGGGGCCCU
miR-2467-3p	AGCAGAGGCAGAGAGGCUCAGG
miR-4524b-5p	AUAGCAGCAUAAGCCUGUCUC
miR-6075	ACGGCCCAGGCGGCAUUGGUG
miR-6722-3p	UGCAGGGGUCGGGUGGGCCAGG
miR-6848-5p	UGGGGGCUGGGAUGGGCCAUGGU
miR-6858-5p	GUGAGGAGGGGCUGGCAGGGAC
miR-6880-5p	UGGUGGAGGAAGAGGGCAGCUC
miR-6885-5p	AGGGGGGCACUGCGCAAGCAAAGCC
miR-7112-3p	UGCAUCACAGCCUUUGGCCCUAG
miR-4433b-3p	CAGGAGUGGGGGGUGGGACGU
miR-1909-3p	CGCAGGGGCCGGGUGCUCACCG
miR-1914-3p	GGAGGGGUCCCGCACUGGGAGG
miR-4433a-3p	ACAGGAGUGGGGGUGGGACAU
miR-4640-5p	UGGGCCAGGGAGCAGCUGGUGGG
miR-4758-5p	GUGAGUGGGAGCCGGUGGGGCUG
miR-5787	GGGCUGGGGCGCGGGGAGGU
miR-6791-5p	CCCCUGGGGCUGGGCAGGCGGA
miR-6847-5p	ACAGAGGACAGUGGAGUGUGAGC

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Table 4. Predicted interactions between cystic fibrosis transmembrane conductance regulator (*CFTR*) and miRNAs through miRWalk.

Таблица 4. Прогнозируемые взаимодействия между геном регулятора трансмембранной проводимости при муковисцидозе (*CFTR*) и микроРНК с использованием базы данных miRWalk.

miRNA id Идентификатор микроРНК	Duplex Дуплекс
miR-145-5p	GUCCAGUUUUCCCAGGAAUCCCU
miR-370-3p	GCCUGCUGGGGUGGAACCUGGU
miR-383-5p	AGAUCAGAAGGUGAUUGUGGCU
miR-671-5p	AGGAAGCCCUGGAGGGGCUGGAG
miR-1471	GCCCGCGUGUGGAGCCAGGUGU
miR-3619-3p	GGGACCAUCCUGCCUGCUGUGG
miR-3620-5p	GUGGGCUGGGCUGGGCUGGGCC
miR-3663-5p	GCUGGUCUGCGUGGUGCUCGG
miR-1343-3p	CUCCUGGGGCCCGCACUCUCGC
miR-4726-3p	ACCCAGGUUCCCUCUGGCCGCA
miR-4769-5p	GGUGGGAUGGAGAGAAGGUAUGAG
miR-5195-3p	AUCCAGUUCUCUGAGGGGGCU
miR-6089	GGAGGCCGGGGUGGGGCGGGGCGG
miR-6749-5p	UCGGGCCUGGGGUUGGGGGAGC
miR-6750-5p	CAGGGAACAGCUGGGUGAGCUGCU
miR-6775-5p	UCGGGGCAUGGGGGAGGGAGGCUGG
miR-6810-3p	UCCCCUGCUCCCUUGUUCCCCAG
miR-6821-5p	GUGCGUGGUGGCUCGAGGCGGGG

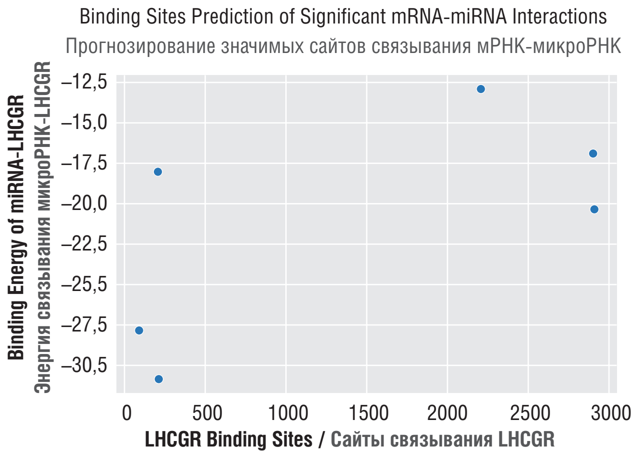

Figure 2. Scatter plot representation of significant miRNA-LHCGR interactions for shortlisting of gene regions.

Note: *LHCGR – luteinizing hormone choriogonadotropin receptor; miRNA – microRNA.*

Рисунок 2. Диаграмма рассеяния значимых взаимодействий микроРНК-LHCGR для определения окончательного набора областей генов.

Примечание: *LHCGR – рецептор лютеинизирующего гормона/хориогонадотропина; miRNA – микроРНК.*

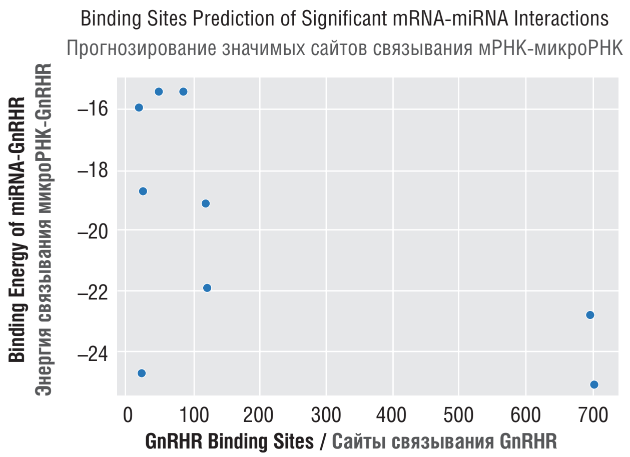

Figure 3. Scatter plot representation of significant miRNA-GnRHR interactions for shortlisting of gene regions.

Note: *GnRHR – gonadotropin-releasing hormone receptor; miRNA – microRNA.*

Рисунок 3. Диаграмма рассеяния значимых взаимодействий микроРНК-GnRHR для определения окончательного набора областей генов.

Примечание: *GnRHR – рецептор гонадотропин-рилизинг гормона; miRNA – микроРНК.*

interactions between shortlisted miRNAs and their corresponding gene (*GnRHR*) regions. Additionally miRNA-FSHR alignment analysis revealed that 55 microRNAs were significantly bound with *FSHR* however 17 miRNA-CFTR interactions were obtained through miRNA-CFTR alignment analysis. Alignments between miRNAs and fertility-related genes elucidated miRNA binding sites on their respective genes indicating low folding energy and high significance.

