Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

New insights into the expression profile of MicroRNA-34c and P53 in infertile men spermatozoa and testicular tissue

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Received April 14, 2017; **Accepted** August 11, 2017; **Published** August 30, 2017

Doi: http://dx.doi.org/10.14715/cmb/2017.63.8.17

Abstract: Spermatogenesis is proliferation and differentiation processes of stem spermatogonia into mature spermatozoa controlled by the genes responsible for transcription and post transcription levels. MicroRNAs (miRNA) are the key factors during gene expression in RNA silencing and post-transcriptional regulation. They play main roles in regulation of early and late spermatogenesis, and reproduction. In this study, we investigate the role of miRNAs in infertile males.
The patients were assigned to five groups based on semen analysis (n=55), including normozoospermic (N), moderate oligoasthenoteratozoospermic (MOAT), severe oligoasthenoteratozoospermic (SOAT), obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). Quantitative RT-PCR was recruited to study the expression of miR-34c and tumor suppressor p53 gene. In addition, malondialdehyde (MDA) and DNA fragmentation was measured. Network analysis was performed using Pathway Studio web tool (Elsevier). Our results revealed statistically significant increased expression of miR-34c in moderate oligoasthenoteratozoospermic, non-obstructive azoospermia and an increased expression of p53 in MOAT, SOAT and NOA males. Also, the percentage of DNA fragmentation and oxidative stress was significantly higher in infertile groups (MOAT and SOAT) than other groups. These findings provide a novel molecular mechanism of gene regulation during cell-cycle and apoptosis in sperm, which gives a new regulatory insight into male infertility in terms of molecular diagnosis.

Key words: Sperm, MicroRNAs; Normozoospermic; Oligoasthenoteratozoospermia; Pathway Studio.

Introduction

Infertility according to WHO is inability to conceive after twelve months of unprotected sexual intercourse (1). Approximately 10–15% of couples worldwide are infertile and male factors can be diagnosed in almost half of the cases (2). Non-coding molecular RNAs ranging from 20-25 nt in length called MicroRNAs (miRNAs) are post-transcriptional regulators of the target genes (3). The miRNAs because of regulating target genes involved in cell-cycle, cell growth, development and apoptosis are important in different biological processes (4). In addition, some researches have shown that miRNAs may also have key roles in male germ cells and mammalian spermatogenesis (5, 6). Moreover, the evaluation of miRNAs in semen gives a novel, noninvasive biomarkers for diagnosing male infertility (7).

The members of miR-34 group have remarkable functions in the infertility, such as miR-34c, miR-34a and miR-34b. MiR-34 as one of the short non-coding RNAs was first identified in C. elegans. It encodes an evolutionary conserved miRNA in several vertebrates (8). Meanwhile, miR-34c is located at chromosome 11q23.1 (9).

Reproductive tissues of bovine are the place where miR-34 family is expressed. MiR-34c, as one of the members of this family, has been mostly found in the testis and sperm of bovine (10). Moreover, It has the highest expression in mouse testis which is involved in spermatogenesis, and also in the adult mouse pachytene spermatocytes and round spermatid (11).

The miR-34 family has different purposes, one of which is to target several important factors regulating cell cycle. (12). The family induces repression of a large number of target mRNAs such as cell cycle proteins including Cyclin D1 and BCL_2 (13). Moreover, it regulates apoptosis and by its anti-proliferative and pro-apoptotic effects, it can affect different tissues such

as basal-like breast cancer cells and can impede G2/M phase cell cycle and mortality in breast cancer cells (14). Cell cycle arrest is induced through atopic expression of miR-34 in both primary and tumor-derived cells (15). In signaling pathway of p53 gene, the miR-34 has a vital role (13). DNA damage directly stimulates P53 which consequently contributes to up-regulated expression of the miR-34b and miR-34c (13, 16, 17).

