Study of Small Non-Coding RNA (miRNA) Expression Pattern of Fertile/Infertile Male Semen

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ABSTRACT

Background: Infertility is a serious health issue that affects people all around the world. One of the most common reasons for male infertility is sperm abnormalities. Researchers and scientists have been searching for a novel genetic marker to detect or recognize the genetic malfunction that causes sperm abnormalities. Micro-RNA (miRNAs) are small non-coded RNA molecules that present intra and extra-cellular and regulate gene expression.

Objective: This study began to search for a relation between miRNA expression levels and other diseases that may be related to them, considering that the main role of miRNAs was the down-regulation of genes.

Methods: The main technique used in this study was to synthesize a complementary DNA (cDNA) (reverse transcription method) of extracted total RNA by TRIzol then amplification of candidates’ miRNAs genes by Reverse Transcriptase Quantitative Polymerase Chain Reaction RT-qPCR.

Results: Studies found that miRNAs have a role in defining sperm qualities such as sperm count, motility, and shape. In this study, we chose the most miRNAs referred to in the previous study as a potential seminal fluid marker (miR-10a, miR-10b, miR-135a and miR-135b) to test them as potential infertility-related miRNAs markers (Asthenospermia AS, Oligospermia OS, Astheno-Oligospermia ASOS) in addition to health normal sperm NS.

Conclusion: The main aim of this study was to find the miRNAs expression pattern to find a way to help scientists track the genetic causes of male infertility issues and a novel method to distinguish infertility genetically diseases. The findings may serve as a potential genetic marker for male infertility and provide a background for future research that targeted miRNAs as a molecular marker for medical and forensic fields, also as an infertility disease potential treatment.

Keywords: MicroRNAs, Infertility, miRNAs expression, Spermatogenesis, Spermatogonial stem cells SSCs.

1. BACKGROUND

Micro RNA is tiny in size (18-23 nucleotides), tissue-specific expression, and non-coded RNA molecules, and their main role is expression regulation. It constitutes a kind of molecular marker for tissue and body fluids identification. Several studies have demonstrated that semen can be distinguished from other related body fluids by employing semen-specific miRNAs evaluated by RT-PCR. Infertility becoming a global health problem, affecting 15%–23% of couples globally, with half of these cases, accounting for male factors (1).

Micro RNA which is composed mainly of 18-23 nucleotides, regulate gene expression in a range of biological processes (2), by attaching to the 3'OH untranslated region of targeted mRNAs inhibiting translation and/or triggering mRNAs degradation acting as a node blocking ribosome from continuing translation moreover, miRNAs are highly conserved molecules among species due to its essential role in many biological processes like cell proliferation, cell differentiation, protein synthesis, etc. (3, 4).

Due to infertility becoming more and more common, biomedical experts found themselves obligated to investigate the influence of infertility on a molecular level, also the forensic experts to find a way to distinguish between...
semen fluids at the crime scene, as it can contribute significantly as evidence of conviction if the semen is distinguished if it belongs to a healthy person or suffers from infertility issues, and this has a role in reducing the number of suspects and restricting it to a specific category, especially in Cases of proving parentage or rape crimes (5, 6).

According to statistics, ‘male-factor infertility’ accounts for 40%-50% of infertility couples’ challenges, affecting one-sixth of all couples globally and the molecular pathways causing these issues to remain unclear (7).

Spermatogenesis is a highly complex and regulated process that produces millions of sperms daily, several pieces of evidence have shown that miRNA play important role in spermatogenesis during mitotic and meiotic stages of spermatogenesis through the regulation of targeted gene expression (8). An RNase III endonuclease enzyme called Dicer plays an essential role in miRNAs biogenesis, previous studies improve that the testes reduced their size and that the spermatogenesis process was delayed or negatively affected after Dicer removal (9). These studies also proved that the Dicer removal at the early stages of development of germ cell line led to producing infertile sperms, as a result of multiple defects effect directly on meiotic division (10), meiosis of spermatogenesis was delayed, the number of successfully divided sperms cells was decreased, apoptotic spermatocytes increased and the matured successfully sperms created were decreased dramatically with abnormal motility and morphological defects (9, 10).