Shortlisting of miRNA-binding regions of fertility-related genes / Окончательный набор связывающих микроРНК областей генов, ассоциированных с фертильностью

MicroRNA-binding regions of fertility-related genes indicating maximum binding interactions with microRNAs were shortlisted through scatterplot function that generated scatter plots for miRNAs-genes interactions (**Fig. 2–5**). It was observed that *LHCGR, GnRHR, FSHR* contained 2 such regions that showed maximum binding interactions with shortlisted miRNAs however, a total of 3 regions were identified for *CFTR* having maximum interactions with miRNAs (**Table 5**). For gene *LHCGR*, out of 6 interactions, 3 miRNAs (hsa-miR-3135b, hsa-miR-4739, hsa-miR-6777-5p) were shortlisted on the basis of maximum interactions (**Table 6**). It was observed that all of three shortlisted microRNAs (hsa-miR-3135b, hsa-miR-4739 and hsa-miR-6777-5p) were significantly bound with region 1 of gene LHCGR nevertheless 2 of aforementioned microRNAs also showed significant interactions (hsa-miR-3135b, hsa-miR-4739) with region 2 of *LHCGR*. For gene *GnRHR*, a total of 5 microRNAs (hsa-miR-4749-5p, hsa-miR-4685-5p, hsa-miR-6857-5p, hsa-miR-6752-5p, hsa-miR-8069) out of 18 interactions have

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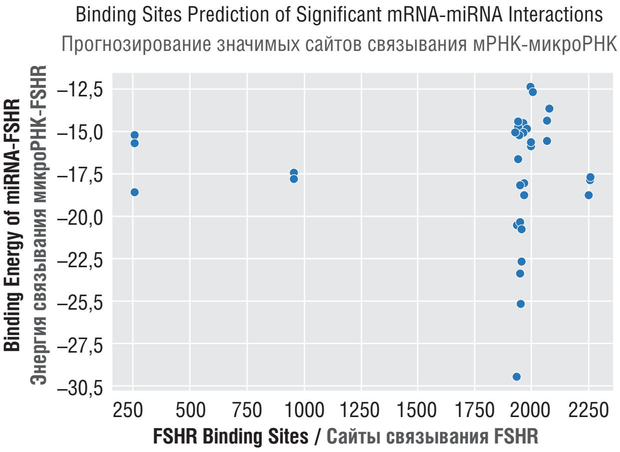

Figure 4. Scatter plot representation of significant miRNA-FSHR interactions for shortlisting of gene regions.

Note: *FSHR – follicle-stimulating hormone receptor; miRNA – microRNA.*

Рисунок 4. Диаграмма рассеяния значимых взаимодействий микроРНК-FSHR для определения окончательного набора областей генов

Примечание: *FSHR – рецептор фолликулостимулирующего гормона; miRNA – микроРНК.*

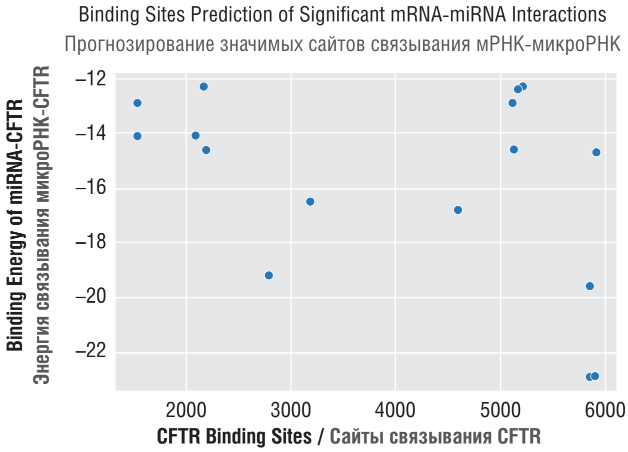

Figure 5. Scatter plot representation of significant miRNA-CFTR interactions for shortlisting of gene regions.

Note: *CFTR – cystic fibrosis transmembrane conductance regulator; miRNA – microRNA.*

Рисунок 5. Диаграмма рассеяния значимых взаимодействий микроРНК-CFTR для определения окончательного набора областей генов.

Примечание: *CFTR – регулятор трансмембранной проводимости при муковисцидозе; miRNA – miRNA – микроРНК.*

Table 5. Shortlisted regions of fertility-related genes.

Таблица 5. Окончательный набор областей генов, связанных с фертильностью.

Gene / Ген	Region Область	Nucleotide length Длина, нуклеотиды
LHCGR (luteinizing hormone choriogonadotropin receptor) *LHCGR* (рецептор лютеинизирующего гормона/хориогонадотропина)	Region 1 / Область 1	1–500
	Region 2 / Область 2	2501–3000
GnRHR (gonadotropin-releasing hormone receptor) *GnRHR* (рецептор гонадотропин-рилизинг-гормона)	Region 1 / Область 1	1–500
	Region 2 / Область 2	501–1000
FSHR (follicle-stimulating hormone receptor) *FSHR* (рецептор фолликулостимулирующего гормона)	Region 1 / Область 1	1–500
	Region 2 / Область 2	1801–2300
CFTR (cystic fibrosis transmembrane conductance regulator) *CFTR* (регулятор трансмембранной проводимости при муковисцидозе)	Region 1 / Область 1	1801-2300
	Region 2 / Область 2	5001–5500
	Region 3 / Область 3	5501–6000

Table 6. Significant binding interactions between shortlisted miRNAs and luteinizing hormone choriogonadotropin receptor (*LHCGR*) regions.

Таблица 6. Значимые связывающие взаимодействия между окончательным набором микроРНК и областями рецептора лютеинизирующего гормона/хориогонадотропина (*LHCGR*).

Gene region Область гена	miRNA микроРНК	Binding sites Сайты связывания	Folding energy Энергия связывания	p-value Значение p
Region 1 Область 1	miR-3135b	88	-27.8	0.0354
	miR-4739	208	-18.0	0.0318
	miR-6777-5p	212	-30.8	0.0318
Region 2 Область 2	miR-3135b	2905	-16.9	0.0215
	miR-4739	2909	-20.3	0.0215

been determined with maximum bindings (**Table 7**). All of the 5 shortlisted microRNAs showed significant interactions with region 1 of *GnRHR* however significant interactions of 2 aforementioned microRNAs (hsa-miR-3135b, hsa-miR-4739) have been also observed with region 2 of *GnRHR*. Moreover, scatterplot of miRNA-FSHR alignments revealed that 21 microRNAs out of 55 interactions have been significantly bound with *FSHR* indicating maximum interactions (**Table 8**). However, 3 (hsa-miR-762, hsa-miR-296-3p, hsa-miR-4651) out of 21 microRNAs were significantly bound with region 1 while all of 21 aforestated microRNAs have shown interaction with

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Table 7. Significant binding interactions between shortlisted miRNAs and gonadotropin-releasing hormone receptor (*GnRHR*) regions.

Таблица 7. Значимые связывающие взаимодействия между окончательным набором микроРНК и областями рецептора гонадотропин-рилизинг-гормона (*GnRHR*).

Gene region Область гена	miRNA микроРНК	Binding sites Сайты связывания	Folding energy Энергия связывания	p-value Значение p
Region 1 Область 1	miR-4749-5p	19	-15.9	0.0165
	miR-4685-5p	23	-24.7	0.0165
	miR-6857-5p	25	-18.7	0.0165
	miR-6752-5p	49	-15.4	0.0165
	miR-8069	85	-15.4	0.0165
	miR-6857-5p	119	-19.1	0.0165
	miR-4749-5p	122	-21.9	0.0165
Region 2 Область 2	miR-8069	697	-22.8	0.0489
	miR-6752-5p	703	-25.1	0.0489

Table 8. Significant binding interactions between shortlisted miRNAs and follicle-stimulating hormone receptor (*FSHR*) regions.

Таблица 8. Значимые связывающие взаимодействия между окончательным набором микроРНК и областями рецептора фолликулостимулирующего гормона (*FSHR*).