Some studies have shown that miR-34c overexpression through P53 pathway significantly stimulates apoptosis and contributes to up-regulation of Caspase 3 and down-regulation of Cyclin D1 in male germline stem-cell (mGSCs) in dairy goat (18). MiR-34 suppresses silent mating type information regulation 2 homolog 1 (SIRT1) gene through its specific binding site in the 3' untranslated reign of SIRT1, causing high expression of transcriptional targets of p53 including P21 and PUMA, controlling cell cycle and inducing apoptotic processes. Another transcriptional target of P53 is MiR-34a, altogether indicating a positive feedback circle between P53 and miR-34a (19). P53 gene regulates expression of three miR-34 families in cell lines and tissues (20). MiR-34 reduces cell growth rate, arrests cells in G1 phase, enhances Caspase-3 activation and specifically, inhibits tumor-sphere formation and cell division. MiR-34 has been determined as a P53 target, and as capable of down-regulation of genes affecting cell cycle progression (15). According to results of a study, apoptosis and cell-cycle can be arrested in the G1 phase through miR-34c as a direct target of P53 (12). It induces down-regulated expression level of Cyclin D1, resulting in inhibition of G1/S transfer during cell cycle in mGSCs (18)**.**

Based on different studies, several genes which are regulated by miR-34c in some cancers have been identified. The role of microRNAs in the process of sperm disorders is not quite understood in infertile men. Here, we designed the present study to investigate miR-34c expression and its role in regulating P53 genes and miR-34c relationship with oxidative stress and DNA fragmentation in sperm of infertile men, as non-invasive biomarkers for the diagnosis of male infertility.

Materials and Methods

Sample collection

The study samples were obtained from 55 males, aged 20–35 (mean 27.5) years, who underwent infertility treatment at the IVF lab of Al-Zahra Hospital (Tabriz, Iran). Ejaculated samples and testicular tissue samples were provided by infertile couples after clarifying the study purpose. In addition, the subjects signed informed consent forms in accordance with the ethic guideline of Tabriz University of Medical Sciences. The ethics committee approved this consent procedure (registered number 66000116 at ethic committee of TUMA).

Thirty to fifty-five semen samples were obtained from infertile men with a spermiogram showing severe (concentrations less than 5 million sperm/mL) and moderate (concentrations 5 million – 10 million sperm/mL) oligoasthenoteratozoospermia (a combination of reduced sperm number in semen with decreased motility and abnormal morphology). Fifteen of the samples were assembled from healthy men with normal sperm parameters. Patient specimens with known medical reasons for their infertility including genetic abnormalities (chromosomal defect and Y-chromosome microdeletions), varicocele and hydrocele were excluded from the study.

Testicular biopsies were obtained from 10 azoospermia male patients, and all the specimens were assessed histologically. Then, they were classified into two principal groups, namely obstructive azoospermia (blockage of the genital ducts) and non-obstructive azoospermia (NOA) (lack of testicular production).

The procedure for collecting the ejaculated samples was by masturbation, and the samples were collected into a sterile and non-toxic collection cup after 2–5 days of sexual abstinence. The sample container was located in an incubator (37 °C) while the semen was liquefied. After liquefaction, the quality of the specimens was assessed by evaluating liquefaction time, viscosity, sperm count, volume, pH, motility, and morphology according to WHO manual.

The specimens were processed by the double density gradient centrifugation technique; accordingly, one milliliter of the semen sample was loaded onto 45%–90% discontinuous Suprasperm (model: Origie, MediCult, Copenhagen, Denmark) and centrifugation was performed at 300g for 20 minutes at room temperature. After centrifugation, the pellet was washed twice (300 g, 10 min) in 5 ml of pre-warmed sperm preparation medium and resuspended in 200 µl of the sperm preparation medium. The procedure was completed according to the manufacturer's recommendations. The supernatant was evaluated for sperm concentration, motility and morphology.

RNA isolation

Isolation of total RNA, including miRNAs, from the sperm samples and testicular tissue

Total cellular RNA, including miRNAs, was purified from sperm pellets and testicular tissue by use of the Qiagen miRNeasy Mini Kit (Qiagen, Germany) with some modifications. Briefly, 700 µl QIAzol Lysis Reagent (Qiagen) was added to the sperm pellets and testis tissue and homogenized. Next, 140 µl chloroform was added to the solution which was vortexed for 15 s. After that, the mixture was incubated and centrifuged. The upper aqueous phase was conveyed to a new collection tube, into which 1.5 volumes (usually 525 µl) of 100% ethanol was added and mixed completely by pipetting. Finally, the following processes in protocol were conducted with respect to the instruction of manufacturer. The specimens were evaluated with use of a Nanodrop ND-2000 spectrophotometer to determine the purity of RNA (Thermo Fisher Scientific).

