Original Article

Epigenetic Dysregulation of *BRDT* Gene in Testis Tissues of Infertile Men: Case-Control Study

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Abstract

Objective: Bromodomain testis associated (BRDT), a testis-specific member of the Bromo- and Extra-Terrminal domain (BET) protein family, is involved in spermatogenesis and, more specifically, chromatin remodeling. In the post-meiotic spermatogenic cells, BRDT protein binds to the hyperacetylated histones and facilitates their replacement with transition proteins (TPs), particularly protamines, which are essential for chromatin condensation. The current research was conducted to assess the expression and epigenetic profile of *BRDT* in the testis tissues of infertile men.

Materials and Methods: In this case-control study, three groups were included: positive control group: obstructive azoospermia (OA, n=10), round spermatid maturation arrest group (SMA, n=10) and negative control group: sertoli cell-only syndrome (SCOS, n=10). Using quantitative real-time polymerase chain reaction (PCR), the expression profile of *BRDT* was generated. Also, ChIP-real time PCR was used to measure the following histone marks: H3K9ac, H3K9me3, H3K4me3, H3K27me3 on the promoter region of *BRDT*.

Results: Our data indicated that *BRDT* expression decreased in the SMA group in comparison with the positive control group and this finding is in line with the ChIP results obtained in this group.

Conclusion: Based on these data, we postulate that *BRDT* gene has a vital role in the spermatogenesis and its decreased expression due to an aberrant epigenetic signaling might be associated with male infertility.

Keywords: *BRDT*, Epigenetics, Histone Modification, Spermatogenesis Failure

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Introduction

Spermatogenesis is a dynamic process in which undifferentiated diploid cells pass a series of mitotic and meiotic divisions to produce spermatozoa cells. During the last stages of this process, spermatogenic cells' core histones are hyperacetylated and then replaced by transition proteins (TPs) and protamines (PRMs) (1, 2). Bromodomain testis associated (BRDT) binding to the acetylated histone H4, emerges a hyperacetylation signal via a BRDT-dependent replacement of histones by TPs and PRMs (3, 4).

BRDT protein, a member of bromodomain and extra-terminal (BET) protein family, is expressed in the spermatocytes as well as in the round and elongating spermatids (5). BRDT plays two types of essential roles during spermatogenesis: first, BRDT attaches to acetylated histones at promoters of meiotic and post-meiotic genes through its bromodomains to facilitate the activity of these genes in an appropriate time frame. Second, BRDT attach to hyperacetylated histones during post-meiotic phase of spermatogenesis and help their removal and substitution (6). It should be mentioned that histone hyperacetylation is an important process during histone removal and substitution in the elongating spermatids (7).

Epigenetic modifications such as histone acetylation and methylation are essential regulators in the spermatogenesis and testis genes expression. For instance, acetylation lysine 9 of histone H3 (H3K9ac) mediates gene transcriptional activity, while methylation of lysine

9 of histone H3 (H3K9me3) mediates gene transcriptional repression (8).

Furthermore, there are two other histone marks, trimethylated lysine 4 on histone H3 (H3K4me3) and trimethylated lysine 27 on histone H3 (H3K27me3), which are associated with promoters of the developmental regulator genes. H3K27me3 mediates gene silencing, while H3K4me3 is associated with the gene transcriptional activity. Contemporary presence of H3K27me3 and H3K4me3 on the genes in the stem cells, identifies a category of bivalent silent developmentally regulated genes (9).

Using testicular biopsies of infertile men, this study highlights the occurrence of an aberrant epigenetic signaling associated with a decreased expression of *BRDT*. For the first time, we made more attention to the importance of *BRDT* expression that may lead to spermatogenesis failure and men infertility.

Materials and Methods

Subjects

In this case-control study, testicular biopsy specimens were obtained from 30 infertile patients who referred to Royan Institute, Tehran, Iran. All samples were collected from testicular sperm extraction (TESE) operation in order to obtain sperm for intracytoplasmic sperm injection (ICSI). Reproductive Biomedicine Research Center and the Ethics Committee of Royan Institute, Tehran, Iran approved this study (IR.ACECR.ROYAN.REC.1394.135). Following obtain written consents from all volunteer patients, residuals of their therapeutic/diagnostic samples were used in the present study.

The samples of 3 patient groups provided our 3 study groups, including: obstructive azoospermia (OA): positive control group, round spermatid maturation arrest (SMA): SMA group and sertoli cell only syndrome (SCOS): negative control group.

RNA isolation and cDNA synthesis

In order to evaluate *BRDT* gene expression, RNA was extracted from tissue samples by using TRIzol reagent (Cat No.15596018, Ambion, USA). To eliminate genomic contaminations, the extracted RNAs were treated using Recombinant DNase I kit (RNase-free), (Cat No. 2270A, Takara, Japan). Then, cDNA was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Cat No.k1632, Thermofisher, USA) according manufacturers' instruction. Also, this synthesized cDNA was used for polymerase chain reaction (PCR) amplification, and the results were normalized by PCR reaction by using specific primers for *GAPDH* gene. All gene expression and epigenetic tests performed individually.

