

EXPRESSION PROFILING OF CREM GENE IN TESTIS WITH NORMAL AND IMPAIRED SPERMATOGENESIS IN EGYPTIAN MALES

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Testes development is a result of the interaction of hormones, cell signaling and genetic control all these factors come together to direct cellular differentiation of the testicular stem cells to form a mature testes tissue. Any defect in this network can affect the final structure of the testis. There are so many causes of testicular failure or which better described as spermatogenic arrest at one of the spermatogenesis stages. One of the most important factors is the genetic factor. When one or more of the spermatogenesis genes are missed or mutated. This can lead to spermatogenic arrest like the deletion of AZF regions on the Y chromosome.

cAMP responsive element modulator (CREM) is one of the most important transcription factors in the cAMP-mediated signal transduction in male testes which connect the extra cellular signals to gene regulation (Sassone-Corsi, 1998). The fate of spermatogenesis is to produce the haploid spermatozoa from the diploid spermatogonia. During this

process CREM proteins are highly expressed in postmeiotic germ cells (Weinbauer *et al.*, 1998; Behr and Weinbauer, 1999). CREM expression switches from repressors to activator in the testes during spermatogenesis (Foulkes *et al.*, 1992; Nantel and Sassone-Corsi, 1996).

This work aims to profile the different expression patterns of CREM activators and repressors in testicular biopsies of Egyptian males with normal and impaired spermatogenesis.

MATERIALS AND METHODS

Samples

Eighty eight Egyptian males presented with non-obstructive azoospermia were included in this study. Hormonal and genetic factors influencing testicular dysfunction were excluded.

Inclusion criteria: Normal karyotype, normal Y chromosome microdeletion analysis (AZF region), normal hormonal profile (LH, FSH and Testoste-

rone). Testicular biopsy samples were collected in (Al-Kamal Hospital for Fertility Care and Assisted Fertilization, Cairo, Egypt) from azoospermic patients opting for TESE-ICSI. All patients signed written informed consent. Each collected testicular biopsy was divided into two pieces. One piece was fixed in Bouin's fixative for histological examination and the other piece was frozen at -80°C for RNA extraction and molecular analysis.

Histological examination

Tissue was fixed in Bouin's fixative, processed for the preparation of 5 microns paraffin section using SLEE@ Microtome, United Kingdom, then stained with Hematoxylin and Eosin (Bancroft and Gamble, 2007). Stained slides were examined under light microscope to identify the testicular pattern of the studied patients using Nikon Eclipse E400 compound microscope, USA. Patients scoring for the evaluation of spermatogenesis were performed according to the method of Holstein *et al.* (2003).

RNA extraction and cDNA synthesis

Total RNA was extracted from testicular biopsies by tri-reagent (triazol, china) according to manufacture instructions. Total RNA concentration was determined using shimadzu[®] 1800 UV-spectrophotometer. First cDNA strand was synthesized using RevertAid First Strand cDNA Synthesis Kit according to kit protocol (#K1622, Thermo Scientific, USA). Reaction components were mixed as follows: 2 μg of RNA, 0.5 μl (10 mmol) Oli-

go dt, and 0.5 μl (10 mmol) random dimer. Then completed to 12.5 μl using DEPC treated water and mixed well then incubated for 5 min at 70°C to remove RNA secondary structure then chilled in ice and 2 μl (10 mmol) dNTPs, 4 μl 5X reaction buffer, 0.5 μl Ribolock and 1 μl reverse transcriptase were added, final reaction volume was 20 μl , reaction mixture spun down and incubated at room temperature for 10 min then 42°C for 1 h followed by 10 min at 70°C as a termination step. cDNA samples were stored at -20°C for further use.