Generation of cDNA

Universal cDNA synthesis Kit (Exiqon) was applied to carry out reverse transcription of RNA for cDNA synthesis. RT reaction was prepared at final volume of 10 µl containing 5× reaction buffer (2 µl), enzyme mix (1 µl), template total RNA (2µL) and nuclease-free water (5 µl), followed by 42 °C for 60 min and 95 °C for 5 min.

Table 1. Oligonucleotides used in this study.

Primer set name	Real time quantitative PCR primer
U6 snRNA	AACGCTTCACGAATTTGCGT
Hsa-Mir-34c	AGGCAGUGUAGUUAGCUGAUUGC

Quantitative real time-PCR

The quantitative RT-PCR (qRT-PCR) for miRNAs was carried out using the ExiLENT SYBR Green Maser Mix Kit (Exiqon). Table 1 indicates the primer sequences used for qRT-PCR. All reactions with a final volume of 20 µl were provided for 45 amplification cycles consisting 95°C for 10 s and 60°C for 1 min. As the endogenous control for normalizing, U6 small nuclear RNA was the outcome. The ΔΔCt method was employed to analyze the obtained data.

Moreover, quantitative real-time PCR was carried out with some modifications , namely use of 10 µl of PCR Master Mix (Thermo Scientific, Mumbai, India), 1.5 µl extracted DNA and 1 µl of each primer in 20 µl total volume, to detect P53 mRNA expression (21). The reaction was run with the following settings: (i) holding at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 sec, (ii) annealing at 56°C for 30 sec, and (iii) extending at 72°C for 30 sec. The last cycle was followed by a final extension step of holding at 72°C for 5 min. Beta-actin was employed for normalization of gene expression. All PCR reactions were repeated three times. The specificity of real-time PCR products was controlled using melting curve analysis. Further, relative miRNA or mRNA expression was performed using the ΔΔCt. The primers are shown in Table 2.

Network analysis of Mir-34c based on literature mining

Using miR-34c as input entity, network analysis was performed using Pathway Studio web tool (Elsevier) (22). Databased of gene, protein, microRNA, and small molecule interaction of Pathway Studiois onstructed using Medscan language programming (23). Additionally, cellular location of proteins was identified based on cellular component term of Gene Ontology classification, derived from Gene Ontology Consortium (http://geneontology.org/).

Measurement of sperm MDA levels

Malondialdehyde (MDA) is an oxidative stress marker, for estimation of which the thiobarbituric acid method was used (24). Briefly, the sperm pellet was homogenized and 100 µl of it was added to 900 µl distilled water in a glass tube. In the next step, the tubes after adding 500 µl of thiobarbituric acid reagent were centrifuged at 4000g for 10 min. After centrifuging, content of MDA was read on a spectrophotometer at excitation 515 nm and emission 553 nm. The results were expressed as nmoL MDA/10^6 cells.

DNA fragmentation evaluation

To determine the sperm DNA fragmentation, the sperm chromatin dispersion (SCD) test was used, and phosphate-buffered saline (PBS) was utilized to dilute aliquots of the washed semen samples up to 5-10 million per mL. The semen sample was mixed with gelled aliquots of low-melting point agarose (Sigma-Aldrich chemical Company, St. Louis, MO, USA). Fifty microliters of the semen–agarose mix was pipetted on a slide already precoated with standard agarose and covered with a 22- by 22- millimeter coverslip. The slides were placed in the refrigerator (4°C) for 5 min, and the coverslips were intently removed. Next, the slides were immersed in an acid solution immediately, incubated horizontally in lysing solution 1 for 5 min and lysing solution 2 for 10 min, after washing for 2 min in Tris – borate – EDTA buffer were dehydrated respectively in ethanol 70%, 90%, and 100% for 2 min, and then dried at room temperature. The slides were stained with DAPI (4′, 6-diamidino-2-phenylindole) (2 µg/ml), and 300 sperm cells were counted and classified as fragmented or nonfragmented DNA using a fluorescence microscope. There are five SCD patterns as follows. Large halo nuclei: the halo width is the same or higher than the minor diameter of the core; medium-sized-halo nuclei: the halo size is between halos with high and small; sperm cells with small-sized-halo nuclei: the halo width is equal to or less than 1.3 of the minor diameter of the core; without a halo; and sperm cells without halos and degraded nuclei.