Gene expression analysis of *BRDT* gene by reverse transcription real-time quantitative polymerase chain reaction

The cDNA samples were quantified with RT-qPCR in the groups by using SYBR Green PCR master mix (Applied Biosystems, USA) on a Step One Plus Real-Time PCR System (Applied Biosystem, USA). Preventing DNA contaminations, primer pairs were designed using Perl Primer (v1.1.19) software from an exon-exon junction area of the genes (Table 1).

Therefore, cDNA amplification was carried out with the following profile: initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 1 minute. Each 25 µl reaction contained 2 µl of template cDNA (12.5 ng/µl), 1 µl of each 5 pmol/µl primer (Sinaclone, Iran), 12 µl of SYBR Green PCR master mix and 9 µl dH_2O.

Two replicates were carried out for each sample and relative gene expression level was quantified by using the $2^{-\Delta\Delta Ct}$ quantitative method (10) and the results were normalized with *GAPDH* gene.

Chromatin immunoprecipitation-quantitative polymerase chain reaction

Presence of acetylated histone H3K9, trimethyl H3K9, trimethyl H3K27 and trimethyl H3K4 on the promoter region of *BRDT* was evaluated by Chromatin immunoprecipitation using the Histone ChIP kit (Cat No. kch-orgHIS-012, Diagenode, Belgium) followed by real time PCR in the three studied groups, including OA (n=5), SMA (n=5) and SCOS (n=5). Anti-H3K9ac, anti-H3K9me3, anti- H3K27me, and anti- H3K4me3antibodies (Cat No. ab4441, ab8898, ab6002, ab1012 respectively, Abcam, UK) were exploited in this method. qPCR method was used in order to amplify immunoprecipitated DNA and input control DNA on a real time PCR system (Step One Plus Real time PCR system, AB Applied Biosystems, USA) by using SYBR Green master mix (Cat No. 4367659, Power SYBR Green PCR Master, Applied Biosystems, USA) and primer pairs designed using Perl primer software to cover the transcriptional start site (TSS) (-96 to +14) of the *BRDT* gene (Table 1). Also, qPCR reaction was carried out with the following profile: initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 1 minutes. The obtained results were normalized to input DNA and presented as a percentage of input DNA.

Statistical analysis

Statistical analyses, among the three groups were performed using Kruskal-Wallis test. version 8.0.2 for Windows, GraphPad Software, La Jolla California USA). Differences between groups were considered to be statistically significant at $P \leq 0.05$.

Table 1: Primer pairs used in this study

Gene	Primer sequence (5′-3′)	Annealing temperature (ºC)	Product size (bp)
RT-PCR primers			
GAPDH	F: CTCATTTCCTGGTATGACAACGA	60	122
	R: CTTCCTCTTGTGCTCTTGCT		
BRDT	F: AGAACAGGCGTCACACAGAT	58.4	109
	R: GGTGGTGATTTGGTGGCATT		
ChIP-qPCR primers			
BRDT	F: GGCTCAGACTCCTACACCTTTT	62.1	110
	R: CAGGCGCTTTTATAGAAGACCC		

RT-PCR; Reverse transcription polymerase chain reaction and ChIP-qPCR; Chromatin Immunoprecipitation quantitative real-time PCR.

Results

Expression pattern of the *BRDT* gene

The mRNA expression level of the *BRDT* gene was evaluated by using real time-RT-qPCR. Amongst the three groups, there was a decrease in the *BRDT* gene expression in the SMA group in comparison with the OA control group while, SCOS group showed less expression level (Fig.1).

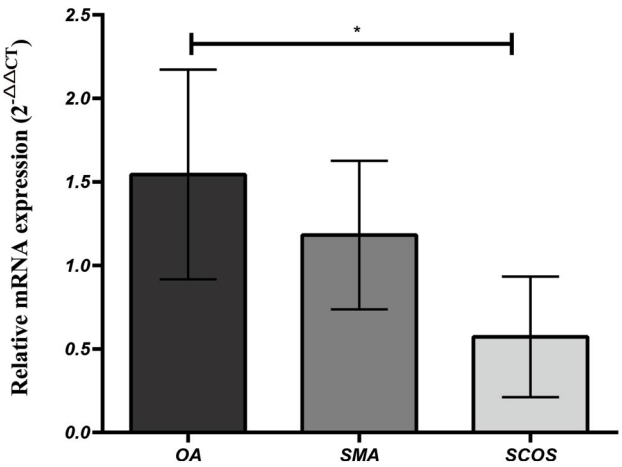

Fig.1: Relative mRNA expression of *BRDT* gene normalized to *GAPDH* in testicular samples with OA (n=10), SMA (n=10) and SCOS (n=10). Values are mean ± SEM. *; P≤0.05, OA; Obstructive azoospermia, SMA; Round spermatid maturation arrest, and SCOS; Sertoli cell-only syndrome.