Gene region Область гена	miRNA микроРНК	Binding sites Сайты связывания	Folding energy Энергия связывания	p-value Значение p
Region 1 Область 1	miR-762	253	-15.2	0.0401
	miR-296-3p	254	-15.6	0.0401
	miR-4651	254	-18.5	0.0401
Region 2 Область 2	miR-4433b-3p	1935	-29.4	0.0123
	miR-6880-5p	1938	-15.1	0.0123
	miR-6847-5p	1938	-14.4	0.0123
	miR-6722-3p	1939	-20.4	0.0123
	miR-1909-3p	1939	-14.7	0.0123
	miR-211-3p	1940	-16.54	0.0123
	miR-762	1946	-20.5	0.0123
	miR-4739	1948	-23.3	0.0123
	miR-1207-5p	1949	-20.3	0.0123
	miR-1914-3p	1951	-25.1	0.0123
	miR-1233-5p	1953	-22.6	0.0123
	miR-6848-5p	1953	-20.7	0.0123
	miR-296-3p	1964	-14.5	0.0123
	miR-1343-5p	1965	-15.0	0.0123
	miR-6858-5p	1967	-18.7	0.0123
	miR-4651	1999	-15.8	0.0123
	miR-2467-3p	1999	-15.6	0.0123
	miR-4524b-5p	2002	-12.6	0.0123
	miR-4656	2064	-15.5	0.0186
	miR-939-5p	2251	-18.7	0.0469
	miR-4758-5p	2257	-17.7	0.0469

region 2 of *FSHR* indicating 3 common microRNAs between both regions. Furthermore, a total of 8 microRNAs were shortlisted for miRNA-CFTR alignments with respect to maximum interactions (**Table 9**). Out of 8 shortlisted microRNAs, 2 microRNAs (hsa-miR-145-5p, hsa-miR-4769-5p) were bound with region 1 while 3 unique microRNAs (hsa-miR-1343-3p, hsa-miR-6775-5p, hsa-miR-1471) including common hsa-miR-4769-5p of region 1 have been found to show significant interactions with region 2. Additionally, significant interactions of 3 unique microRNAs (hsa-miR-6821-5p, hsa-miR-3620-5p, hsa-miR-3619-3p) have been identified against

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Table 9. Significant binding interactions between shortlisted miRNAs and cystic fibrosis transmembrane conductance regulator (*CFTR*) regions.

Таблица 9. Значимые связывающие взаимодействия между окончательным набором микроРНК и областями регулятора трансмембранной проводимости при муковисцидозе (*CFTR*).

Gene region Область гена	miRNA микроРНК	Binding sites Сайты связывания	Folding energy Энергия связывания	p-value Значение p
Region 1 Область 1	miR-145-5p	2087	-14.1	0.00619
	miR-4769-5p	2162	-12.3	0.00619
	miR-4769-5p	2188	-14.6	0.00619
Region 2 Область 2	miR-1343-3p	5114	-12.9	0.0431
	miR-6775-5p	5128	-14.59	0.0431
	miR-4769-5p	5160	-12.4	0.0431
	miR-1471	5208	-12.3	0.0431
Region 3 Область 3	miR-6821-5p	5849	-19.6	0.00749
	miR-3620-5p	5860	-22.9	0.00749
	miR-6775-5p	5904	-22.9	0.00749
	miR-3619-3p	5916	-14.7	0.00749

region 3 including 1 common microRNA hsa-miR-6775-5p of region 2.

Molecular docking analysis between 3D structures of shortlisted miRNAs and gene regions / Молекулярный докинг-анализ между трехмерными структурами окончательного набора микроРНК и областями генов

3D structures of shortlisted microRNAs and gene regions were modeled through Vfold Pipeline and RNA-Composer respectively to perform molecular docking analysis. Subsequently predicted models of shortlisted miRNAs and fertility related genes were docked through Hdock-server for integrated protein–protein docking. Molecular docking generated 10 complexes for each miRNA-gene interaction; however only the top complex in terms of least docking score was shortlisted for further analysis. Out of 5, 7, 24 and 10 docked complexes for each interaction between shortlisted miRNAs and gene regions of *LHCGR, GnRHR, FSHR* and *CFTR* individually, only top docked complex was selected with respect to least affinity score (docking score) and highest confidence score indicating preferred and stable orientation of docked complex (**Table 10**). The docking results for each fertility-related gene were merged together and sorted on the basis of least docking score and highest confidence score in order to extract top 3 complexes out of all miRNAs-genes complexes. It was identified that miRNAs interactions with region 2 of *FSHR* majorly miR-6880-5p-R2, miR-2467-3-R2 and miR-211-3p-R2 were highly stable among all complexes with least docking score of -566.39, -554.35, -528.01 and highest confidence score of 0.9998, 0.9997 and 0.9995 respectively (**Table 11**).

Visualization of shortlisted complexes / Визуализация окончательного набора комплексов

The shortlisted miRNA-mRNA complexes namely miR-4739-R1, miR-6857-5p-R1, miR-6880-5p-R2, miR-4769-5p-R2 were visualized through PyMOL to identify the interaction sites between shortlisted miRNAs and gene regions. A total of 20 interactions were observed between

Table 10. Shortlisted docked complexes with respect to least docking score and highest confidence score.

Таблица 10. Окончательный набор пристыкованных комплексов с учетом минимального показателя стыковки и максимального показателя достоверности.

Gene / Ген	Complex Комплекс	Docking score Показатель стыковки	Confidence score Показатель достоверности
LHCGR (luteinizing hormone choriogonadotropin receptor) *LHCGR* (рецептор лютеинизирующего гормона/хориогонадотропина)	miR-4739-R1	-451.05	0.9976
GnRHR (gonadotropin-releasing hormone receptor) *GnRHR* (рецептор гонадотропин-рилизинг-гормона)	miR-6857-5p-R1	-423.13	0.9958
FSHR (follicle-stimulating hormone receptor) *FSHR* (рецептор фолликулостимулирующего гормона)	miR-6880-5p-R2	-566.39	0.9998
CFTR (cystic fibrosis transmembrane conductance regulator) *CFTR* (регулятор трансмембранной проводимости при муковисцидозе)	miR-4769-5p-R2	-494.32	0.999

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Table 11. Shortlisted top 3 docked complexes from merged docking results.

Таблица 11. Три лучших окончательных набора стыковочных комплексов по объединенным результатам стыковки.

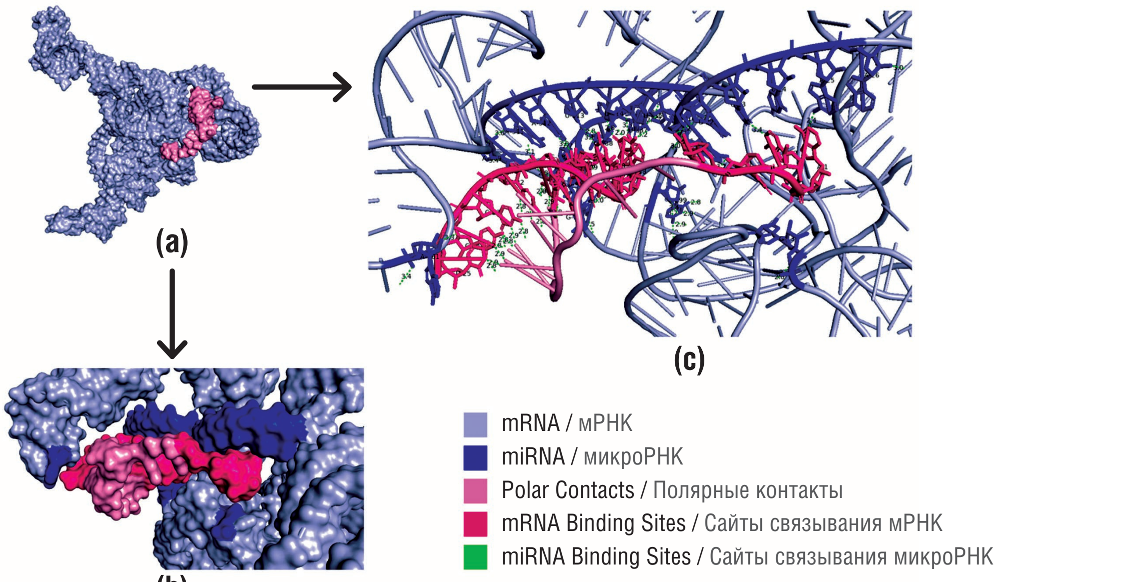
Gene / Ген	Complex Комплекс	Docking score Показатель стыковки	Confidence score Показатель достоверности
FSHR (follicle-stimulating hormone receptor) *FSHR* (рецептор фолликулостимулирующего гормона)	miR-6880-5p-R2	-566.39	0.9998
	miR-2467-3p-R2	-554.35	0.9997
	miR-211-3p-R2	-528.01	0.9995