CREM profile analysis

In order to amplify any expected CREM variants in tested samples, the following primers were used: forward primer: 5'-ATGACCATGGAAACAGTTGAATC-3' positioned in CREM exon B from 1-23 of the human cDNA sequence and the reverse primer: 5'-CTGTAATCAGTTCATAGTTAAA-TATTT CTA-3' positioned on CREM 3'UTR reverse position 1429-1400 of the human cDNA sequence (Masquillier *et al.*, 1993). These primers amplified from the 5' of exon B to the 5' of exon I_b. The nomenclature of exonic organization of the CREM is based upon that provided by Walker and Habener (1996)

PCR

Approximately 0.2 μg cDNA template was used in a 25 μl total volume PCR mix (Takara[®] max 2X PCR master mix) and 20 pmol of each primer. Cycling

conditions: 3 min at 94°C as initial denaturation followed by 30 cycles at 94°C for 30s, 56°C for 40s and 72°C for 1.3 min. Then followed by final elongation at 72°C for 10 min. 12 µl of each tube were separated on 2% w/v agarose gel using promega® TAE 1X running buffer and the gel was stained with ethidium bromide and visualized under UV-transilluminator.

RESULTS

According to histological data, patients were classified into three main groups:

1. Normal spermatogenesis (control group)
2. Impaired spermatogenesis (Tested group)
3. Sertoli cell only (Negative control group)

The 2nd group was sub grouped into two sub groups:

- a- Hypospermatogenesis
- b- Round spermatid maturation arrest

PCR results and fragment analysis

CREM profile in the normal spermatogenesis

Gel electrophoresis of the resulted RT-PCR for the Normal spermatogenesis group which was representing 25% from the total studied samples, gave two sharp bands, one at 882 bp which corresponds to CREM τ 2 activator and the second band was at 641 bp which represented gamma repressor isoform as shown in Fig. (1).

CREM profile in the impaired spermatogenesis

a) Hypospermatogenesis

Patients with hypospermatogenesis represented 27% from the total studied samples. Amplification of testicular tissue cDNA from these patients resulted in three products: 882 bp represented CREM τ 2, 641 bp and 1131 bp represented CREM repressors, the last one considered to be variant 19 (alpha repressor isoform) as shown in Fig. (1).

b) Round spermatids maturation arrest

Round spermatids group represented 28% from the total studied samples. Ninety percent of this group exhibits the 1095 bp and the 452 bp PCR products which represented repressors isoforms as shown in Fig. (1). Only 10% of this group showed also 882 bp and 1284 bp bands which represented CREM τ 2 activator in addition to repressor isoform (data not shown).

CREM profile in sertoli cell only

Sertoli-cell-only group, which represented 20% from the studied samples, didn't show any CREM bands. These results were expected as CREM repressors/activators expression is restricted to germ cells only, except for Induced cAMP Early Repressor (ICER) which is considered the only CREM isoform expressed in Sertoli cells (Sassone-Corsi, 1997) and it was not targeted by the used primers in this study (Fig. 1).

DISCUSSION

cAMP responsive element modulator (CREM) isoforms switches during male germ cell development from repressors in premeiotic cells to an activator form (CREM τ) in post-meiotic cells, based on alternative splicing, alternative polyadenylation and alternative translation initiation (Kosir *et al.*, 2012). CREM activators specifically expressed in haploid germ cell prior to spermatid elongation (Behr and Weinbauer, 1999). Homozygous CREM knocked out mice exhibit complete arrest of spermatogenesis at the stage of round spermatids and several fold increase in the number of apoptotic germ cells (Blendy *et al.*, 1996). More recent studies have reported that the absence of CREM deregulates over 4700 genes in CREM knocked out testis. Among them there are 101 genes associated with spermatogenesis, 41 of which are directly regulated by CREM (Kosir *et al.*, 2012).

The present study represents the first trial to profile the CREM transcription pattern in Egyptian males with spermatogenic arrest.

Results showed that the normal testis contained two transcript isoforms of CREM gene; one activator CREM τ_2 and one repressor isoform (gamma repressor). This proof that the CREM repressors didn't completely switch off during activator production but it is still expressed in adult testis with normal spermatogenesis. This was also reported by Behr and Weinbauer (2000). This could be a kind of reg-

ulation control through the affinity between repressors and activators to cAMP Responsive Element (CRE) binding sites. Behr and Weinbauer (2000), in their RT-PCR, yielded five distinct bands for this group, one activator 882 bp and four repressors 852 bp, 693 bp, 641 bp and 452 bp. While the present study found the 882 bp activator and the 641 bp repressor only in the Normal spermatogenesis group. This discrepancy could be attributed to the difference in histological classification. Behr and Weinbauer (2000) considered in their study the Hypospermatogenesis and Normal spermatogenesis as one group, but the present study categorized the Hypospermatogenesis and Normal spermatogenesis into two different groups.

