MOLECULAR ANALYSIS OF GUANIDINOACETATE-N-METHYLTRANSFERASE (*GAMT*) AND CREATINE TRANSPORTER (*SLC6A8*) GENE BY USING DENATURING HIGH PRESSURE LIQUID CHROMATOGRAPHY (DHPLC) AS A POSSIBLE SOURCE OF HUMAN MALE INFERTILITY

FURHAN IQBAL[1, 2 *], CHIKE BELLARMINE ITEM[2], RENE RATSCHMANN[2], MUHAMMAD ALI[3], EUGEN PLAS[4] AND OLAF BODAMER[2, 5]

[1]*Institute of Pure and Applied Biology, Zoology Division. Bahauddin Zakariya University, Multan, Pakistan*
[2]*Department of Pediatrics and Adolescent Medicine, Laboratory for Inherited Metabolic Disorders, Medical University of Vienna, Austria*
[3]*Institute of Biotechnology, Bahauddin Zakariya University, Multan, Pakistan*
[4]*Department of Urology, KH Heitzing mit Neurologischem Zentrum. Rosenhuge 1, Wolkersbergenstrasse 1, 1130, Viienna, Austria*
[5]*Institute for Inherited Metabolic Diseases, Paracelsus Medical School Salzburg, Austria*

ABSTRACT

The creatine/phosphocreatine system is essential for cellular phosphate coupled energy storage and production, particularly in tissues subject to high metabolic demands. Male factor infertility is a common condition with unknown etiology in most of the cases. Sperm abnormalities could possibly lead to infertility. As sperm motility depends on intact mitochondrial function and energy levels. Thus reduced intracellular creatine stores may contribute to decreased sperm motility leading to male infertility as creatine /phosphocreatine system plays major role in making and breaking of ATP, thus in energy kinetics. We developed and validated a denaturing high performance liquid chromatograph (DHPLC) method for the molecular analysis of *SLC6A8* and *GAMT* genes involve in creatine biosynthesis and transport as a possible source of human male infertility by analyzing DNA from 64, clinically confirmed, infertile men. No mutation/polymorphism was detected in the exonic regions of both genes in all the patients and in fertile healthy controls indicating that *SLC6A8* and *GAMT* genes may not be directly involved in human male infertility.

Keywords: Male infertility, creatine transporter, *GAMT*, DHPLC.

INTRODUCTION

Creatine provides a readily available source of energy in the form of a high energy phosphate bond. It functions both a reservoir for ATP derived energy and as a mean for moving energy from the site of production in the mitochondria to the sites of consumption, such as muscle contractile apparatus, by a mechanism that has been termed the phosphocreatine circuit (Wallimann *et al*., 1993).The human pool of creatine is composed by external dietary supply and endogenous production in the cytosol, requesting two enzymes; L-arginine:glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT), in kidney (AGAT>GAMT), liver (GAMT>AGAT) and pancreas (high levels of both enzymes) (Wyss *et. al*., 2000). The role of Na^+ and Cl^- dependent creatine transporter (CrT) is to take up the creatine from blood and transport in to the creatine requiring tissues against large concentration gradient (Schulze, 2003).

**Corresponding author*: e-mail: furhan.iqbal@bzu.edu.pk

Disorders of the creatine metabolism can be classified into two groups, either caused by disorder of the creatine synthesis, GAMT-deficiency (McKusick 601240) (Stockler, 1996) and AGAT-deficiency (McKusick 602360) (Battini *et. al*., 2002; Item, 2001), or caused by a disorder of the creatine transporter (SLC6A8) deficiency (McKusick 300352) (Salomons, 2001).

SLC6 protein family has another member *SLC6A10,* generally known as pseudo gene of creatine transporter or creatine transporter 2. In opposite of *SLC6A8* mRNA which can be found in several tissues (Chen *et. al*., 2004; Salomons *et. al*., 2001), *SLC6A10* mRNA has exclusively been detected together with mRNA of *SLC6A8* in testis (Iyer, 1996). The frequency of creatine and related enzymes in the organs of the reproductive tract imply the importance of creatine for a well functioning male fertility (van Dorsten *et al.,* 1997; Lee, 1998). In spermatozoa, creatine/phoshpocreatine system is present to transfer energy from mitochondria to the flagellum, which is essential for the swimming of sperms. A deficiency of creatine may explain low sperm motality in some infertile men. Deficiencies of enzymes in the creatine synthesis

pathway are known to occur (Newsholme and Leech, 2010).