Statistical analysis

For statistical analysis, we used the Statistical Package for Social Sciences software, version 22 (SPSS Inc., USA). The parametric data analyses were accomplished with one-way ANOVA test and t-tests and the non-parametric data were analyzed with Kruskal-Wallis test. The REST 2009 software (Qiagen) was used to analyze the respective gene and miRNA expression levels and the statistically significance level was $P < 0.05$.

Results

Sperm parameters and DNA Fragmentation

The characteristics of forty-five semen samples of infertile men groups and control group are presented in Table 3 That into three groups classified: normozoospermia (n=15), severe oligoasthenoteratozoospermia

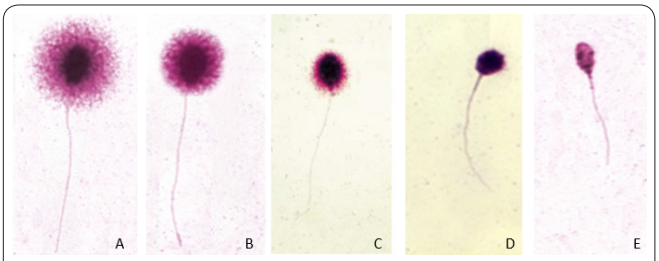

Figure 1. Sperm chromatin dispersion (SCD) test. Sperm classified into two groups: Sperm which produce a big halo (A) or medium-sized halo (B) contain intact DNA. However, spermatozoa with fragmented DNA produce a small halo (C), no halo (D), or a degraded nuclei (E).

Table 2. The primer sequences.

Gene	Forward primer	Reverse primer
B-actin	GCGGCATCCACGAAACTAC	TGATCTCCTCTGCATCCTGTC
P53	GCCCAACAACACCAGCTCCT	CCTGGGCATCCTTGAGTTCC

Table 3. Semen parameters and DNA fragmentation in different groups. Characteristic sperm test (mean ± SE) from normozoospermic patients (N), moderate oligo astheno teratozoospermic patients (MOAT) and severe oligo astheno teratozoospermic patients (SOAT).

Characteristic	N (n=15)	MOAT (n=15)	SOAT (n=15)	*p* Value
Concentration (10^6/ml)	103.73±15.73	8.20±1.37**	4.33±0.97**	0.000
Progressive motility (%)	43.13±7.1	7±3.6**	6.8±5.07**	0.000
Normal morphology (%)	22.63±7.75	2.50±0.62**	1.53±0.79**	0.000

DNA fragmentation with SCD test (mean ± SE)

	Big halo	medium halo	small halo	without	degrade	Total fragmented
N (n=15)	67.13±11.3	10.2±5.30	6.27±3.77	14.2±5.42	2.13±1.59	22.67
MOAT (n=15)	30.47±4.83*	17.6±4.32	24.73±2.98**	22.33±3.90*	5.73±1.71**	51.93**
SOAT (n=15)	28.2±5.19**	14.2±3.38	31.6±4.33**	20.87±5.23*	5.13±3.11**	57.6**

Correlation coefficient of DNA fragmentation and Conventional Semen

	r(MOAT)	*p* Value	r(SOAT)	*p* Value(SOAT)
Concentration	-o.870*	0.00	-0.870*	0.00
Progressive motility	-0.881*	0.001	-0.859*	0.00
Normal morphology	-0.772*	0.00	-0.7.73*	0.01

Total fragmented sperm (degraded cell+Without halo+Small halo) ; * Significant at (p<0.05) ; **significant at (p<0.01).

(SOAT) (n=15) moderate oligoasthenoteratozoospermia (MOAT) (n=15). There was a significantly different in means of spermatozoa motility, sperm morphology and sperm Concentration (P values <.001) between infertile couples and control group (Table 3).

Human spermatozoa processed with SCD test that five patterns were observed: cells with big halo, cells with medium-sized halo, cells with small halo, cells without halos and cells without halo – degraded. Sperm with small halo, without halo, and without halo-degraded, correspond with fragmented DNA (Figure 1). The groups of men with MOAT and SOAT was compared to control group men. The percentage of sperm cells whit big halo was significantly decreased in MOAT (30.47±4.83) and SOAT (28.2±5.19). In contrast, the percentage of sperm cells with small-sized halo, without halo, and degraded were significantly increased in MOAT and SOAT (p<0.01) (Table 3). Also, we found negative Correlations between fragmentation of chromatin and sperm Concentration and motility and normal morphology in group MOAT [r=-0.870(p<0.01), r=-0.881(p<0.01), r=-0.772(p<0.01), respectively] and in group SOAT [r=-0870(p<0.01), r=-.859(p<0.01), r=-0.7.73(p<0.01), respectively] (Table 3).

