Original Article

Comparison between *SPATA18* and *P53* Gene Expressions in The Sperm Cells Obtained from Normospermic and Asthenospermic Samples: A Case-Control Study

Alireza Panahi, M.Sc., Sina Mirza Ahmadi, Ph.D.*, Golnaz Asaadi Tehrani, Ph.D.

Department of the Genetics, Zanjan Branch, Islamic Azad University, Zanjan, Iran

Abstract

Background: Improving sperm motility results in increasing the success of a treatment cycle. Recently, sperm RNA has been used for diagnostic purposes such as whole seminal fluid, sperm analysis, and sperm quality test in patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI). SPATA18-P53 pathway is considered an essential pathway related to sperm mitochondria, which controls mitochondrial quality by eliminating its oxidative proteins. Oxidative stress may decrease sperm motility and affect sperm quality negatively due to an increase in P53 expression. SPATA18 protein is found in satellite fibers related to outer dense fibers in the middle piece of sperm. The downregulation of SPATA18 in the asthenospermia group can represent this gene's critical function in sperm motility and fertility. The present study aimed to assess the relationship between *SPATA18* and *P53* gene expression in sperm cells obtained from normospermia and asthenospermia.

Materials and Methods: In this case-control study, the quantitative real-time polymerase chain reaction (RT-PCR) technique was used to measure the *SPATA18* and *P53* gene expression level in sperm samples collected from 21 patients and 63 healthy individuals. Further, the sperm DNA fragmentation assay (SDFA) kit was applied to determine the relative apoptosis level in cells and evaluate the biochemical information related to the patients' sperm samples. Furthermore, all the participants completed the consent form, and the ethics committee confirmed the study.

Results: Based on the results, the *P53* and *SPATA18* gene expression levels in most of the samples, in which motility was less than 40%, increased and decreased ($P \leq 0.001$), respectively.

Conclusion: The *SPATA18* and *P53* gene expression levels increased and decreased in the asthenospermic patients, respectively, compared to the control group. Thus, the *P53* and *SPATA18* expression levels can be used as an appropriate marker for diagnosing sperm motility in males.

Keywords: Apoptosis, Asthenosperm, Normosperm, *P53*, *SPATA18*

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Introduction

Recently, sperm RNA has been applied for diagnostic purposes such as whole seminal fluid, sperm analysis, and sperm quality test in patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI). Sperm RNA is considered a potential marker for diagnosing sperm abnormalities and fertility capability in infertility clinics (1). Based on the latest information provided by the World Health Organization, normal sperm parameters are considered 15 million sperms with 4% normal morphology and 40% motile sperm or at least 32% with progressive motility per ml of seminal fluid (2). The infertility distribution caused by male factors varies between 20-70%, and the percentage of infertile males ranges between 2.5-12% (3-9).

Asthenospermia is a common reason for male infertility, which is diagnosed by reduced sperm motility in new ejaculation (8). The maximum frequency of genetic factors recognized in male infertility (25%) is observed in asthenospermia (10). Three main factors, including decreased sperm count, motility power, and abnormal sperm morphology, are raised in male infertility. Selecting normal and mature sperms is considered useful in assisted reproduction techniques (11-13).

Mieap is considered another name for *SPATA18*. The genomic position for *SPATA18* gene is on the chromosome 4 (GRCh38/hg38), with the 46,002 bp linear DNA length and the 14 exons. The genomic position for *P53* gene is on the chromosome 17 (GRCh38.p13), with the length of 19149 bp linear DNA and 12 exons. It is assumed that *SPATA18* is a *P53* inducible protein, the transcription of which is directly regulated by the *P53* tumor suppresser. Mieap controls mitochondrial

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***Corresponding Address: P.O.Box: 58145-45156, Department of the Genetics, Zanjan Branch, Islamic Azad University, Zanjan, Iran**
Email: sinacanmir@yahoo.com

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quality by repairing or removing unhealthy mitochondria through the Mieap-induced accumulation of lysosome-like organelles within mitochondria (MALM) or Mieap-induced vacuole (MLV), respectively (14, 15). Inactivating *P53* or *Mieap* disturbs MALM and MLV severely, leading to the accumulation of unhealthy mitochondria. Mitochondria are necessary for intracellular signaling and cellular energy supply after stress (16, 17).