miR-4739 and region 1 of *LHCGR* with least binding affinity of -451.05 (**Fig. 6**) while 23 interactions were identified between miR-6857-5p and region 1 of *GnRHR* indicating least binding score of -423.13 (**Fig. 7**). However visualization analysis revealed that miR-6880-5p interacted with region 2 of *FSHR* at 20 different binding sites with least binding score i.e. -566.39 (**Fig. 8**) whereas 46 interactions were identified between miR-4769-5p and *CFTR* docked complex representing highest stability with least docking score of -494.32 (**Fig. 9**). Additionally visualization analysis of shortlisted complexes from merged docking analysis revealed highly stable complexes including aforementioned docked complex miR-6880-5p-R2 of *FSHR* (**Fig. 8**) and other 2 highly stable complexes namely miR-2467-3p-R2 and miR-211-3p-R2 of *FSHR* elucidating 46 and 22 stable interactions respectively (**Fig. 10, 11**).

Discussion / Обсуждение

Infertility is a highly prevalent global health problem affecting the reproductivity of 8–12 % of the couples having unprotected intercourse [34]. However, a global survey has reported a rapid increase in male infertility cases over the past decades ranging from 20 to 70 % [35]. It has been identified that males with reported infertility ailments are more prone to other diseases including metabolic disorders and several cancers affecting the overall wellbeing of the patients and posing financial pressure on patients and healthcare systems [2]. Infertility arises in men as a consequence of several risk factors: considerably acquired factors (radiations, severe disorders, hypogonadism and varicocele), idiopathic actors (chemical exposure, smoking, over-age, psychological problems)

miR-4739-mRNA (*LHCGR*: Region 1) Interaction Sites / Сайты взаимодействия miR-4739-мРНК (*LHCGR*: область 1)



After Region 1 1 nt-500 nt / После области 1 1 нт-500 нт

Figure 6. Surface representation of docked complex – miR-4739-R1 of luteinizing hormone choriogonadotropin receptor (LHCGR) indicating stable interactions between miR-4739 and region 1 of *LHCGR*: **(a)** surface representation of docked complex; **(b)** miRNA is shown in light pink color while mRNA is represented in light purple however miRNA binding sites and mRNA binding sites are indicated in hot pink and dark blue colors respectively; **(c)** polar contacts between ligand (miRNA) and receptor (mRNA) are shown in yellow dotted lines [drawn by the authors].

Рисунок 6. Изображение поверхности пристыкованного комплекса – miR-4739-R1 рецептора лютеинизирующего гормона/хориогонадотропина (LHCGR) указывает на стабильные взаимодействия между miR-4739 и областью 1 *LHCGR*: **(a)** изображение поверхности пристыкованного комплекса; **(b)** микроРНК показана светло-розовым цветом, мРНК – светло-фиолетовым цветом; сайты связывания микроРНК и сайты связывания мРНК обозначены ярко-розовым и темно-синим цветом соответственно; **(c)** полярные контакты между лигандом (микроРНК) и рецептором (мРНК) показаны желтыми пунктирными линиями [рисунок авторов].

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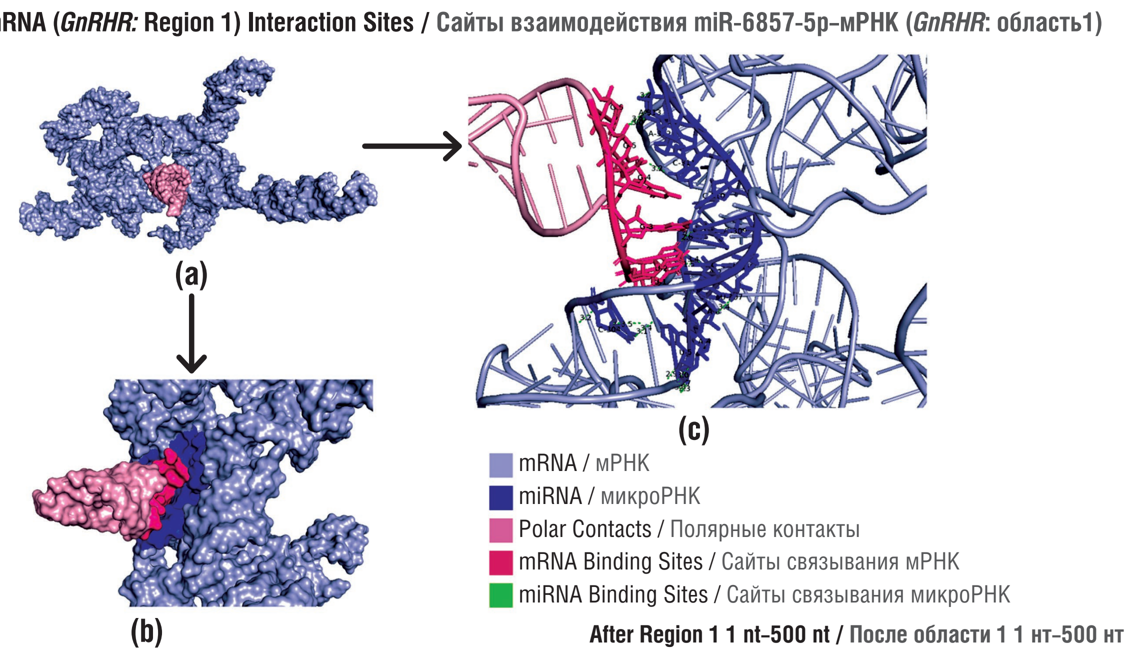

Figure 7. Surface representation of docked complex – miR-6857-R1 of gonadotropin-releasing hormone receptor (GnRHR) indicating stable interactions between miR-6857 and region 1 of *GnRHR*: **(a)** surface representation of docked complex; **(b)** miRNA is shown in light pink color while mRNA is represented in light purple however miRNA binding sites and mRNA binding sites are indicated in hot pink and dark blue colors respectively; **(c)** polar contacts between ligand (miRNA) and receptor (mRNA) are shown in yellow dotted lines [drawn by the authors].