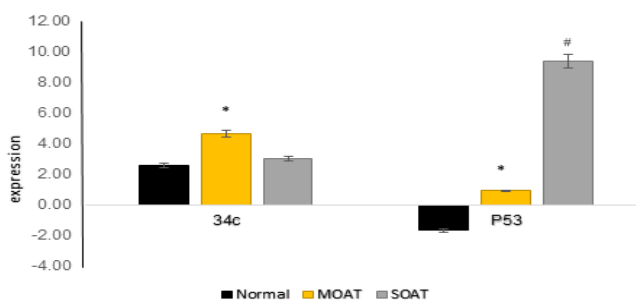

Figure 2. miR-34 and P53 gene expression in oligoasthenoteratozoospermic (MOAT), severe oligoasthenoteratozoospermic patients (SOAT) and normozoospermic patients (N). Expression of miR-34c in MOAT patients was significantly increased compared with control group. Also P53 gene had a statistically significant increase in MOAT and SOAT groups compared with normal group. * Significant at (p<0.05) ; # significant at (p<0.01).

Differentially Expressed of Mir-34c and P53 in Study Subjects

Quantitative RT- PCR shows significantly increased expression of miR-34c and p53 in infertile couples (n=25). Expression of miR-34c in SOAT patients was lower compared with MOAT patients. Whereas miR-34c had a statistically significant increase in MOAT group compared with control group men. P53 gene was highly expressed in SOAT patients compared with MOAT patients and normal individuals in our experiment (Figure 2). There were statistically significant Correlations between the expression of miR-34c and P53 gene in MOAT and SOAT patients: r=-0.85 (p=0.04).

Our results showed significantly up-regulated miR-34c and p53 gene in the NOA group men compared with OA group. Also, we found significant correlation between the amount of miR-34c and p53 gene in non-obstructive azoospermia (NOA) men and obstructive azoospermia men: r= 0.46(p=0.014) (Figure 3).

DNA fragmentation, malondialdehyde (MDA) and the expression of Mir-34c and P53

The ΔCt values of miRNA-34c showed significant correlation with sperm concentration: r=-0.429(p=0.020), sperm motility: r=-0.423(p=0.022) and sperm normal morphology: r=-0.393 (p=0.035). As well, we were observed significant correlation between the amounts of p53 ΔCt and sperm concentration: r=-0.587 (p=0.008), In contrast, no significant correlation

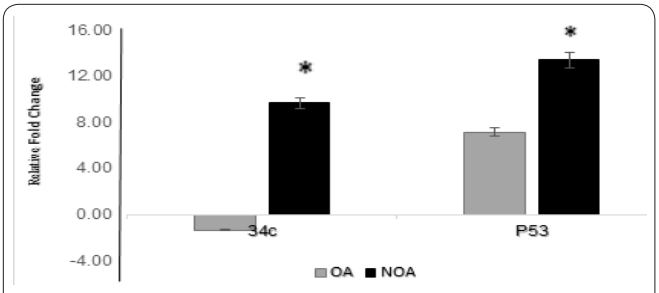

Figure 3. miR-34 and P53 gene expression in obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). MiR-34 and P53 gene expression were significantly increased in the NOA men compared with OA men. * Significant at (p<0.05).

Table 4. Correlation coefficient of miRNA ΔCt and P53 with semen parameters in moderate oligoasthenoteratozoospermic patients (MOAT) and severe oligoasthenoteratozoospermic patients (SOAT).

	r(Concentration)	*p* Value (Concentration)	r(motility)	*p* Value(motility)	r(morphology)	*p* Value (morphology)
Mir-34c	-0.429*	0.020	-0.423*	0.022	-0.3938*	0.035
P53	-0.587**	0.008	-0.410	0.081	-0.352	0.140

Correlation is *Significant at (p<0.05) ; **significant at (p<0.01).

Table 5. Correlation coefficient of ΔCt values of miRNA and P53 with DNA fragmentation in moderate oligoasthenoteratozoospermic patients (MOAT) and severe oligoasthenoteratozoospermic patients (SOAT).

	r(MOAT)	*p* Value (MOAT)	r(SOAT)	*p* Value(SOAT)
Mir-34c	0.81	0.677	0.433*	0.019
P53	0.576*	0.011	0.176	0.410

Correlation is *Significant at (p<0.05) ; **significant at (p<0.01).

was observed among p53 gene, morphology and motility (Table 4).