Crosstalk is available between the nucleus and mitochondria during stress events (16). Reactive oxygen species (ROS) are produced as side-products during the oxidative phosphorylation process, the overproduction of which can play a role in mitochondrial damage and stress (16, 18). Mitophagy is considered an effective mechanism for controlling mitochondrial quality since it can optionally remove unwanted or damaged mitochondria (16, 19). Mitophagy plays a role in basal mitochondrial turnover and eliminates damaged mitochondria under stress (16, 20).

Additionally, these organelles are regarded a major source of intracellular ROS, including highly reactive free oxygen radicals like hydroxyl radical (OH•) and superoxide anion (O2•−), as well as stable non-radical oxidants such as hydrogen peroxide (H_2O_2) (21). ROS is commonly created as the by-products of oxidative phosphorylation (21, 22). The generation of excessive ROS in mitochondria (mtROS) causes oxidative damage to lipids, proteins, and DNA and may lead to apoptosis (21, 23). Further, ROS accumulation can cause various diseases like degenerative disorders and cancer. Based on recent reports, elevated levels of mtROS can increase cancer cell invasion and metastasis through activating different major signaling pathways and transcription factors (21). Increasing oxidative stress, such as oxygen free radicals and ROS, can negatively affect sperm quality (24). Mitochondria include cardiolipin (CL), which is considered an organelle-specific phospholipid that carries 40 fatty acids with a strong preference for unsaturated chains (25-27). Further, TPCL is associated with acrosome, a sperm-specific organelle, during spermiogenesis, along with a subset of authentic mitochondrial proteins such as Suox, Ant4, and *SPATA18* (27).

Proteins such as caspase -1, 3, 7, 8, and 9 and aquaporin-7 are involved in regulating mitochondrial function in the apoptotic pathway and decreasing the sperm volume. Apoptosis may be associated with decreasing sperm motility (28). *SPATA18* protein is found in satellite fibers related to outer dense fibers in the middle piece of sperm. Decreasing *SPATA18* expression in the asthenospermia group can represent this gene's critical function in sperm motility and fertility (29, 30).

Based on Dan et al. (16), *SPATA18* expression is an essential player in the mitophagy process after DNA damage.

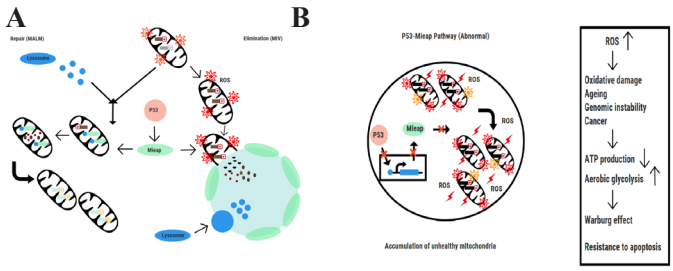

Fig.1: The Cross talk between *P53* and Mieap in mitochondria. **A.** The hypothetical model of *P53*-Mieap pathway for mitochondrial quality by which Mieap controls mitochondrial quality by repairing or removing unhealthy mitochondria through generating MALM or MIV, respectively (14, 15), and inactivating *P53* or Mieap disturbs MALM, and MLV severely leads to the accumulation of unhealthy mitochondria and **B.** Hypothetical model for inactivating *P53*-Mieap pathway by which ROS surfaces increase under severe oxidative stress such as oxygen free radicals, leading to DNA damage and apoptosis induction, and finally the disruption in the function of *P53*-Mieap (14). MALM; Mieap-induced accumulation of lysosome-like organelles within mitochondria, MIV; Mieap-induced vacuole, and ROS; Reactive oxygen species.

Considering the changes in *SPATA18* gene expression in normospermic and asthenospermic cells and the effect of the gene expression on *P53*-induced apoptosis, the present study aimed to assess the possibility of gene expression and its effect in generating apoptosis in sperm cells.

Materials and Methods

Sampling, classification, and characterization of samples

In this case-control study, the count and motility of the sperm samples of the 84 participants, who were referred to Bu Ali Laboratory in Zanjan for 6 months from June-November of 2020, were written on the day of sample preparation and the data were sorted from minimum motility to maximum one. From the total 84 samples were assessed in the present study, of which 21 and 63 were related to the patients and healthy individuals, respectively. Also, to appropriately compare gene expression levels between the asthenospermia and normospermia samples, the normospermic samples were divided into three subgroups, each of them including 21 samples based on their motility range, including [41-55] subgroup A, [55-69] subgroup B, and [69-83] subgroup C. Since the population included 21 asthenospermic samples and 63 normospermic ones, the control group with 63 members was divided into three subgroups to compare the 21-member asthenospermia group with each of the control subgroups statistically.