Рисунок 7. Изображение поверхности пристыкованного комплекса – miR-6857-R1 рецептора гонадотропин-рилизинг гормона (GnRHR) указывает на стабильные взаимодействия между miR-6857 и областью 1 *GnRHR*: **(a)** изображение поверхности пристыкованного комплекса; **(b)** микроРНК показана светло-розовым цветом, мРНК – светло-фиолетовым цветом; сайты связывания микроРНК и сайты связывания мРНК обозначены ярко-розовым и темно-синим цветом соответственно; **(c)** полярные контакты между лигандом (микроРНК) и рецептором (мРНК) показаны желтыми пунктирными линиями [рисунок авторов].

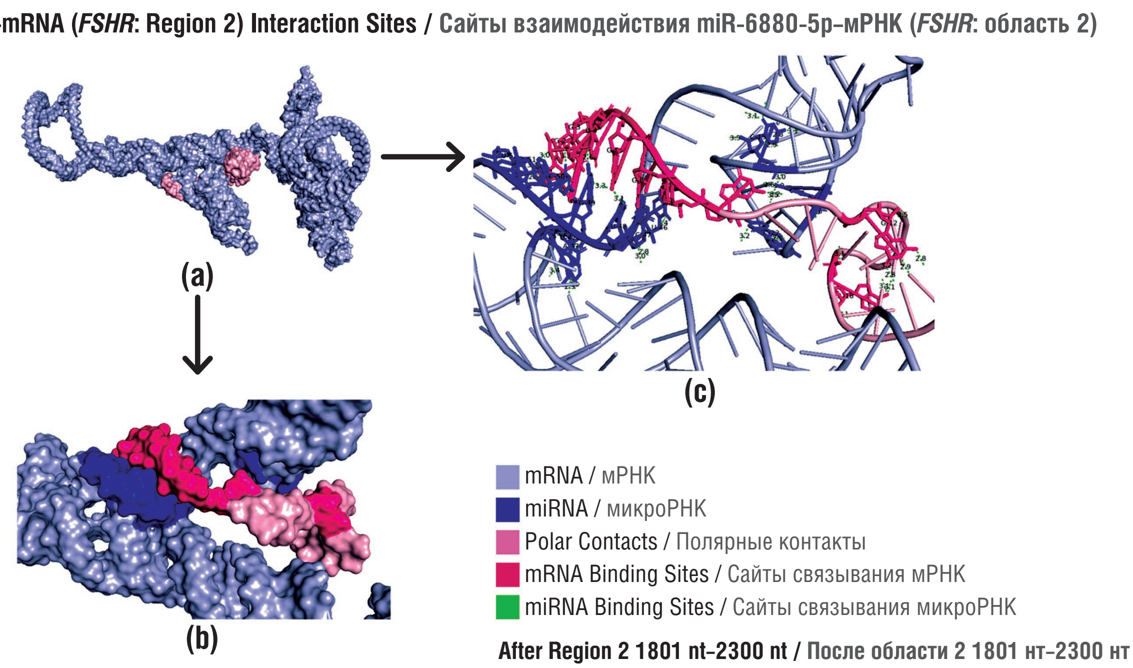

Figure 8. Surface representation of docked complex – miR-6880-R2 of follicle-stimulating hormone receptor (FSHR) indicating stable interactions between miR-6880 and region 2 of *FSHR*: **(a)** surface representation of docked complex; **(b)** miRNA is shown in light pink color while mRNA is represented in light purple however miRNA binding sites and mRNA binding sites are indicated in hot pink and dark blue colors respectively; (c) polar contacts between ligand (miRNA) and receptor (mRNA) are shown in yellow dotted lines [drawn by the authors].

Рисунок 8. Изображение поверхности пристыкованного комплекса – miR-6880-R2 рецептора фолликулостимулирующего гормона (FSHR) указывает на стабильные взаимодействия между miR-6880 и областью 2 *FSHR*: **(a)** изображение поверхности пристыкованного комплекса; **(b)** микроРНК показана светло-розовым цветом цвет, мРНК – светло-фиолетовым цветом; сайты связывания микроРНК и сайты связывания мРНК обозначены ярко-розовым и темно-синим цветом соответственно; **(c)** полярные контакты между лигандом (микроРНК) и рецептором (мРНК) показаны желтыми пунктирными линиями [рисунок авторов].

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miR-4769-5p-mRNA (*CFTR*: Region 2) Interaction Sites / Сайты взаимодействия miR-4769-5p-мРНК (*CFTR*: область 2)

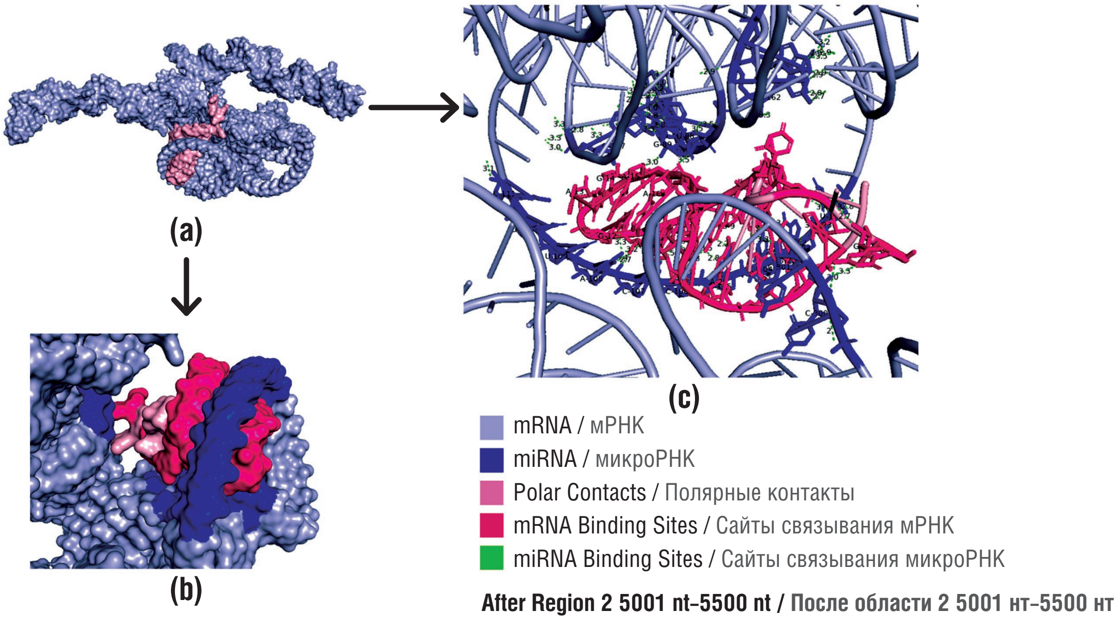

Figure 9. Surface representation of docked complex – miR-4769-R2 of cystic fibrosis transmembrane conductance regulator (CFTR) indicating stable interactions between miR-4769 and region 2 of *CFTR*: **(a)** surface representation of docked complex; **(b)** miRNA is shown in light pink color while mRNA is represented in light purple however miRNA binding sites and mRNA binding sites are indicated in hot pink and dark blue colors respectively; **(c)** polar contacts between ligand (miRNA) and receptor (mRNA) are shown in yellow dotted lines [drawn by the authors].

