Genetic Markers of Male Infertility: Y Chromosome Microdeletions and Cystic Fibrosis Transmembrane Conductance Gene Mutations

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Today, approximately 15% of couples have reduced fertility. In most cases the reason is male infertility, usually of genetic origin. Thus, in the context of research in genes involved in reproduction and sex determination, genetic defects in gametogenesis are being extensively studied. The most frequent pathogenic causes of male infertility are Y chromosomal microdeletions and obstructive azoospermia due to congenital absence of the vas deferens (CAVD) in the presence of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. We have investigated the most common CFTR gene alterations in Croatian men with CAVD, using Roche research prototype assays. Results revealed that the 5T variant was present in 27% of the subjects. The F508 deletion was found in 21% of the subjects. It was the most frequent mutation, although its incidence was much lower than among patients with cystic fibrosis. The prevalence of microdeletions in the azoospermia factor region (AZF) of the Y chromosome in Croatia was 4.5%. This is the first report of Y microdeletions in the Croatian population. Genetic counseling of all couples with the diagnosis of male infertility is recommended before intrauterine insemination, in vitro fertilization, and intracytoplasmic sperm injection, and should also include AZF and CFTR genotyping. Couples requesting assisted reproductive treatment should be offered molecular analysis of the CFTR gene, if male infertility due to obstructive azoospermia is the underlying cause. Also, men with severe oligozoospermia or non-obstructive azoospermia seeking assisted reproductive treatment should be screened for deletions in the Y chromosome.

Key words: cystic fibrosis transmembrane conductance regulator; gene deletion; infertility, male; mutation; Y chromosome

Y Chromosomal Microdeletions

Genes located on the euchromatic region of the long arm of the Y chromosome, such as those in azoospermia factor (AZF) regions, play an essential role in spermatogenesis. There are four regions in AZF (AZFa, AZFb, AZFc, and AZFf) on the Y chromosome, and gene deletions in these regions have been shown to be pathogenically involved in male infertility associated with azoospermia or severe oligozoospermia (1). Since the type of the deletion has a prognostic value, screening for complete or partial microdeletion before assisted reproduction treatment is recommended (2). Men with proximal deletions, which include AZFa and AZFb regions, show severe defects in spermatogenesis, with high prevalence of Sertoli cell-only syndrome, whereas deletions of the distal AZFb and of the AZFc region can be compatible with residual spermatogenesis (3). Men with deleted AZFc and their partners are candidates for intracytoplasmic sperm injection. Deletion of the AZFc region on the Y chromosome is the most frequent molecularly defined cause of spermatogenic failure. Y chromosome microdeletions have been reported to account for 2-20% of infertile men (4, 5). The prevalence of Y-microdeletions in a Swedish population in infertile azoospermic men was 3% (4), whereas in New Zealand microdeletions were detected in 20% of men (5). Wide variations in deletion frequency reported in the published work could be caused by ethnic differences, different patient selection criteria, and partly methodological aspect (6). We analyzed microdeletions in the AZF region of the Y chromosome in infertile men in Croatia, using a multiplex polymerase chain reaction (PCR), and found microdeletions in 4.5% (N=67) of azoospermic men. Genetic algorithm including AZFa, AZFb, or AZFc DNA deletion analysis gives genotype results that can be used as a potential prognostic factor for intrauterine insemination, in vitro fertilization, or intracytoplasmic sperm injection. However, Y chromosome deletion analysis is still not performed routinely in all laboratories.

A series of Y chromosome-specific sequence target sites (STS) has been characterized, as well as the primer sequences selected to cover deletions. These primer sequences indicate the deletion pattern with...
the presence or absence of STSs as germinal elements on distal Yq chromosome between euchromatin and heterochromatin. Diagnostic testing of deletions should be performed by multiplex PCR amplification of STS locus in AZFa, AZFb, and AZFc regions of the Y chromosome, by use of an in-house method or a commercial product. In addition, the analysis of two STS loci in each region increases diagnostic accuracy, because deletions usually involve more than one STS loci. The set of PCR primers that should be used in multiplex PCR reaction for diagnosing microdeletions in the AZFa, AZFb, and AZFc region includes the following markers: sY84, sY86, sY127, sY134, sY252, and sY255 (7). The use of this primer set allows the detection of over 90% deletions in the three major AZF regions. Guidelines for diagnostic testing region, according to European Academy of Andrology, include STS primers for AZFa, AZFb, and AZFc region as the first choice, and primers for detecting proximal and distal borderlines of each AZF region as the second choice (8). The choice of the primers for extended analysis is left to the individual laboratory.

### Cystic Fibrosis Transmembrane Conductance Regulator Gene Mutations

Various genetic disorders are associated with reproductive failure or transmissible to the offspring, or both (9). Chromosomal abnormalities associated with spermatogenic failure have been documented, including Klinefelter’s syndrome and Robertsonian translocation.