Primer design

Table 1 presents the sequence of primers using Oligo 7 software. In this table, the content of each reaction and the time and temperature of each cycle is shown.

In Table 2, the *P53* and *SPATA18* gene expression levels are compared between the asthenospermia group with the asthenospermic samples and the three normospermia subgroups. Table 3 shows the sperm DNA fragmentation assay (SDFA) results concerning gene expression in two groups of fair to low and good fertility potential.

The sperm samples were analyzed using HFTCASA Computer Aided Semen Analysis System software, 8.00 (31). A t test was implemented to compare the results using SPSS 22 (IBM Company, USA). Which was evaluated using the Kolmogorov-Smirnov test. To compare two groups from t test and to compare 3 groups from one-way analysis of variance. The abbreviation ANOVA is used. The significance level was considered 0.05, and the SDFA results were calculated using REST 2009 software. In this software, REST RG mode is used for data analysis.

Total RNA was extracted using EZ-10 Spin Column Total RNA Mini-Preps Kit (BioBasic Inc., USA) based on the Sperm RNA Company's guidelines.

cDNA synthesis

The cDNA complementary strand was created using the RNA extracted by the Takara kit based on the company's guidelines. Additionally, cDNA was synthesized immediately after extracting RNA based on the Takara Company's kit protocol (cat.no RR037Q).

Real-time polymerase chain reaction

The Rotor-Gene (Q) real-time PCR machine (QIAGEN) was used in the present study.

Steps of real-time polymerase chain reaction

Real-time PCR was conducted for the target and control genes in two separate, paired tubes. The mixture volume is provided in Table 1.

Halosperm® G2 kit (HT-HSG2, halotech)

Of the 84 samples, 24 were randomly assessed using the SDFA kit. Additionally, the Halosperm® G2 kit (HT-HSG2, Halotech) was used based on the company's guidelines for sperm DNA fragmentation assay (SDFA).

Reference rage of SDFA

• Samples with SDF<15%: These samples were assayed to have a deficient fragmentation level and ranked good.
• Samples with 15%<SDF<30%: These samples were assayed to have a low fragmentation level and ranked medium.
• Samples with 30%<SDF: These samples were assayed to have a high fragmentation level and ranked abnormal.

Results

A significant difference was observed regarding the *P53* and *SPATA18* gene expression levels between the asthenospermia group and the subgroups A, B, and C of normospermic samples. As shown in Table 2A, by decreasing motility rates in the asthenospermic samples led to lower *SPATA18* and higher *P53* gene expression levels compared to the normospermic samples.

Table 1: The sequence of primers and the condition of optimized Real-Time PCR reaction

Primer	Primer length (5´-3´)	Length of created piece (bp)
GAPDH	F: GGTCATCATCTCTGCCCCCT R: AGGCAGGGATGATGTTCTGG	276
SPATA18	F: GTTCAGCGATTCCTATTCCCAGGC R: TCGACCCCACATAAGATGGTGTCA	192
P53	F: ATAGTGTGGTGGTGCCCTATGAGC R: TTCCAGTGTGATGATGGTGAGGAT	134

Component	Vol./reaction (µl)	Final concentration (µM)
2X Master Mix RealQ Plus	10	1x
Forward primer	0.5 (0.25-2.5)	0.1 (0.05-0.5)
Reverse primer	0.5 (0.25-2.5)	0.1 (0.05-0.5)
PCR-grade H_2O	7	-
Template cDNA	2	0.1
Total	20	-

Cycles	Duration of cycle	Temperature (°C)
1 for activation TEMPase	15 minutes	95
40	15 seconds	95
	30 seconds	52
	30 seconds	72

Table 2: The obtained P value of *P53* and *SPATA18* gene expression levels in the studied groups to determining statistical significance. The comparison of the *P53* and *SPATA18* gene expression levels between the asthenospermia and three normospermic subgroups samples, and the simultaneous assessment of significance of expression *P53*, *SPATA18* genes in all the three subgroups (a, b, c) and total samples (d)

	Gene	P value	Gene	P value
		P53		*SPATA18*
a. The mean difference of the asthenospermia and normospermia subgroup A	12.86 $_{\Delta CT}$	0.023	-14.06 $_{\Delta CT}$	0.001
b. The mean difference of the asthenospermia and normospermia subgroup B	36.72 $_{\Delta CT}$	0.025	-14.10 $_{\Delta CT}$	0.001
c. The mean difference of the asthenospermia and normospermia subgroup C	33.90 $_{\Delta CT}$	0.004	-14.05 $_{\Delta CT}$	0.001
d. The mean difference of the asthenospermia and normospermia total subgroups	12.86 $_{\Delta CT}$	0.023	-14.07 $_{\Delta CT}$	0.00000*