Рисунок 9. Изображение поверхности пристыкованного комплекса – miR-4769-R2 регулятора трансмембранной проводимости при муковисцидозе (CFTR) указывает на стабильные взаимодействия между miR-4769 и областью 2 *CFTR*: **(a)** изображение поверхности пристыкованного комплекса; **(b)** микроРНК показана светло-розовым цветом, мРНК – светло-фиолетовым цветом; сайты связывания микроРНК и сайты связывания мРНК обозначены ярко-розовым и темно-синим цветом соответственно; **(c)** полярные контакты между лигандом (микроРНК) и рецептором (мРНК) показаны желтыми пунктирными линиями [рисунок авторов].

miR-4769-5p-mRNA (*CFTR*: Region 2) Interaction Sites / Сайты взаимодействия miR-4769-5p-мРНК (*CFTR*: область 2)

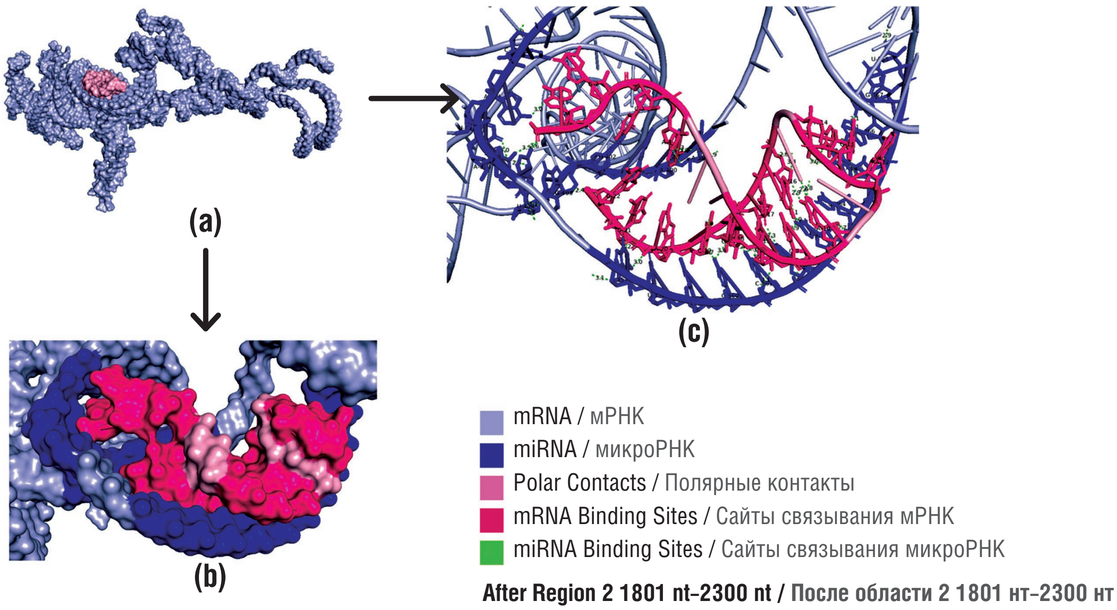

Figure 10. Surface representation of docked complex – miR-2467-R2 of follicle-stimulating hormone receptor (FSHR) indicating stable interactions between miR-2467 and region 2 of *FSHR*: **(a)** surface representation of docked complex; **(b)** miRNA is shown in light pink color while mRNA is represented in light purple however miRNA binding sites and mRNA binding sites are indicated in hot pink and dark blue colors respectively; **(c)** polar contacts between ligand (miRNA) and receptor (mRNA) are shown in yellow dotted lines [drawn by the authors].

Рисунок 10. Изображение поверхности пристыкованного комплекса – miR-2467-R2 рецептора фолликулостимулирующего гормона (FSHR) указывает на стабильные взаимодействия между miR-2467 и областью 2 *FSHR*: **(a)** изображение поверхности пристыкованного комплекса; **(b)** микроРНК показана светло-розовым цветом, мРНК – светло-фиолетовым цветом; сайты связывания микроРНК и сайты связывания мРНК обозначены ярко-розовым и темно-синим цветом соответственно; **(c)** полярные контакты между лигандом (микроРНК) и рецептором (мРНК) показаны желтыми пунктирными линиями [рисунок авторов].

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miR-211-3p-mRNA (*FSHR*: Region 2) Interaction Sites / Сайты взаимодействия miR-211-3p-мРНК (*FSHR*: область 2)

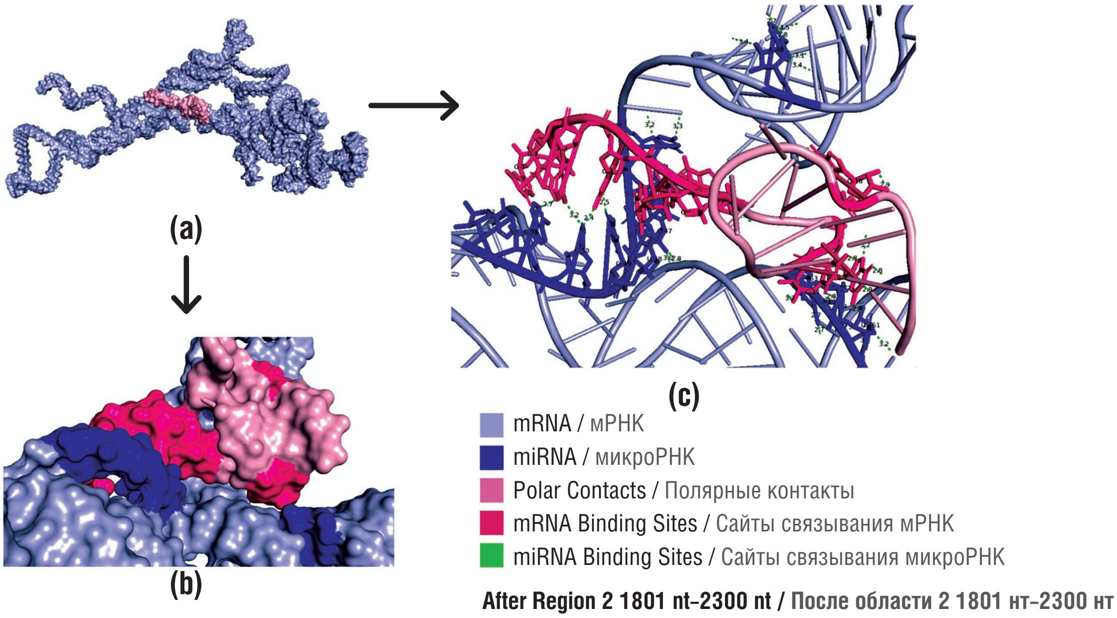

Figure 11. Surface representation of docked complex – miR-211-R2 of follicle-stimulating hormone receptor (FSHR) indicating stable interactions between miR-211 and region 2 of *FSHR*: **(a)** surface representation of docked complex; **(b)** miRNA is shown in light pink color while mRNA is represented in light purple however miRNA binding sites and mRNA binding sites are indicated in hot pink and dark blue colors respectively; **(c)** polar contacts between ligand (miRNA) and receptor (mRNA) are shown in yellow dotted lines [drawn by the authors].