At the gene level, reproductive failure has been linked with CF, Y-chromosomal microdeletions, and changes in the polymorphic trinucleotide repeat in the androgen receptor gene (10). Cystic fibrosis (CF MIM 219700) is a very heterogeneous genetic disease, with more than 800 mutations (11). The disease is characterized by abnormal secretion of electrolytes and fluid across the epithelial membranes of most exocrine organs. Clinical hallmarks include pulmonary obstruction, pancreatic insufficiency, diabetes mellitus, elevated sweat electrolytes, and male infertility (12). More than 95% of adult man with CF are infertile because of obstructive azoospermia. Mutations in cystic fibrosis transmembrane regulator (CFTR) gene are a relatively frequent cause of male infertility. Congenital absence of the vas deferens (CAVD), a form of male sterility, is a frequent cause of obstructive azoospermia (13). The observation that men presenting with CAVD have mutations in CFTR gene has lead to a hypothesis that CAVD may be primarily a genital form of CF. Recent studies show that the 5T variant within intron 8 in the CFTR gene may be considered a mild set of mutation specifically associated with male sterility (14). However, the molecular basis of CAVD has not been completely understood. The CFTR DNA screening in men with CAVD is recommended for the prevention of CF in couples with reproductive problems. We used Linear Array Panel (LAp-31, Roche Molecular Systems, Alameda, CA, USA) for polyT genotyping analysis (N = 76), and found that in Croatia polyT 7/7 genotype was the most frequent (34.2%), followed by T5/9, T7/9, and T5/5 (Fig. 1). In addition, the results revealed that 27.0% of patients have 5T allele (Fig. 2). These are the first data on polyT genotype in Croatia, and are consistent with results in other European countries (15). The F508 deletion was found in 21.0% of the subjects, which is less frequent than in CF patients (64.5%). The distribution and ethnic/regional origin of 101 CF mutations in Central and Eastern European population were studied (16-18) within a long-term collaborative project aimed to evaluate the distribution of the most common CF mutations in 17 Central and Eastern European countries. Twenty participating CF centres or laboratories ascertained a representative cohort of 4,296 patients with CF. The distribution of CFTR mutations reflects the regional ethnic composition, and inclusion of respective alleles in routine screening panels will improve molecular genetic diagnosis. CFTR or the small conductance cAMP-activated chlo-

![Figure 1](image1.png) Comparison of poly T genotype between congenital absence of vas deference (CAVD) and the control group.

![Figure 2](image2.png) Frequency of poly-T alleles in intron 8 of the CFTR gene in men with congenital absence of vas deference (CAVD) and patients with cystic fibrosis (CF).
ride channel encoded by the CFTR gene have been shown to play an important role in the formation of the epididymal fluid microenvironment (19). CFTR mutations are responsible for poor sperm quality in healthy men with CF and CAVD. The CFTR gene has an effect on human reproduction. Given the widespread role of this membrane protein in male reproduction, screening of testis-specific CFTR inhibitors can provide a new avenue of research into the development of novel male contraceptives. Due to the importance of CFTR in the formation of the epididymal fluid, pharmacological intervention in CFTR activity could lead to the alteration of sperm microenvironment. As with other ion channels on cell membrane, CFTR is amenable to regulation by pharmacological agents, thus providing a new strategy for male contraception (19).

PCR is one of the several molecular techniques used in the diagnostics of CFTR mutations (20). This assay is more sensitive and faster than karyotyping, in situ hybridization, fluorescence in situ hybridization, and Southern blotting. There are several forms of PCR specifically designed for mutation analysis, but they are labor-intensive and costly because post-PCR processing by restriction enzyme digestion and gel electrophoresis analysis is necessary. The new real-time technology involves combined microliter volume thermal cycler and fluorometer suitable for fast real-time fluorescence PCR. This system, designed for mutation detection by melting point analysis, applies fluorescence energy transfer (FRET) principle. The LightCycler procedure to detect major CFTR mutation F508 deletion has recently been developed and implemented in routine work (21). Strategy for CFTR testing is to organize services on two levels: local diagnostic laboratory (level I), at which rapid, standardized, and cheap assays should be performed, and level II laboratory, at which a database on patients and mutations could be set up. New techniques are evaluated for the implementation at the level I testing laboratory, and for CFTR research at the international level (22). Level I laboratory uses a wide range of techniques for CFTR mutation detection, such as electrophoresis, LightCycler procedure, and reverse line-blots. There is no gold standard for routine testing. The Lap CF-31 contains 28 different 5’biotin-labeled primers that simultaneously amplify 14 different regions of CFTR gene. Selective amplification of target DNA from a specimen in the assay is achieved by the use of uracil-glycosylase. The biotin labeled DNA sequences are then hybridized to the specific probes onto the membrane. The detection is performed by binding streptavidin-horseradish peroxidase conjugate to the biotin, followed by colored reaction with hydrogen peroxide and tetramethylbenzidine. Lap CF-31 includes the following mutations and polymorphisms: exon 3, G85E, 405+3A>C; exon 4, R117H; intron 4, 621+1G>T; intron 5, 711+1G>T; exon 7, R334, R347P, 1078 del T, intron 8, A455E; exon 10, G480C, Δ507, ΔF508, (F508C, 1507V, 1506V); intron 10 1717-1G>A; exon 11, G542X, G551D, R553X, A559T, R560T; exon 13, 2307 ins A; intron 14b, 2789+5G>A; intron 16, 3120+1G>A; exon 19, R1162X, 3659 del C; intron 19, 3849+10kb C>T; exon 20, S1255X, W1282X; and exon 21, N1303K. Lap-31 assay is an accurate, simple, rapid, and reliable novel non-isotopic method, suitable for the screening of 31 CFTR mutations and polymorphisms.