There are more than 780 known miRNAs found expressed in testes and more than 264 miRNAs found in seminal fluid (11), but not all these miRNAs have an essential role in spermatogenesis, several studies mentioned that several miRNAs interfere with the spermatogenesis process such as miR-20 and miR-106a which influence differentiation of spermatogonia stem cells (SCCs) at the post-transcriptional level by targeting Ccnd1 and Stat3 genes (12), miRNA miR-135a interfere spermatogonia stem cells maintenance via Foxol activity modulation (13), also miR21 which regulate the ETV5 transcriptional factors via SSCs process, so miR21 play important role in SSCs process main maintenance (14). Other miRNAs mentioned in previous studies that play role in the spermatogenesis process are listed in Table 1.

miR-135b and miR-135a were studied well by several studies and their role in spermatogenesis was explained intensively (22, 24), miR-135b along with miR-205 and miR-146a play important role in the regulation of sperm maturation, development, motility, proliferation and survival, by effects on the regulation of Foxol gene which localized to spermatogonia stem cells, forehead box protein O1 Foxol act as a transcriptional factor increased in the stem cell nucleus and deceased in undescended testes (25), while miR-20, miR-17 and miR-209 play important role in the regulation of Signal Transducer and Activator of Transcription (Stat3) in Sertoli cells gene which responsible for the majority of transducing of cytokine for somatic cyst cell regulation (26).

Huszar and colleagues reported that the miR-146 was highly expressed in undifferentiated sperm cells which means that miR-146 downregulates the regulation of retinoid spermatogonia (27), many miRNAs were mentioned by many studies especially miR-153a and miR-153b but poor knowledge about miR-10a and miR10b although the fact that the miR-10 was reported by many studies as a unique miR-10 along with other miRNAs for seminal fluids and vary in expression between infertile sperm that refers to its role in the spermatogenesis process (28, 29).

Forensically, biological samples such as blood, menstrual blood, seminal fluid, vaginal secretions, saliva etc., in crime scenes, play an important role and may carry the convincing evidence depending on the genetic material that these fluids carry inside (30, 31), however, these samples may not be in the best condition, intangible and unaffected by many factors, including heat, humidity and light, which have a significant negative effect on the DNA, especially in cases where crime scenes have occurred for a long time before it was reported, in these cases, the genetic material is often completely or partially destroyed and it is very difficult to adopt it as evidence of conviction through DNA fingerprinting, but several studies have proven the effectiveness of miRNA as forensic evidence that can remain intact for three years (32, 33).

Also, many studies have demonstrated the specificity of

| Micro RNA name | Predicted target gene-related to spermatogenesis | Function | References |
|----------------|-----------------------------------------------|---------|-----------|
| miR-376a | CDK2, ACO2 | Germ cell morphology, sperm apoptosis | (15) |
| miR-367 | RUNX1, SMAD6, LATS2 | Germ cell line Proliferation and survival | (16) |
| miR-17 | Bcl2l11, Socs3, Stat3 | The proliferation of germ cell line | (17) |
| miR-146 | Med1 | Cell adhesion | (18) |
| miR-21 | ETV5 | ETV5, Essential transformation transcriptional factor family, effect Proliferation and invasion | (19) |
| miR-302 | NRF2F2 | Transcriptional regulation in haploid germ cell line | (20) |
| miR-290 | Stat3, Ccnd1 | Spermatogenesis renewing and maintenance | (21) |
| miR-135a | Foxo1 | Foxo1, Forehead box protein O1, affect germ cell line Proliferation and survival | (16) |
| miR-106 | Bcl2l11, Socs3, Stat3, Ccnd1 | The proliferation of germ cell line | (17) |
| miR-181a | CDK11B, CDK9 | Cyclin-dependent kinase 1B transcription factor synthesis affects SSC differentiation and programmed apoptosis | (22) |
| miR-15b | IDH3A | Sperm motility and morphology | (22) |
| miR-7-3p | PIK3R3 | Spermatозoon development and differentiation | (22) |
| miR-34a | NOTCH1 | Germ cell line differentiation and survival | (23) |
| miR-141 | CB1, TGF8 | Germ cell line differentiation and programmed apoptosis | (22) |
| miR-20 | Ccnd1, Stat3 | Spermatogenesis renewing and maintenance | (21) |

Table 1: Micro-RNAs and their predicted target genes which interfere with the spermatogenesis process and its function.
miRNAs in aged and damaged DNA materials’ body fluids even in post-mortem cases, however, most forensic studies have been focused on identifying normal physiological fluids but have not considered the investigation of abnormalities in seminal fluids in infertility diseases (34). Forensic investigators encounter a variety of unresolved issues: First, is infertile sperm’s expression identical to that of normal sperm? If not, how does this affect sperm’s capacity to be distinguished from other body fluids? Second, given the availability of infertile sperm samples at the crime scene, might the body fluids identification range be utilized to detect abnormal sperm? (35).