Incorporation of histone marks: H3K9ac, H3K9me3, H3K4me3 and H3K27me3 on the *BRDT* gene promoter

Incorporation of H3K9ac, H3K9me3, H3K4me3, H3K27me3 on the promoter region of *BRDT* gene was evaluated by ChIP-qPCR assay. Compared to the OA control group, in the SMA group, the active histone marks of H3K9ac and H3K4me3 decreased on the *BRDT* gene promoter, while the repressive marks of H3K9me3 and H3K27me3 showed some increase in this group. All results are presented in Figure 2.

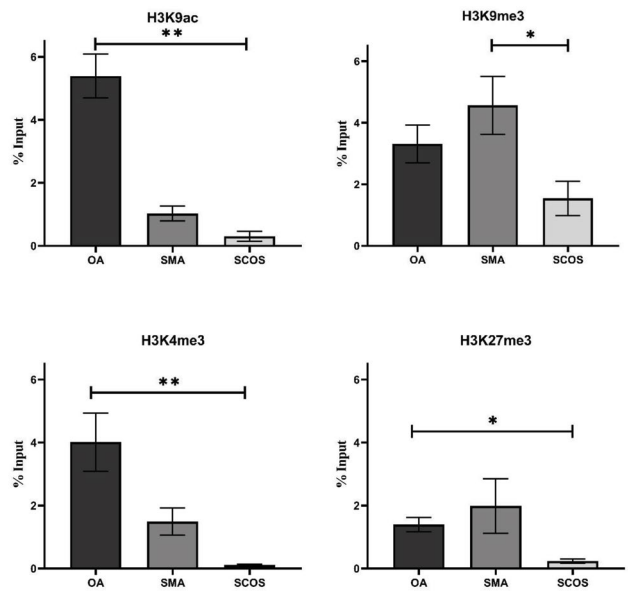

Fig.2: Incorporation of H3K9ac, H3K9me3, H3K4me3, H3K27me3 on the regulatory region of *BRDT* gene in patients of different groups: OA (n=5), SMA (n=5) and SCOS (n=5). Values are mean ± SEM. **; P≤0.01, *; P≤0.05, OA; Obstructive azoospermia, SMA; Round spermatid maturation arrest, and SCOS; Sertoli cell-only syndrome.

Discussion

Spermiogenesis is one of the most important stages of spermatogenesis. During spermiogenesis, the haploid spermatids completely transform and differentiate to mature spermatozoa which contain a highly condensed genome (11).

BRDT plays an important role in the acetylation-dependent histone substitution during spermiogenesis

(3, 4, 6) and its reduced expression level might cause spermatogenesis failure (12). Omitting first bromodomain of Brdt, Shang et al. observed defects during spermiogenesis in a mouse model (7). In parallel with these findings, Gaucher et al. (6) concluded by *Brdt* knock-out mice that Brdt is required at both meiotic and post-meiotic stages.

Our findings showed that in the infertile men with post-meiotic defects, the *BRDT* expression level is decreased in comparison with the positive control group. Also, our ChIP results indicated that the reduced expression of *BRDT* in these patients could be due to a wrong epigenetic signaling.

Indeed, association H3K9ac at the regulatory regions of *BRDT* with its active expression, a decrease in may lead to a lower the *BRDT* expression level in the SMA group. In addition, the occurrence H3K9me3 on the *BRDT* regulatory regions could prompt gene silencing in the SMA group.

Furthermore, histone methylations of H3K4me3 and H3K27me3 on the *BRDT* promoter can modulate gene expression and gene silencing, respectively. Our ChIP results showed that H3K4me3 was decreased in the SMA group, while H3K27me3 was slightly higher in the same group in compared with the positive control group. These results are completely in line with our conclusions on the epigenetic perturbation of *BRDT* genes in the SMA group. It is worthwhile to mention that in order to assess this matter more accurately, further investigations with a larger number of patients including more epigenetic marks are required.

Conclusion

It can be assumed that epigenetic regulation of *BRDT* gene and its deregulated expression is associated with male infertility in patients with round spermatid maturation arrest.

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The authors certify that all procedures contributing to this work comply with the ethical standards of the relevant national guidelines on human experimentation (testicular sperm extraction operation) and the Helsinki Declaration of 1975, 2008 revised version.

Authors' Contributions

F.K.; Study performance and manuscript drafting. P.J.; Statistical analysis, manuscript drafting and revision. R.F.; Technical performance supporter and data analysis. M.A.S.G.; Urologist and the administrative supporter for collecting samples. S.M.M.; Research supervisor. M.S.; Study designer and supervisor and manuscript revision collaborator. All authors read and approved the final manuscript.

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