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ARTICLE

Methylation pattern of methylene tetrahydrofolate reductase and small nuclear ribonucleoprotein polypeptide N promoters in oligoasthenospermia: a case-control study

Anca Botezatu [a], Razvan Socolov [b,*], Demetra Socolov [b], Iulia Virginia Iancu [a], Gabriela Anton [a]

[a] *Stefan S. Nicolau-Institute of Virology, Bucharest, Romania;* [b] *Grigore T. Popa-University of Medicine and Pharmacy, Iassy, Romania*

[*] Corresponding author. *E-mail address:* razvan.socolov@yahoo.com (R Socolov).

Anca Botezatu has a PhD in the Department of Viral Genetics from Institute of Virology, Bucharest. As a specialist in molecular biology and genetics, she was involved in projects focused on viral and cell mechanisms involved in oncogenesis. Her research area also includes epigenetics changes in various pathological conditions (analysis of global genome methylation status, tumour suppressor gene promoter hypermethylation, including miRNA, and the role and functions of DNA methyltransferases). Also, key enzymes involved in folate metabolism are directions of her research area.

Abstract Alterations in DNA methylation patterns in several genes may lead to abnormal male sexual development and infertility. This study investigated the promoter methylation status of *MTHFR* and *SNRPN* in infertile men from Romania by quantitative methylation-specific PCR in order to investigate possible correlations with sperm abnormalities. The study groups included patients ($n = 27$) with a median age of 31 years (range 26–41 years) as well as controls ($n = 11$) with a median age of 30 years (range 24–37 years) recruited from couples seeking advice for infertility. DNA was isolated from sperm samples and promoter methylation was assessed using direct. Significant trends were detected for both genes that indicate a tendency towards promoter hypermethylation in spermatozoa with low motility (*MTHFR* $P = 0.0032$, $r = 0.23$; *SNRPN* $P = 0.0003$, $r = 0.32$) and poor morphology (*MTHFR* $P = 0.0012$, $r = 0.27$; *SNRPN* $P = 0.0003$, $r = 0.33$) but no trend was found in cases of low sperm count (*MTHFR* $r = 0.007$; *SNRPN* $r = 0.06$). The data indicate that the methylation patterns of the promoters of *MTHFR* and *SNRPN* are associated with changes in sperm motility and morphology, which could lead to male infertility.

KEYWORDS: DNA hypermethylation, epigenetics, male infertility, MTHFR, quantitative methylation-specific PCR, SNRPN

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Introduction

Male infertility refers to the inability of a male to achieve a pregnancy in a fertile female and accounts for 40—50% of infertility cases (Brugh and Lipshultz, 2004). Despite the progress in the understanding of human reproductive physiology, the causes of male infertility have not been fully elucidated. The aetiology of impaired sperm production and function can be related to different congenital or acquired factors acting at the testicular level (World Health Organization, 2010). Traditionally, the diagnosis of male infertility is based upon a semen profile, according to World Health Organization (WHO) guidelines. The evaluation of male infertility relies upon a physical examination as well as semen and hormone analyses. Although these evaluations are quick and relatively inexpensive, they often do not explain the cause of infertility or predict the usefulness of various therapeutic interventions. Therefore, new approaches are needed to identify the aetiology of male infertility.

In recent studies there has been considerable focus on epigenetic modifications as significant contributors to reproductive pathology in the human male. Properly functioning epigenetic processes are required to ensure both appropriate sperm function and embryonic development (Rajender et al., 2011). The most studied epigenetic mechanism is DNA methylation, which occurs at cytosine bases in CpG islands and plays an important role in cellular processes including embryonic development, genomic imprinting, and X-chromosome inactivation (Paoloni-Giacobino and Chaillet, 2004; Reik et al., 2001). The DNA methylation pattern is the result of complex interactions between de-novo methylation and the maintenance of pre-existing methylation. In the course of gametogenesis DNA methylation patterns are re-established in a sex-specific manner (Rajender et al., 2011).

The effects of DNA methylation on the expression of genes that affect the development of male reproductive organs, spermatogenesis and male sexual behaviour has been previously investigated (Cisneros, 2004; Filipponi and Feil, 2009; Trasler, 2009). Hypermethylation of several genes was associated with impaired sperm parameters and male infertility. Among these genes are those that encode methylene tetrahydrofolate reductase (*MTHFR*) and small nuclear ribonucleo-protein associated protein M (*SNRPN*). *MTHFR* codes for an enzyme with an important role in maintaining the pool of methyl donors, while *SNRPN* is a maternal imprinted gene. Moreover, *MTHFR* is a good example to highlight the role of gene—environment interaction in the development of phenotype.

Methylene tetrahydrofolate reductase promoter methylation has been shown in recent years to exhibit the most promising association with azoospemia (Khazamipour et al., 2009) and recurrent spontaneous abortion (Rotondo et al., 2012), but few studies have been performed on small nuclear ribonucleoprotein polypeptide N methylation, and the association with oligoasthenospermia. The present study aims to investigate the methylation status of the promoters of *MTHFR* and *SNRPN* in infertile males from Romania and to examine correlations with sperm abnormalities.

Materials and methods

Study groups

The patients included in this study ($n = 27$, median age 31 years, range 26—41 years) were recruited from couples presenting at the Cuza Voda Hospital and at a private medical centre who were seeking advice for infertility between January 1 and 1 April 2013. The exclusion criteria for this study referred to previous or current maldescended testes, physical injury to the testes, the absence of secondary sexual characters, varicocele, chromosomal or known genetic aberrations, infection, use of medications that impair spermatogenesis and/or fertility, impairment of the endocrine system, any other causes of male infertility. All patients had been married for a minimum of two years and did not use contraception.

The control group ($n = 11$, median age 30 years, range 24—37 years) consisted of semen from donors with normal semen parameters who had also presented with problems conceiving, but where the infertility was ultimately attributed to the female. The inclusion criteria for controls were therefore: normal semen parameters (according to the WHO standards of 2010 (World Health Organization, 2010), matched for age, and signing the informed consent to participate in the study; exclusion criteria were sperm donors with an inadequate period of abstention, with known genetic illnesses or not accepting to sign the consent form. The seminal fluid was collected after 3—4 days of sexual abstinence.

Semen analysis

Analysis of sperm count, motility and morphology was carried out according to the guidelines of WHO. The study was approved by ethics committee of the University of Medicine and Pharmacy, Iassy, Romania (reference no. 4335, approved on 13 March 2013).

DNA isolation

DNA was isolated using a High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The DNA concentration and purity of all samples was evaluated using a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA).

Unmethylated C-residue conversion

Unmethylated C-residue conversion was performed via bisulphite treatment using an EpiTect Bisulfite kit (Qiagen, Valencia, California) according to the manufacturer's instructions. Each DNA sample (700 ng/μl) was treated with sodium bisulphite, which leads to the conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. Positive and negative controls (CpGenome Universal Methylated DNA and CpGenome Universal Unmethylated DNA, respectively; Millipore, Billerica, MA, USA) were included. Aliquots of bisulphite treated samples were stored at −80°C.