2. OBJECTIVE

This study aimed to determine if the four miRNAs that separate sperm from other forensics-related body fluids are the most appropriate and best markers for recognizing sperm when infertile samples are detected on the forensic scene. It may also give some information to medical experts investigating reproductive issues at the genetic level, this research looked at normal sperm (NS) and three forms of infertile sperm samples: asthenospermia (AS), oligospermia (OS), and asthenospermia plus oligospermia (ASOS).

The levels of expression of four semen-specific miRNA markers (miR-10a, miR-10b, miR-135a, and miR-135b) were determined using a real-time quantitative RT-PCR technique with a Bright green fluorescent dye.

3. MATERIAL AND METHODS

Samples collection and preparation

Seminal fluids samples were collected from healthy males and males with infertility diseases such as Asthenospermia AS, Oligospermia OS and Asthenospermia Oligospermia ASOS, 120 male subjects (30 samples for each group) donated freshly ejaculated seminal fluids (2ml) in sterile sealed falcon tubes, which were subsequently transferred to sterile cotton swabs.

Every volunteer has given written informed permission. This research received ethical approval (DSM/HO-16642) for scientific research from the Ministry of Health MOH and Ministry of Higher Education and Scientific Research MOHESR ethics committees in Iraq.

Table 2. The following is a list of previously described semen-specific miRNAs with their sequences.

| miRNA name | Sequences of mature miRNAs | References |
|------------|-----------------------------|------------|
| has-miR-10b | UACCCUGUAAUCGGAUUUGUG       | (38)       |
| has-miR-135a | UAUGGCUUUUAUUCUUAUGUGA      | (35)       |
| has-miR-135b | UAUGGCUUUUAUUCUUAUGUGA      | (30, 39)  |
| has-miR-10a | UACCCUGUAAUCGGAUUUGUG       | (40)       |
| RNU6-2     | GTGTCGCTCTCGCAGCCACATCTA    | (40)       |

Health Organization WHO laboratory manual, all primary semen parameters were evaluated using the micro-cell site and computer-aided semen analysis (WHO, 2010. (CASA, SSA-II) formalized paraphrase.

Micro-RNAs selection

miR-10a, miR-10b, miR-135a and miR-135b were chosen in this study depending on the variety in their expression in seminal fluids between infertile disease, also for their uniqueness as a seminal fluid’s biomarker, rather than their role in interfering with several spermatogenesis pathways (24, 36).

RNU6-2 chose as a reference gene for normalizing miRNAs expression in all samples, sequences of four candidate miRNAs genes and RNU6-2 information listed in Table 2, these sequences served as forwarding primers while the reverse primer was a universal RT-primer for all miRNAs genes which added poly-Adenosine at the end of mature miRNA sequence during the complementary DNA synthesis (37).

Total RNA Extraction and purity determination

According to manufacturer’s instructions, the total RNA was extracted by RNAzol RT reagent (Sigma-Aldrich/USA). Integrity and yields of RNA were measured by micro/nano spectrophotometer (Biodrop/UK) to determine sample concentration and purity at 260/280 nm.

Complementary DNA synthesis

Complementary DNA was obtained by two methods: 2-steps to perform Polymerase Chain Reaction PCR which includes synthesizing complementary DNA by All-In-One cDNA synthesis kit from abm/USA then performed PCR step in another separated step, and 1-step RT-qPCR which performed each cDNA synthesis and polymerase chain reaction process in the same step.
Reverse Transcription Polymerase Chain Reaction RT-qPCR

RT-qPCR was performed by Bright-Green qPCR Master Mix (abm/USA). RT-qPCR test was carried out by Exicyc-lerTM 96 Thermocycler (pioneer/USA) the expression level of all samples and reference genes were assessed in triplicates. RT-PCR mixture content of 20 µl reaction volume as 300nM of concentration of primers and 3 µl of RNA templates, PCR program set at 95°C for 10 min. before being cycled 40 times at 95°C for 10 sec. as denature temperature, then 63°C for 15 sec. as annealing step, then 72°C for 30 sec. extension step.