We hypothesized that as sperm motility depends upon intact mitochondrial function and energy storage, reduced intracellular creatine levels either because of abnormal creatine production (*GAMT* deficiency) or transport (*SLC6A8* deficiency) may contribute to human male infertility. We have developed a DHPLC method for the molecular analysis of *SLC6A8* and validated DHPLC method for *GAMT* gene, already established by Item et al,. (2005), for their molecular analysis in order to detect genetic changes in these two genes as possible source of human male infertility.

MATERIALS AND METHODS

Patients

Molecular analysis of *SLC6A8* and *GAMT* was done in 64 Austrian patients with infertility referred by Department of Urology, University General Hospital Vienna along with healthy fertile controls.

Reagents

Oligonucleotides were synthesized by VBC Genomics (Vienna, Austria) and Taq DNA polymerase was purchased from Transgenomic (UK).

DNA extraction

DNA extraction was carried out from dry blood spot filter paper samples and from EDTA blood using Qiagen DNA extraction kit (Qiagen, U.S.A.) following standard procedures.

Polymerase chain reaction

Genomic DNA of all infertile men and healthy fertile controls was subjected to a polymerase chain reaction (PCR) using primer pairs covering all 13 exons of *SLC6A8* and 6 exons of *GAMT* including the adjacent intronic boundaries. PCR products of *GAMT* exons were put to heteroduplex formation which consist of mixing of equal volumes of control and patient exon specific PCR amplicon followed by denaturation and reannealing of patient and control alleles forming a heteroduplex which will latter be detected by DHPLC. Creatine transporter gene is located on X chromosome (deGrauw, 2003) so it was not put to heteroduplex formation and PCR amplicon were directly run on DHPLC. The details of primer for *SLC6A8* and DHPLC conditions are given in table while for *GAMT* we used the primers and DHPLC conditions already established by Item *et al*. (2005).

DHPLC

DHPLC analysis for *SLC6A8* and *GAMT* was carried out using a Wave DNA Fragment Analysis System (Transgenomic Ltd., UK) equipped with a high pressure pump, a column heater and a UV-detector. The samples were kept at 4^{o}C until 5µl of sample was applied to a preheated C18 reversed phase column (DNASep cartridge, Transgenomic Ltd. UK). DNA was eluted at a flow rate of 0.9 mL/min with in a linear acetonitrile gradient consisting of buffer A (0.1M triethylammonium acetate; TEAA)/buffer B (0.1 M TEAA, 25% acetonitrile). The retention time was measured online via the UV absorption of the eluate that was monitored at 260 nm. The temperature at which Amplicon detection occurred was deducted from the Transgenomic software (Wavemaker 3.4) which analyses the melting profile of the specific DNA fragment.

DNA Sequencing and mutation analysis

In case of abnormal DHPLC histogram sequencing of the affected DNA was carried out along with the control. Sequencing was performed by a commercial sequencing service (VBC Genomics, Vienna, Austria). Chromas pro (Version 1.41) software was used for the mutational analysis of all the DNA sequences.

RESULTS

Molecular analysis of 64 infertile men on DHPLC revealed no mutation/polmorphism in DNA sequences of both genes when compared with the healthy fertile men. Results were confirmed by direct sequencing of selected PCR amplicons of both genes from patients and controls revealing the same genetic sequence without any change. These findings indicates that creatine synthesis and transport machinery is intact in the infertile men understudy and for atleast these patients these two genes do not plays any role in human male infertility.