*** P value decreases to zero to five decimal places**

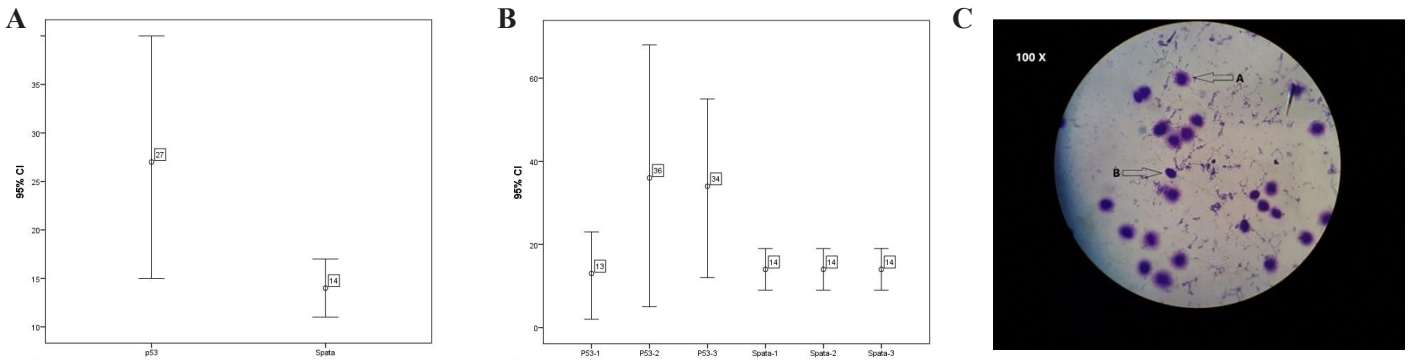

Fig.2: The results of the comparison of *P53* and *SPATA18* gene expressions obtained by both RT-PCR and SDFA methods. **A.** The mean difference of the asthenospermia and normospermia groups regarding *P53* and *SPATA18* genes in all the three subgroups. **B.** The mean difference of the asthenospermia and normospermia groups in *P53* and *SPATA18* genes in each of the three subgroups. **C.** Sperms (A) with halo (healthy and without DNA fragmentation) and (B) without halo (with DNA fragmentation) with ×100 magnification. CI; Confidence Interval of mean, SDFA; Sperm DNA fragmentation assay, and RT-PCR; Real-time polymerase chain reaction.

Table 3: The SDFA results concerning gene expression in two groups of fair to low and good fertility potential by using REST 2009 software

Gene	Reaction efficiency	Expression	P value
GAPDH	0.7124	1.000	
P53	0.6548	1.617	0.748
SPATA18	0.6014	7.012	0.078

The *P53* and *SPATA18* gene expression levels were compared between the asthenospermia group and the three subgroups of normospermic samples. Based on the real-time PCR technique results, the *P53* gene expression level increased (P≤0.01 up, especially Table 2 subgroup C) whereas the *SPATA18* expression level decreased (P≤0.01 down, Table 2 all subgroups). Furthermore, based on the analysis of expression level of total normospermic with asthenospermic samples, the P≤0.05 and P<0.001 were obtained for *P53* and *SPATA18*, respectively, and the difference was significant due to the high sample size (Table 2). However, no significant difference was observed concerning gene expression between the good and fair to low fertility potential groups (Table 2). In the healthy groups, the possibility of asthenosperima in males increased by decreasing their motility range to 40. Comparing the data related to the three groups demonstrated that the *P53* and *SPATA18* expression levels in patients with motility between 12.2 and 30 increased and decreased, respectively.

Thus, the *SPATA18* gene expression level hypothesis was accepted in normospermic and asthenospermic cells by considering the effect of its expression in generating *P53*-induced apoptosis and assessing the *SPATA18* and *P53* gene expression levels, which was different in sperm cells.

Based on the results, a significant difference was observed between the asthenospermia and normospermia groups. Figures 2A and 2B display the mean difference of the asthenospermia and normospermia groups regarding *P53* and *SPATA18* genes in all and each of the three subgroups, respectively.