Our data indicate a statistically significant correlations between the ΔCt values of miR-34c and sperm DNA fragmentation in severe oligoasthenoteratozoospermia group(r=0.433, p<0.01) and There were significantly correlated between the ΔCt of P53 and sperm DNA fragmentation in moderate oligoasthenoteratozoospermia group(r=0.576, p<0.01) (Table 5).

MDA content in the spermatozoa of MOAT and SOAT patients was significantly higher than in normozoospermic samples (0.86 ± 0.21 and 1.02 ± 0.024 nmoL/10^6 spermatozoa, respectively) (Table 6). We showed significant association between oxidative stress and the expression of miR-34c in infertile groups; MOAT: r=0.530(p=0.017) and SOAT: r=0.39 (0.040). As significant association was observed between MDA and P53 in MOAT: r=-0.564(p=0.012) (Table 6).

Network analysis

Network analysis of miR-34c according to literature mining is presented in (Figure 4) and the underlying relationships of this network is presented in Supplementary.1 As it can be inferred from network, miR-34c has a considerable number of interactions with genes located on different organelles. In particular, miR-34c has considerable number of reported interactions with RUNX2, MYC, MET, BCL2, SIRT1, TP53, CRHR1, CDK6, KITLG, ATF1, TGIF2, MAPT, BMF, NOTCH1, NOTCH2, NOTCH4, CCNE1, PDGFRA, SEMA4B, and TTC19.

Discussion

The present research was carried out to assess the miR-34c and P53 gene expression, DNA fragmentation and oxidative stress between infertile subjects and those with normal fertility. Of interest was also to determine miR-34c relations with P53 gene, DNA fragmentation and oxidative stress in infertile males. Our study indicates that miR-34c level significantly increased in patients with moderate oligoasthenoteratozoospermia compared with fertile control individuals, and the DNA fragmentation level and sperm MDA content in infertile men were elevated significantly. In addition, in non-obstructive azoospermia patients, expression of miR-34c was observed to be significantly different compared with obstructive azoospermia patients. Our network analysis revealed that NOTCH1, NOTCH2, NOTCH4 are important targets of miR-34c. It seems that miR-34c is an important player in Notch signaling pathway. In Adult rhesus monkeys, NOTCH1 is many expressed and is essential for differentiation and survival of germ cells in mouse testis (25, 26).

Within the spermatogenesis, haploid spermatozoa are differentiated from diploid spermatogonia, and this procedure is regulated by MiRNAs at the post-trans-

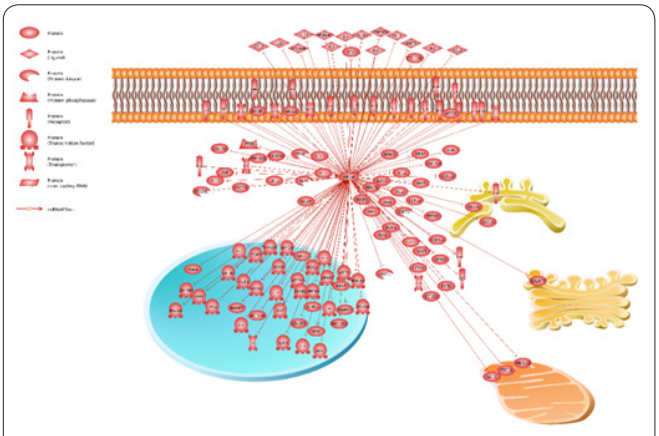

Figure 4. Network analysis of miR-34c. MiR-34c has considerable number of reported interactions with Different genes.

Table 6. Malondialdehyde levels (MDA) (mean ± SE) in sperm (moderate oligoasthenoteratozoospermic patients (MOAT) and severe oligoasthenoteratozoospermic patients (SOAT)) and its Correlation with miRNA ΔCt and P53.

	N (n=15)	MOAT(n=15)	SOAT (n=14)	
MDA (nmoL/10^6spermatozoa)	0.32±0.24	0.86±0.21**	1.02±0.24**	
Correlation among miRNA ΔCt and P53 and MDA				
	r(MOAT)	*p* Value (MOAT)	r(SOAT)	*p* Value(SOAT)
Mir-34c	0.530*	0.017	0.391*	0.040
P53	0.564*	0.012	0.014	0.95

*Significant at (p<0.05) ; **significant at (p<0.01).

criptional level.