DISCUSSION

Denaturating High Performance Liquid Chromatography (DHPLC) is a semi-automated technique for screening mismatches in DNA, and is increasingly being used to test for disease causing mutations (Xiao and Oefner, 2001; Bodamer *et. al*., 2009; Kasper *et al*., 2010). Genetic mutations and single nucleotide polymorphisms (SNPs) detection is based on DNA heteroduplex formation and separation of heteroduplex from homoduplex molecular species under partial denaturing conditions by means of ion-pair reverse phase HPLC. Single-base substitutions, small insertions or deletions can be distinguished by their denaturing profiles compared to their corresponding normal homoduplex. After identifying the precise mutations, amplicons with abnormal DHPLC profiles can then be sequenced as a following step (Iqbal *et. al*., 2010). Due to the complexity of the fragment-specific melting curve we developed a protocol using GC-clamps at the 5' or 3' ends of the primers that converts the target DNA sequence into a single low melting domain. As a result only one temperature is required for the complete analysis of each fragment.

Table: Primers for PCR amplification and DHPLC conditions for molecular analysis of *SLC6A8*.

DNA Fragments	Primer Sequence	Amplcon Size (bp)	PCR Annealing Temperature (°C)	DHPLC Temperature (°C)
Exon 1A	5´TCGTGGACTGCTTCTGACTG3´ 5´ACCTCCGCCGTTCTTGTAG3´	466	66	67.8
Exon 1B	5´GCCAGATGGACTTCACTATG 3´ 5' CACGCTCTCCTCCCCGGTGT 3'	295	60	65.8
Exon 2A	5´TGGGCCTGGGCAGCCTGG 3´ 5´GGAGCCAGCTGGGAGAAGGG 3´	244	66	62.6
Exon 2B	5'-(GC)- TGCCATCTGATAACAGACTA-3' 5'-GGGGAGACGGCATGAAGTC-3'	180	66	57.7
Exon 3	5'-(GC)- TCGGGAGGTGGCCAGGGAAGA-3' 5´GCAACATGAGGGAGACCTGGG 3´	381	66	63.1
Exon 4	5´GCTCCATCCTCTGCTGGCAC 3´ 5'-(GC)- CGACATTTGTGTTGAACTGG-3'	235	66	63.4
Exon 5	5´CCACAGCCTCCGCTGAGCAG 3´ 5'-(GC)- CCTCCACCTCACCTGAGGGG-3'	220	66	64.4
Exon 6	5'-(GC)- CCCCTCATGCCTGCGCTCTC-3' 5´CAGGGCAGGACAGGGCACGG 3´	119	66	64
Exon 7	5'-(GC)- ACTCTGGCCCCTCCACCCCT-3' 5´GCACTCGCTGCTCTGGAGGC 3´	230	66	64.4
Exon 8	5´CACAGGGCAGGACATCGGCT 3´ 5´TGCCTGCAGCCCTCCCTC 3´	223	66	65.1
Exon 9	5´GGCTGCAGGCAAGGAAAGGG 3´ 5'-(GC)- GATGGCAGACGGCCACCAGA-3'	277	66	63.8
Exon 10	5´CCTCTGGTGGCCGTCTGC 3´ 5´GCTGGAGACCTGCCTTCCC 3´	209	66	65.1
Exon 11	5´TGGCTGAGGGCTGGGCTG 3´ 5´ACCAGAATGCTGCGGTTAATGGG 3´	258	66	64.6
Exon 12	5´TCTGCATGGTAAGGGCTGGG 3´ 5´GGAAGCAGGCTGGGTTGAATG 3´	328	61.4	63.8
Exon 13	5´GTGGGAACCGGAGAGAGGCA 3´ 5'-(GC)- TCCCGCCGCCCCCGCCCG-3'	360	66	64.2

(GC) = CGCCCGCCGCGCCCCGCGCCGTCCCGCCGCCCCCGCCCG

The etiopathogenesis of testicular failure remains unknown in about half of the cases and is referred to as "idiopathic infertility". Idiopathic testicular failure is of probable genetic origin since numerous genes are involved in human spermatogenesis and only a small proportion of them have been identified and screened in infertile men (Tanaka *et al.*, 2007).