Bioanalytical statistics

Expression level calculated according to Pfaffl analysis (41), which was carried out by calculating Cycle Threshold CT value of each candidate miRNAs genes in addition to reference gene in each sample, reference gene was acquired for qPCR normalization by subtracting CT value of the corresponding miRNA from the reference gene (RNU6-2) CT value for each sample as follows:

\[ \Delta Ct = \text{CT (targeted miRNA)} - \text{CT (Endogenous control or reference gene (RNU6-2))} \]

Then calculate \( \Delta Ct \) value for each candidate miRNAs as the following equation:

\[ \Delta \Delta Ct = (\Delta Ct \text{ sample}) - (\Delta Ct \text{ control}) \]

To find the Relative Quantification RQ or gene fold (expression) as follow:

\[ \text{RQ} = 2^{-\Delta \Delta Ct} \]

IBM (version 21.0 for Windows, SPSS, Chicago, IL, USA) and Prism were used to compute and evaluate Ct values’ mean and standard error (version 5, GraphPad software). RNU6-2 was used to compare the investigated miRNA targets, and P-value 0.05 was regarded as statistically significant (42).

4. RESULTS AND DISCUSSION

CT and CT value of Micro-RNA analysis

RNU6-2 was used to compare miRNAs candidate genes, and the average CT value of triplicates was calculated. Table 3 shows the mean and standard deviation SD of CT and \( \Delta \text{CT} \) of candidate miRNAs (miR-10a, miR-10b, miR-135a and miR-135b) in each Asthenospermia AS, Oligospermia OS, Astheno-Oligospermia ASOS and Normal spermen NS, to determine the samples’ relative quantification, a housekeeping gene or reference gene was employed for normalization the gene expression (41). The results show that the miR-10b, miR-135b, miR-10a and miR-135a have stable and normal CT values in normal sperm about 23-24 CT value, while miR-135a CT value decreased in asthenospermia AS and oligospermia OS (19.45 and 13.18, respectively), and as we know the relation between CT value and expression level is an inverse relation, in another word as CT value increased as expression level will be decreased and vice versa, due to the CT value refer to the number of cycles needed by targeted gene to emit fluorescent signal can be detected by a UV detector. Whereas, the miR-135b CT decreased widely in ASOS samples. Figure 1 depicts a scatter plot of candidate \( \Delta \text{CT} \) miRNAs values in all samples (fertile and infertile samples).

Level of expression of miRNAs in four fertile and infertile seminal fluids

The expression level or gene fold of candidate miRNAs showed in Figure 2. The results show significant differences between four miRNAs expression between samples at a P-value of 0.05. Furthermore, as shown in Figure 2, candidate miRNAs were subsequently down-regulated and up-regulated in infertile semen samples compared to normal semen NS, Tian and colleagues identified miR-135b, miR-135a and miR-888 downregulation (43). The same results were obtained by Moritoki and colleagues, a significant decrease in expression of miR-135a in ASOS patients referred to the direct effect of miR-135a on the expression of Foxol (25).

miR-135 family (which include 135a, 135b, 135a-3p and 135-3p) have been found generally highly expressed in infertile seminal fluids, which means there is an mRNA gene that will be inhibited or down-regulated by miR-135a, and since the expression of miR-135a was high in each AS and OS cases separately, and since the defect of low sperms number occurred as a result of OS case, and the defect of abnormal shape of sperms occurred as a result of AS case, that’s mean the miR-135a role for OS case, particularly in the spermatogenesis process and for AS case may occur in spermato-
genesis but stages of sperm construction, in another word, effect on transcriptional factors coded genes (44). Previous studies referred to down-regulate of miR-135a to Foxo1 which localized to spermatogonia stem cells, forehead box protein O1, Foxo1 acts as a transcriptional factor increased in the stem cell nucleus and deceased in undescended testes to regulate the sperm maturation, capacitation, and motility (25). While in AS case the miR-135a and miR-135b down-regulate Ccnd1 and Stat3 genes, Ccnd1 also known as Cyclin D1 which is responsible for cell cycle progression, Denise and colleagues found the blocked or lack of Ccnd1 lead to failure of germ cells to enter G1/G0 phases during mitotic: meiotic switch, and arrested at this stage, lead to decreased number of sperms (45). Stat3, known as Signal Transducer and Activator of Transcription, plays an important role in Sertoli cell signalling, Sertoli cells, which are epithelial supporting cells of seminiferous tubules where mitosis and meiosis’s division occur to produce haploid gametes, blocking the Stat3 gene lead to sperm undifferentiation (26).