Discussion

In the recent decade, the recognition of male reproductive function and the effect of malefactors on infertility has progressed significantly. Based on previous studies, germ cells, anatomic and hormonal disorders, and genetic abnormalities can be considered reasons for infertility in males (32). Sperm abnormalities can emerge in different forms, such as azoospermia (seminal fluid with no sperm), oligospermia (a low concentration of sperm), asthenospermia (low sperm motility), teratozoospermia (decreased sperms with normal morphology), or a combination of them (33).

The motility level is inversely and directly related to *P53* and *SPATA18* expression, respectively. Accordingly, low motility results in increased and decreased *P53* and *SPATA18* gene expression, respectively. Low motility hinders sperm motility, and *P53* prevents *SPATA18* activation and directs cells toward cell death by increasing their expression.

Thus, inactivating *SPATA18* and increasing *P53* expression during infertility can probably intensify the condition.

Male *P53* -/- knockout mice in Zalzali et al.'s (34) study showed a decreased sperm count and abnormal sperm motility and morphology. Their results implicated the central role of cell cycle gene *P53* in some events like sperm development and differentiation. However, unknown pathways and the absence of probable known downstream effectors like Cdkn1a highlight the complicated roles of these genes in sperm biology. Nakamura and Arakawa (35) examined the *SPATA18* role in mice fertility. They found that although the *SPATA18* -/- knockout mice were fertile in vivo, the sperm of these mice was severely impaired *in vitro* because of sperm motility failure and the oxidized proteins were dramatically accumulated in the midpiece of *SPATA18* -/- sperms.

Several studies have been conducted on *P53* and *SPATA18* gene expression, although their results are consistent and inconsistent with those of the present study. Other study declared that *SPATA18* expression was reduced by the direct effect of the *P53* regulatory response. However, almost all the other studies reported that this relationship was inverse (14, 30, 36-40).

After surveying the literature to find out the underlying hypothetical mechanism, the model presented by Kitamura et al. (14) was used to interpret the results. Based on the model, increasing *P53* expression and decreasing *Mieap* resulted in inducing *Mieap* by *P53*. Mieap functions by two methods. Increasing disturbance in mitochondria, they continued with lysosomes by destroying damaged mitochondria or repairing and returning mitochondria to their natural activity. Further, since knocking down the *Mieap* gene disturbs repairing, an increase in *P53* and a decrease in *SPATA18* in these sperms disturb repair of mitochondria. Consequently, destroying mitochondria leads to the reduction of sperm energy and low motility.

According to Moradi et al. (36), the *P53* level increased significantly in asthenospermic samples. The present study results demonstrated that the *P53* and *SPATA18* gene expression levels increased and decreased in the asthenospermic samples, respectively. However, in their study the ROS level in sperm samples and the activity of thioredoxin reductase (TrxR) and identified sperm DNA fragmentation were assessed by using TUNEL assay, while the SDFA kit was used in the present study to assess the DNA fragmentation level.

Ghandehari-Alavijeh et al. (37) provided a model in which hypoxia markers such as *P53* were significant in infertile males due to asthenospermia compared to fertile ones. Moreover, its high expression, such as *P53*, led to apoptosis, which is in line with the present study results showing that *P53* in the asthenospermic samples was high and directed sperm cells toward apoptosis. However, Ghandehari-Alavijeh et al. (37) assessed reactive oxygen species (ROC) in sperm cells, while the SDFA kit was used in the present study to evaluate the DNA fragmentation level.

Furthermore, considering the data obtained by comparing the sperms without and with DNA fragmentation, the *P53* and *SPATA18* expression levels decreased and increased, respectively, although the difference was insignificant.

Belloc et al. (39) reported that the decomposed fragmented sperm DNA level was more in males with asthenospermia (sperm motility defect) than in males with oligozoospermia or teratozoospermia, which is related to motility. Thus, their motility was low, and consequently, their *P53* and *SPATA18* gene expression levels increased and decreased, respectively.

Conclusion

Based on the results, low motility hinders sperm motility and *P53* prevents *SPATA18* from activation and directs cell toward DNA breaking by increasing its expression, which is considered the apoptosis background. Accordingly, *SPATA18* inactivation and increased *P53* expression during infertility can probably intensify the condition. In other words, the motility level is directly and inversely related to *SPATA18* and *P53* expression, respectively. Thus, lower motility leads to increased and decreased *P53* and SPATA18 gene expression, respectively. The viability and motility of sperm samples can be found using the gene expression panel of sperms in the future.

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Authors' Contributions

A.P.; Methodology, formal analysis, investigation, resources, and writing (original draft and editing). S.M.A.; Methodology, conceptualization, and supervision. G.A.T.; Formal analysis and resources. All authors read and approved the final manuscript.

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