Рисунок 11. Изображение поверхности пристыкованного комплекса – miR-211-R2 рецептора фолликулостимулирующего гормона (FSHR)указывает на стабильные взаимодействия между miR-211 и областью 2 *FSHR*: **(a)** изображение поверхности пристыкованного комплекса; **(b)** микроРНК показана светло-розовым цветом, мРНК – светло-фиолетовым цветом; сайты связывания микроРНК и сайты связывания мРНК обозначены ярко-розовым и темно-синим цветом соответственно; **(c)** полярные контакты между лигандом (микроРНК) и рецептором (мРНК) показаны желтыми пунктирными линиями [рисунок авторов].

and congenital factors (genetic factors) [3]. Among various risk factors, genetic factors majorly Y chromosome microdeletions and single gene defects contribute largely to male infertility with a high incidence rate of 15 to 30 % [6]. Gonadotropins (fertility-regulating genes) namely *LHCGR, GnRHR, FSHR* in addition to wild *CFTR* have been reported to be responsible for normal regulation of the reproductive system. However dysregulation, particularly underexpression of aforestated genes may lead to infertility causing abnormalities in sperm parameters and reduced level of testosterone [11]. OncomiRs (upregulated miRNAs) are known hallmarks of male infertility causing downregulation of essential gonadotropins and have been investigated as disease biomarkers [14]. Considering the role of dysregulated gonadotropins as a consequence of interacting oncomiRs, miRNA-mRNA interactions are required to be identified and analyzed.

The miRNA-mRNA interaction analysis identified potential oncomiRs indicating strong interactions with fertility-related genes considerably *LHCGR, GnRHR, FSHR* and *CFTR*. Among miRNA-mRNA interactions, 10 strong interactions were observed for miRNAs-LHCGR, 13 against miRNAs-GnRHR, 31 for miRNAs-FSHR and 18 for miRNAs-CFTR indicating the binding potential of shortlisted microRNAs on aforesaid genes. Additionally binding sites were identified, elucidating that mutations in the binding sites may alter its target specificity and its subsequent impact on gene regulation [36]. However significant interactions between shortlisted miRNAs and fertility-related genes were identified using RNA22 tool that depicted 6, 18, 55 and 17 significant interactions for miRNAs-LHCGR, miRNAs-GnRHR, miRNAs-FSHR and miRNAs-CFTR respectively. RNA22 selects miRNAs on the basis of folding energy and p-value representing significant interactions. It has been reported that the most important factor for the significant miRNA-mRNA interaction is having the perfect homology between the seed sequence of miRNA and the corresponding target gene sequence, that can be acquired through RNA22 [37].

For LHCGR, interacting miRNAs majorly miR-3135b, miR-4739, miR-6777-5p were found to be associated with male infertility and related cancers. Dysregulated expression of predicted miR-3135b has been observed in pancreatic ductal adenocarcinoma causing tumor development and progression [38]. Several studies have confirmed that male infertility has been linked with the emergence of various malignancies majorly breast cancer, pancreatic cancer and testicular germ cell cancer [39, 40]. Thus predicted miR-3135b can be used as a promising target for early diagnosis and prognosis of male infertility. While miR-4739 has been associated with spermatogenic impairment in the male Korean population elucidating its potential interaction with stromal antigen 3 (STAG3), however mutations in *STAG3* effect its protein

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function leading to male infertility due to meiotic arrest [41]. It was observed that dysregulation of miR-6777 negatively modulates LINC00355:8 that in turn activates the Wnt/β-catenin signaling pathway and induces epithelial-mesenchymal transition (EMT) progression leading to hepatocellular carcinoma [42]. However hypogonadism is commonly found in patients having chronic liver disease thus considering the involvement of liver malignancies in inducing male infertility, miR-6777-5p may be considered a diagnostic biomarker and therapeutic target.

For miRNAs-GnRHR interactions, male infertility-related microRNAs have been identified including miR-4749, miR-4685-5p and miR-8069. The oncogenic function of miR-4749 was observed in impairment of tumor suppressor gene p53 that affects DNA binding function bringing about tumor progression and invasiveness [43]. Moreover oncogenic role of another predicted oncomiR miR-4685-5p has been identified in early stage lung adenocarcinoma [44] that reveals that miR-4685-5p may act as a potential signature for early diagnosis and prognosis of male infertility having correlation with CFTR mutations in lung cancer that modulate the risk of male infertility [45]. Elevated level of miR-8069 has been observed in pancreatic cancer that shows association with male infertility suggesting its oncogenic role [46].

For miRNAs-FSHR interactions, miR-762 has been known for its crucial role in mammalian reproduction. It interacts with 3 untranslated region (UTR) of DNA-damage related gene called RNF4 and alters its expression inducing growth and proliferation of porcine immature Sertoli cells inhibits apoptosis by activating DNA damage repair and minimizing the expression of androgen receptor [47]. Another study has confirmed the oncogenic role of miR-762 in non-obstructive azoospermia (NOA) as compared to fertile men, in which overexpression of miR-762 has been associated with NOA disease [48]. The underexpression of predicted miR-296 has been reported in highly fertile men suggesting that overexpression of miR-296 leads to infertility [49]. Additionally, elevated level of miR-296 expression has been also predicted in patients diagnosed with several classes of male factor infertility particularly teratozoospermia, asthenozoospermia, oligozoospermia and normozoospermia indicating characteristic role of miR-296 in inducing infertility [50]. Among impaired spermatogenesis cases of male Korean population, miR-4739-STAG3 interaction has been observed however *STAG3* gene is a known hallmark of male factor infertility therefore predicted miR-4739 can be used as therapeutic target against male infertility [41].

For miRNAs-CFTR interactions, a study investigated the function of miR-145 by predicting its interacting genes in patients with spermatogenesis. It was observed that overexpression of miR-145 downregulates expression of male sex-determining gene *SOX9* that in turn induces male fertility. Therefore the oncogenic role of miR-145 reveals its diagnostics and prognostic value as an infertility biomarker [51]. miR-1471 was found to be overexpressed in patients with sertoli-cell-only syndrome that is characterized by azoospermia therefore miR-1471 can be used as a promising strategy against male infertility [52]. Overexpression of miR-1343 has been identified in capacitated spermatozoa through expression profiling study of capacitated spermatozoa and non-capacitated spermatozoa [53]. However, capacitated spermatozoa refers to essential physiological changes that are required for spermatozoa to undergo therefore miR-1343 can be used as a therapeutic target for mitigating the effect of infertility. Hence predicted microRNAs have shown significant association with male infertility and can be used as therapeutic targets for early diagnosis and prognosis of male infertility.

The mRNA regions of fertility-related genes were shortlisted with respect to maximum binding interactions with miRNAs that resulted in 2 regions for *LHCGR, GnRHR, FSHR* and 3 regions for *CFTR*. It was observed that *LHCGR, GnRHR, FSHR* and *CFTR* showed maximum binding interactions with 3 miRNAs, 5 miRNAs, 21 miRNAs and 8 miRNAs respectively. 3D structures of shortlisted microRNAs and mRNA regions were docked through HDOCK to determine the best conformation of the docked complex in terms of high stability. Molecular docking generated 5 complexes for miRNAs-LHCGR, 7 complexes for miRNAs-GnRHR, 24 complexes for miRNAs-FSHR and 10 complexes for miRNAs-CFTR. However, only the top complex with respect to highest confidence score and least affinity score was considered for further analysis. All of the shortlisted complexes namely miR-4739-LHCGR(R1), miR-6857-5p-GnRHR(R1), miR-6880-FSHR(R2) and miR-4769-5p-CFTR(R2) revealed interaction of miRNAs with coding sequence (CDS) region of respective genes. Moreover top 3 complexes particularly miR-6880-FSHR(R2), miR-2467-3p-FSHR(R2), miR-211-3p-FSHR(R2) were shortlisted from combined docking results containing all shortlisted complexes making up a total of 46 complexes. Among shortlisted complexes, miRNA interaction was observed at the CDS region of corresponding genes. It has been reported that binding regions particularly 3 UTR, 5 UTR and CDS have functional impact on regulatory activity of microRNAs for instance miRNA interaction with 3 UTR region of gene causes mRNA degradation while miRNA binding with 5 UTR or CDS region of genes is responsible for translational repression through mRNA silencing [54]. Therefore, mRNA binding sites are required to be identified in order to predict functional characteristics of miRNAs in male infertility.