**Genetic Counseling Prior to Intracytoplasmic Sperm Injection**

The spermatogenic loci AZFa, AZFb, and AZFc on the Yq11 chromosome control spermatogenesis in men and have an effect on fertility and genomic imbalance. The intracytoplasmic sperm injection technique is rapidly becoming an accepted procedure to assist reproduction in case of male infertility. When intracytoplasmic sperm injection is applied, AZFc deleted spermatozoa are capable of fertilizing oocytes and eliciting full developmental potential. Y chromosome microdeletion screening is important to define the etiology of spermatogenesis. It allows the prediction of whether the surviving boys will be azoospermic or oligozoospermic as adults, and contributes to the discovery of an effective therapeutic intervention. Vertical transmission of AZF subregion defect to male offspring by intracytoplasmic sperm injection has been reported as Y status, because the infertility problem is most probably transmitted to sons, and leads to familiar infertility (23-25). Men with obstructive azoospermia and CAVD should be screened for CFTR mutations, as well as their wives if they are heterozygous. CFTR mutations may result in typical or atypical infertility (23-25). All men with idiopathic obstructive azoospermia have an increased risk of having CF offspring.

**Quality Assessment of Molecular Diagnosis**

In most countries, clinical laboratories offer molecular genetic diagnostic testing. These centres are faced with problems found in other diagnostics – standardization, quality controls, and response time. External quality control includes methodological proficiency testing that is suitable for frequent clinical entities. The heterogeneity of the protocols employed and results published, together with increasing diffusion of this diagnostic procedure, suggested the necessity of quality control program for genetic diseases, such as CF and male infertility. International programs, such as External Quality Assessment (EQA) and European Molecular Genetics Quality Network (EMQN), have been started in 1996 for CF, and in 1997 for Y chromosome microdeletions. EMQN is supported by the European Union, under the Standards Measurements and Testing Programme. The quality assessment schemes are designed to evaluate both practical analysis results and interpretation of data. With the current trend towards accreditation, it is becoming even more important for laboratories to participate in External Quality Assessment schemes and to demonstrate the quality of the genetic testing services they provide. A panel of three or four experts evaluates genotype results, and each participating center receives the report. This procedure contrib-
uted to the generation of the Laboratory guidelines for molecular diagnosis of Y chromosomal microdeletions.

Recommendations for quality improvement in genetic testing for CF by European Concerted Action on Cystic Fibrosis provided general guidelines for molecular genetic testing of CF in patients/individuals, general guidelines for laboratory procedures, internal and external quality assurance, reporting of results, nomenclature for describing mutations and polymorphism, guidelines to implement a quality system in a molecular diagnostic laboratory, guidelines for members connected with a genetic diagnostic laboratory, and frequency of mutations in European countries. Schemes are mostly restricted to the genetic diagnostic laboratories for familiar breast cancer, Charcot-Marie Tooth Diseases, Willi/Angelman syndromes. EMQN also run schemes for fragile-X syndrome, retinoblastoma and Prader deletion. New schemes in 2001 are pilot schemes for diagnosis of Y chromosomal microdeletions. Recommendations for quality improvement in diagnostic laboratories in external Quality Assessment schemes is necessary to introduce consensus guidelines to implement a quality system in each molecular genetic diagnostic laboratory leading to accreditation, centralize mutation analysis for identification of rare mutations, and set up a network with other laboratories in the region.

European Community Concerted Action for Cystic Fibrosis publishes CF-leaflets aimed at patients and their families for better understanding of the disease. The test is based on the manual of the World Health Organization and the International Cystic Fibrosis Association. Laboratory guidelines for the molecular diagnosis of Y chromosomal microdeletions have been developed by the European Academy of Andrology, whereas European Molecular Genetics Quality Network organized External Quality Assessment Scheme for the molecular diagnosis of Y chromosome microdeletions. New schemes in 2001 are pilot schemes for fragile-X syndrome, retinoblastoma and Prader Willi/Angelman syndromes. EMQN also run schemes for familiar breast cancer, Charcot-Marie Tooth Disease, Duchenne Muscular dystrophy, Friedrich ataxia, Huntington disease. These schemes have an educational role and provide participating laboratories with an opportunity to critically review their performance.

References


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