Numerous male mouse models, mutation screening and association studies reported over the last few years reveals the high prevalence of genetic causes of spermatogenic impairment, accounting for 10-15% of severe male infertility, including chromosomal aberrations and single gene mutations. Despite many efforts, only a few clinically relevant polymorphisms have been identified and a large proportion of infertile males are idiopathic, reflecting poor understanding of the basic mechanisms regulating spermatogenesis and sperm function (Ferlin *et al.*, 2007).

The most promising polymorphisms are in genes involved in the endocrine regulation of spermatogenesis and on the Y chromosome, the "gr/gr" deletions. Polymorphisms are generally considered as co-factors. Their final effect on testis function and fertility is probably modulated by the genetic background of each individual and/or by the presence of certain environmental factors (Krausz and Giachini, 2007).

St John *et al* (2007) has described the role of sperm mitochondria in fertilization. According to their study, oxidative phosphorylation plays important role in sperm motility and function. This gave us the idea to analyze *SLC6A8* as creatine transporter might be involved in the transport of creatine and subsequently its conversion into creatine phosphate, which acts as an energy reservoir in the cells with higher energy demands (Bessman and Carpenter, 1985). So anz genetic change in creatine transporter gene could lead to male infertility.

Schmidt *et al* (2004) mentioned that GAMT deficiency in mice is associated with increased neonatal mortality, muscular hypotonia, a non-leptin-mediated life-long reduction in body weight due to reduced body fat mass and decreased male fertility. This led us to the hypothesis that change in human GAMT gene could result in male infertility. A detailed DHPLC analysis of *SLC6A8* and *GAMT* genes followed by DNA sequencing, in infertile men, revealed that these genes has possibly no direct role in male infertility as we were unable to detect any change in DNA sequence of infertile men when compared with the confirmed control samples.

ACKNOWLEDGEMENTS

We are indebted to the physicians who sent us samples for *SLC6A8* and *GAMT* mutation analysis.

REFERENCES

Battini B., Leuzzi V, Carducci C, Tosetti M, Bianchi MC, Item CB, Stockler-Ipsiroglu S and Cioni G (2002). Creatine depletion in a new case with AGAT deficiency: clinical and genetic study in a large pedigree. *Mol Genet Metab*. **77**: 326-331.

Bessman SP and Carpenter CL (1985). The creatine-creatine phosphate energy shuttle. *Annu. Rev. Biochem.* **54**: 831-862.

Bodamer OA, Iqbal F, Mühl A, Hung C, Prayer P, Ratschmann R and Item CB (2009). Low creatinine – The diagnostic clue for a treatable neurological disorder. *Neurol.* **72**: 854 - 855.

Chen NH, Reith ME and Quick MW (2004). Synaptic uptake and beyond: The sodium-and chloride-dependent neurotransmitter transporter family *SLC6. Pflugers. Arch.* **447**: 519-531.

van Dorsten FA, Wyss M, Walliman T and Nicolay K. (1997). Activation of sea urchin sperm motality is accompanied by an increase in the creatine kinase exchange flux. *Biochem. J*. 325: 411-416.

de Grauw TJ, Cecil KM, Byars AW, Salomons GS, Ball WS and Jakobs C (2003). The clinical syndrome of creatine transporter deficiency. *Mol. Cell Biochem.* **244**: 45-48.

Ferlin A, Raicu F, Gatta V, Zuccarello D, Palka G and Foresta C (2007). Male infertility: role of genetic background. *Reprodu. Biomed. Online.* **14**: 734-45.

Item CB, Stockler-Ipsiroglu S, Stomberger C, Muhl A, Alessandri, MG, Bianchi MC, Tosetti M, Fornai F and Cioni C (2001). Arginine:glycine amidinotransferase deficiency: The third inborn error of creatine metabolism in humans. *Am. J. Hum. Genet.,* **69**: 1127-1133.

Item CB, Mercimek-Mahmutoglu S, Battini R, Edlinger-Horvat C, Stromberger C, Bodamer OA, Muhl A, Vilaseca MA, Korall H and Stockler-Ipsiroglu, S (2004). Characterization of seven novel mutations in seven patients with GAMT deficiency. *Hum. Mutat*. **23**: 524.