In this study binding affinities of shortlisted microRNAs were identified against fertility-related genes majorly *LHCGR, GnRHR, FSHR* and *CFTR*. Out of all individual and combined complexes, miR-6880-FSHR(R2) was found to be a highly stable complex indicating least binding affinity (-566.3) and high confidence score (0.999); however miR-4769-5p-CFTR(R2) exhibited high number of inter-

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actions at 46 different sites comparatively with docking score of -494.32 and confidence score of 0.999. Hence strong and potential interactions between miRNAs and mRNAs have determined the stability and potential efficacy of aforementioned complexes.

Conclusively this research has predicted highly significant and stable interactions between oncomiRs and fertility-regulating genes thus unveil the causative determinants of azoospermia. Additionally microRNA-binding regions of genes indicating significantly high interactions with oncomiRs were identified that determines dysregulated expression of gonadotropins. Regulatory role of predicted microRNAs has been investigated in several malignancies including pancreatic ductal adenocarcinoma, hepatocellular carcinoma, lung adenocarcinoma, teratozoospermia, asthenozoospermia, oligozoospermia, normozoospermia and spermatogenic impairment. Additionally characteristic involvement of gonadotropins in fertility has been reported in the regulation of fertility in males therefore suppressing the role of oncomiRs can alter the spermatogenesis impairment that improves the health of patients. Hence this study proposes a promising strategy to identify disease biomarkers that would mitigate infertility issues in men. However wet lab investigations are required to validate the findings of the proposed study.

Conclusion / Заключение

In this study miRNA-mRNA interaction analysis has revealed significant interactions of 6, 18, 55 and 17 between miRNAs and genes particularly *LHCGR, GnRHR, FSHR* and *CFTR* respectively. Subsequently molecular docking between shortlisted mRNA regions and miRNA generated stable complexes however only top one highly stable conformation and top 3 best poses were shortlisted from individual and combined complexes respectively that disclosed miR-6880-FSHR(R2) as a highly stable complex indicating least binding affinity (-566.3) and high confidence score (0.999). Additionally shortlisted complexes revealed that miRNAs interacted with genes at CDS region indicating translational repression. Thus strong interactions between miRNAs and mRNAs have ensured the stability and potential efficacy of aforementioned complexes. Furthermore oncogenic role of predicted miRNAs has been discovered in fertility-related disorders majorly teratozoospermia, asthenozoospermia, oligozoospermia, normozoospermia and spermatogenic impairment. Therefore it has been inferred from the study that predicted miRNAs may act as oncomiRs suppressing the function of gonadotropins. Hence this study proposes key oncomiRs that can be used as potential candidates for infertility diagnosis and prognosis.

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All authors participated equally in the collection, analysis and interpretation of the data.	Все авторы принимали равное участие в сборе, анализе и интерпретации данных.
All authors have read and approved the final version of the manuscript.	Все авторы прочитали и утвердили окончательный вариант рукописи.
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The authors declare no conflict of interests.	Авторы заявляют об отсутствии конфликта интересов.
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Study protocol, statistical analysis plan, analytic code will be available at methodologically sound request. Requests should be sent to the mailbox ahmed.suleiman@uoanbar.edu.iq. In order to gain access, data requesters will need to sign a data access agreement.	Протокол исследования, план статистического анализа, принципы анализа будут доступны по методологически обоснованному запросу. Предложения должны быть направлены на почтовый ящик ahmed.suleiman@uoanbar.edu.iq. Чтобы получить доступ, лица, запрашивающие данные, должны будут подписать соглашение о доступе к данным.
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About the authors:
Noor A. Oohayyed – Assistant Lecturer, Department of Medical Biotechnology, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq.
Mais M. Mohammed – Assistant Lecturer, Ibn Sina University for Medical and Pharmaceutical Sciences, Baghdad, Iraq. ORCID: https://orcid.org/0000-0003-4455-8674. Scopus Author ID: 57561562000.
Aya M. Al-Rahim – Assistant Lecturer, Department of Medical Biotechnology, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq. ORCID: https://orcid.org/0000-0002-7120-7960. Scopus Author ID: 57226461520. Researcher ID: ACZ-6702-2022.
Rawaa N. AlChalabi – Assistant Professor, Dr, Department of Medical Biotechnology, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq. ORCID: https://orcid.org/0000-0002-1570-2537. Scopus Author ID: 57210340073. Researcher ID: ABD-7380-2020.
Semaa A. Shaban – PhD, Assistant Professor, Department of Biology, College of Sciences, Tikrit University, Tikrit, Saladin, Iraq. ORCID: https://orcid.org/0000-0002-0538-8716. Scopus Author ID: 57223083825. Researcher ID: H-1150-2019.
Ahmed A.J. Suleiman – PhD in Biotechnology and Molecular Biology, Professor, Department of Biotechnology, College of Science, University of Anbar, Ramadi, Anbar, Iraq. E-mail: ahmed.suleiman@uoanbar.edu.iq. ORCID: https://orcid.org/0000-0001-7427-4483. Scopus Author ID: 57200943508. Researcher ID: AAF-7727-2019.

Сведения об авторах:
Нур А. Охайед – ассистент кафедры медицинской биотехнологии Колледжа биотехнологии, Университет Аль-Нахрейн, Багдад, Ирак.
Маис М. Мохаммед – ассистент, Университет медицинских и фармацевтических наук Ибн Сины, Багдад, Ирак. ORCID: https://orcid.org/0000-0003-4455-8674. Scopus Author ID: 57561562000.
Ая М. Аль-Рахим – ассистент кафедры медицинской биотехнологии Колледжа биотехнологии, Университет Аль-Нахрейн, Багдад, Ирак. ORCID: https://orcid.org/0000-0002-7120-7960. Scopus Author ID: 57226461520. Researcher ID: ACZ-6702-2022.
Раваа Н. АльЧалаби – доктор наук, доцент кафедры медицинской биотехнологии Колледжа биотехнологии, Университет Аль-Нахрейн, Багдад, Ирак. ORCID: https://orcid.org/0000-0002-1570-2537. Scopus Author ID: 57210340073. Researcher ID: ABD-7380-2020.
Семаа А. Шабан – доктор наук, доцент кафедры биологии Колледжа наук, Университет Тикрита, Тикрит, Саладин, Ирак. ORCID: https://orcid.org/0000-0002-0538-8716. Scopus Author ID: 57223083825. Researcher ID: H-1150-2019.
Ахмед А.Дж. Сулейман – доктор наук (биотехнология и молекулярная биология), профессор кафедры биотехнологии Колледжа наук, Анбарский университет, Рамади, Анбар, Ирак. E-mail: ahmed.suleiman@uoanbar.edu.iq. ORCID: https://orcid.org/0000-0001-7427-4483. Scopus Author ID: 57200943508. Researcher ID: AAF-7727-2019.

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Информацию о репринтах можно получить в редакции. Тел.: +7 (495) 649-54-95; эл. почта: info@irbis-1.ru.