**Micro RNA expression pattern as a molecular maker in forensic science.**

Forensic scenes carry important evidence especially the biological pieces of evidence due to the fact of each biological residue may contain a DNA molecule that carries a lot of information about the crime but these DNA molecules are affected by many factors like temperature, humidity and the time consumed until the crime reported, all these factors may lead to destructive effect to DNA, so miRNA reported as very conservative and can be resistance very long time up to 2 years (46). So, miRNAs, in this case, play an essential role in crime scenes as a witness and at least miRNA can limit the circle of suspects, according to this study we target crime scenes when the seminal fluids are found as evidence (Rape crime, Sexual assault, Paternity proof and Sexual abuse of minors) to prove the alleged testimonies by the perpetrator or the victim, in many cases, a female is framed as having sexually assaulted, and she may now be pregnant and demand financial compensation from that person or really being sexually assaulted. Here comes the role of miRNAs by proving that the suspect is fertile or infertile by taking samples from the neck of the vagina (since the semen can still be extracted and the sperm are still present, since the sperm may remain from two to three days inside the vagina). If the suspect suffers from a fertility disease and the accusation against him is malicious. But if the result indicates that the suspect is fertile, then at least the gene expression pattern of several miRNAs is adopted to prove that the semen present at the crime scene corresponds to the semen of the suspects depending on the pattern of expression of miRNAs genes. This idea still needs extensive studies to prove the possibility of adopting it as forensic evidence or vice versa, as the results in this study showed a large variation between each sample and another in the gene expression of each miRNA and between each type of sample.

Figure 2 shows the candidate’s miRNAs expression pattern between four samples type (AS, OS, ASOS and NS), the results of expression show an obvious variation between miRNAs genes, miR-135b expressed as the remarkably highest gene among all other 3 miRNAs, especially in Asthenospermia AS and Oligospermia OS, separately. miR-10a and miR-10b also show high expression values when compared with control or normal semen NS but not as high value as the miR-135 family, but the significant change was the miR-135 family showed a normal expression when miR-10a and miR-10b being more than its normal expression value.

Pons-Espinal and co-worker also found that miR-135a significantly expression increased in AS and OS but in the current study the miR-135a also increased in ASOS (36), while
miR-10a and miR-10b show no remarkable change between all four types of fertility semen but expressed higher than control or normal semen, Barbu and colleagues also refer that miR-10a and miR-10b as a strong specific seminal fluids miRNA marker since the miR-10a and miR-10b not expressed in another fluid they tested (blood, saliva, vaginal secretion, breast milk and plasma) (35).

In another word, the results show that the means of a pattern in expression changed between each case and between each sample and the specificity of specific miRNAs to specific body fluids, which can be applied as identification for body fluids in the crime scene, and that is exactly what was found by previous studies (22, 30, 34, 39).

**Micro RNA expression pattern as a molecular marker in medical fields.**

In the medicine field, the results in this study may provide valuable information to give a situation at the molecular level that may answer in the future as a treatment, especially miR-135b when give such a significant expression increasing in asthenospermia-oligospermia semen (ASOS) from the other normal and fertile semen, whereas miR-135a also show significant expression increasing in both asthenospermia (AS) and oligospermia (OS) while expression decreased in normal semen (NS) and asthenospermia-oligospermia semen (ASOS), miR-10a and miR-10b show no significant expression changes between all candidate fertile and infertile semen, but still a specific marker for seminal fluids sample to be identified, also rather than its weak changes among other miRNAs but there were changes a pattern of expression of those miRNAs especially when the miR-10a and miR-10b expression increased the other miR-135 family expression dropped to their normal expression which mean there is a pattern of expression between each sample.

5. CONCLUSION

Each of miR-10a, miR-10b, miR-135a and miR-135b were a specific molecular marker for seminal fluids serve as a body fluids identification method in the crime scene, miR-135b was shown as a strong miRNA marker to distinguishing asthenospermia-oligospermia semen (ASOS) from the other normal and fertile semen, whereas miR-135a also show significant expression increasing in both asthenospermia (AS) and oligospermia (OS) while expression decreased in normal semen (NS) and asthenospermia-oligospermia semen (ASOS), miR-10a and miR-10b show no significant expression changes between all candidate fertile and infertile semen, but still a specific marker for seminal fluids sample to be identified, also rather than its weak changes among other miRNAs but there were changes a pattern of expression of those miRNAs especially when the miR-10a and miR-10b expression increased the other miR-135 family expression dropped to their normal expression which mean there is a pattern of expression between each sample.

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