Item CB, Stockler-Ipsiroglu S, Willheim C, Muhl A and. Bodamer OA (2005). Use of denaturing HPLC to provide efficient detection of mutations causing guanidinoacetate methyltransferase deficiency. *Mol. Genet. Metab*. **86**: 328-334.

Iqbal F, Item CB, Vilaseca MA, Jalan A, Mühl A, Couce ML, Duat A, Delgado MP, Bosch J, Puche A, Campistol J, Pineda M and Bodamer OA (2010). The identification of novel mutations in the biotinidase gene using denaturing high pressure liquid chromatography (DHPLC). *Mol. Genet. Metab*., **100**: 42-45.

Iyer GS (1996). Identification of a testis-expressed creatine transporter gene at 16p11.2 and confirmation of the X-linked locus to Xq28. *Genomics*, **34**: 143-146.

Kasper DC, Iqbal F, Dvorakova L, Zeman J, Magner M, Pollak A, Herkner KR and Item CB (2010). Rapid and Accurate Denaturating high performance liquid chromatography protocol for the detection of α-L-Iduronidase mutations causing Mucopolysaccharidosis Type I. *Clinl. Chem. Acta.,* **411**: 345-350.

Krausz C and Giachini C (2007). Genetic risk factors in male infertility. *Arch. Androl*., **53**: 125-133.

Lee HJ, Kim H, Chae YJ, Oga H, Wa M, Lee H and Gerton GL (1998). Creatine synthesis and transport systems in the male rat reproductive tract. *Biol. Reprod.,* **58**: 1437-1444.

Newsholme E and Leech T (2010). Functional Bio-chemistry in health and disease. John Wiley and Sons, UK, pp.194-195.

Salomons GS, van Dooren SJ, Verhoeven NM, Cecil KM, Ball WS, deGrauw TJ and Jakobs C (2001). X-linked creatine-transporter gene (*SLC6A8*) defect: a new creatine-deficiency syndrome. *Am. J. Hum. Genet.,* **68**: 1497-1500.

Schmidt A, Marescau B, Boehm EA, Renema WK, Peco R, Das A, Steinfeld R, Chan S, Wallis J, Davidoff M, Ullrich K, Waldschutz R, Heerschap A, de Deyn PP, Neubauer S and Isbrandt D (2004). Severely altered guanidino compound levels, disturbed body weight homeostasis and impaired fertility in a mouse model of

guanidinoacetate N-methyltransferase (*GAMT*) deficiency. *Hum. Mol. Genet*., **13**: 905-921.

Schulze A (2003). Creatine deficiency syndrome. *Mol. Cell Biochem.,* **244**: 143-150.

St. John JC, Bowles EJ and Amaral A (2007). Sperm mitochondria and fertilization. *Soc. Reprod. Fertil. Suppl*., **65**: 399-416.

Stockler S, Isbrandt D, Hanefeld F, Schmidt AB and von Figura K (1996). Guanidinoacetate methyltransferase deficiency: the first inborn error of creatine metabolism in man. *Am. J. Hum. Genet*., **58**: 914-922.

Tanaka HM, Hirose K, Tokuhiro Y, Matsuoka Y, Miyagawa A, Tsujimura A, Okuyama A and Nishimune Y (2007). Single nucleotide polymorphisms: discovery of the genetic causes of male infertility. *Soc. Reprod. Fertil. Suppl*., **65**: 531-534.

Wallimann T, Wyss M, Brdiczka D, Nicollay K and Eppenberger HM (1993). Significance of intracellular compartmentation, structure and function of creatine kinase isoenzymes for cellular energy homeo-stasis: "The Phospho-Creatine Circuit". *Biochem. J*.. **281**: 21-40.

Wyss M, Kaddurah D and Rima A (2000). Creatine and Creatinine Metabolism. *Physiol. Rev*.. **80**: 1107-1213.

Xiao W and Oefner PJ (2001). Denaturing high-performance liquid chromatography: A review, *Hum. Mutat*., **17**: 439-474.