The role of miR-34c in later steps of spermatogenesis is essential, because it participates in germ cell differentiation and sperm generation (11, 27-29). When miR-34c is down-regulated, it starts to promote apoptosis of primary spermatocytes and male germline stem cells, a process which is crucial in producing normal sperm (27).

The male infertility has relationship with the miR-34c level in seminal plasma, as well. Studies showed that miR-34c level significantly decreased in men with azoospermia) no sperm count), oligoasthenozoospermia or teratozoospermia in comparison with normozoospermic males, but interestingly increased in men with asthenozoospermia (decreased sperm motility) when compared with fertile control individuals (7, 30). Another study revealed that miR-34c expression significantly decreased in patient samples with non-obstructive azoospermia (31) and azoospermic men (32). Also, seminal miRNA-34c-5 was significantly reduced in infertile oligoasthenoteratozoospermic men with varicocele, indicating a significant negative correlation with MDA (33).

According to previous results, the expression of miR-34c in male gonads is significantly independent of P53 gene and showed limited reduction in p53-deficient mouse testis (11); however, other researches revealed that there is a straight link between the miR-34 family and P53 gene. The up-regulated expression of Mir-34c can be seen due to over-expression of P53 in mGSCs. In addition, activation of P53 protein by doxorubicin contributes to increased expression of miR-34c and Mir-34c endorsed apoptosis in mGSC and reduces their proliferative ability. It was claimed that miR-34c is p53-dependent in dairy goat mGSCs (18).

Based on the study result, P53 is known as "the guardian of the cell cycle" (34), and has significant roles in cell growth rate and differentiation abilities. Following DNA damage, P53 can be activated in order to respond to genome instability, contribute to apoptosis, regulate the cell cycle and repair DNA damage (35). The "p53 codon 72 *Arg/*Arg genotype" with its robust apoptotic properties adversely affects spermatic parameters such as sperm motility and leads to male infertility (36).

A thorough investigation of the literature yielded few studies regarding the correlation between P53 and miR-34c in male infertility. Our results showed a significantly increased expression of Mir-34c and P53 during infertility. Expression of miR-34c was low in SOAT patients, while MOAT individuals had high expression which gives a new regulatory insight into the molecular diagnosis of male infertility. Many studies have described increased levels of reactive oxygen species in abnormal conditions (37). DNA damage in reaction or interaction between reactive oxygen species and DNA has been found because of oxidative stress (38). Moreover, the expression of miR-34c and miR-34b in mouse ovarian surface epithelium cells was enhanced following P53 triggered by DNA damage (13). In our study, significant correlation was observed between DNA fragmentation with mir-34c and p53. However, our study showed that p53, which transcriptionally controls gene expression after DNA damage and oxidative stress, has increased in SOAT patients in comparison with MOAT and normal individuals. It is supposed that DNA damage in infertile patients is responsible for this increased expression. Therefore, interaction between miR-34c and P53 needs to be investigated and confirmed with high number of patient samples. The results of this study indicate that high levels of ROS lead to DNA damage, forasmuch as the ROS has a positive relationship with apoptotic protease as the ROS-induced apoptotic protease involved in apoptosis. With increasing levels of ROS, DNA was greater damaged, P53 activity level changed and apoptosis started at the molecular level where we observed a direct correlation between the expressions of P53 Mir-34c. These findings provide a novel molecular mechanism of gene regulation during cell-cycle and apoptosis in sperm/semen samples by miR-34c through targeting p53, and give a new regulatory insight into the molecular diagnosis of male infertility.

Acknowledgments

We would like to thank the Department of Molecular Medicine, Faculty of Advanced Medical Science of Tabriz University, and IVF Center—Al-Zahra Hospital of Tabriz University of Medical Sciences for their assistance in this study. This work was supported by Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran and Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran Grant (Study ID: 4975).

Authors' contributions

MG contributed to the design of study and getting financial support and helped to draft the manuscript, SR participated in the design of experiments, collected samples, performed experiments and preparation of the manuscript. EA and MP carried out molecular studies and participated in drafting the manuscript. VSH, YA and LF helped collect samples. EE performed the statistical analysis. MP participated in the design and helped to draft the manuscript and getting financial support. The authors read and approved the final manuscript.

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