Hypospermatogenesis group showed a longer repressor isoform, the 1131 bp band, which represented alpha repressor, with the presence of gamma repressor, the 641 bp band, and the CREM τ_2 activator, the 882 bp band. This finding may explain the reduced number of sperm per tubules in this group. It also supported the hypotheses, of the synergistic effect between CREM repressors and each other; as well as the antagonistic effect between CREM repressors and activators.

From the obtained data in Hypospermatogenesis group for the CREM gene expression it could be concluded that the presence of some unusual repressor transcripts might alters the CREM τ_2 concentration and activation capacity.

Ninety percent of the round spermatids maturation arrest group showed two types of repressors without any detected signal of CREM activators, which may be caused by the failure in the CREM switch machinery or splicing machinery. This might lead to the missing of the activator isoforms. This 90% results the same finding reported by Behr and Weinbauer (2000). But the other 10% showed signal for the CREM τ activators which is 882 bp for the transcriptions variant 1 and the 1284 bp for the transcriptions variant 21.

SUMMARY

cAMP responsive element modulator (CREM) is one of the most important transcription factors expressed in male testes which connect the extra cellular signals to gene regulation. CREM expression switches from a repressor to an activator in testes during spermatogenesis. Any alteration in this switch leads to different testicular patterns. Methods: Total RNA was extracted from Eighty eight testicular biopsy and analyzed for CREM expression profiling. Normal spermatogenesis showed expression of CREM τ_2 and gamma repressor, the same finding was detected in Hypospermatogenesis group but with another longer repressor belong to alpha repressor type, 95% of Round spermatid maturation arrest group showed two repressor patterns, and 5% showed two activator transcripts of CREM τ_2 activator. Sertoli cells only group didn't show any activator transcripts of CREM. In conclusion studying the mechanisms of interaction between CREM repressors and

activators in the normal and impaired spermatogenesis may lead to better understanding of genetic control of the spermatogenesis and consequently to better treatment options.

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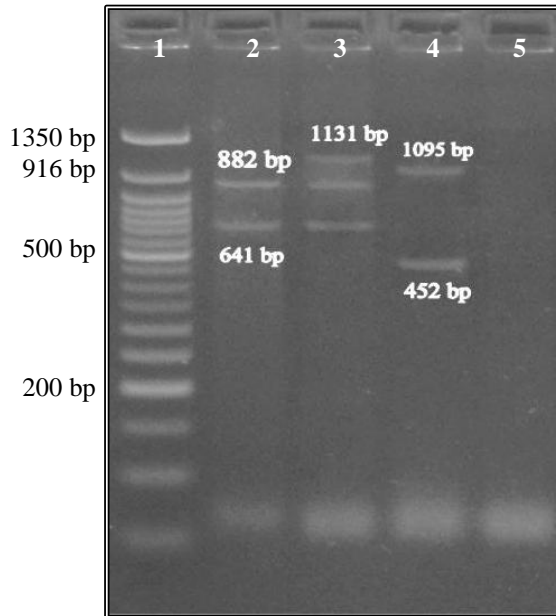


Fig. (1): Agarose gel (2%) demonstrated the different expression pattern of CREM isoforms in normal and impaired spermatogenesis.

Lane 1: represents 50 bp DNA marker.

Lane 2: control group with normal spermatogenesis showing tau2 activator at 882 bp and the CREM delta C-F (gamma) repressor at 641 bp.

Lane 3: hypo-spermatogenesis group showing the same two bands in lane (1) 882 bp, 641 bp and one new repressor 1131 bp which represent type alpha repressor.

Lane 4: Round spermatids maturation arrest showing two bands of CREM repressors 1095 bp and 452 bp.

Lane 5: Sertoli cell only showing no bands.