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Distribution of the *DAZ* gene transcripts in human testis

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Abstract: Involvement of variety of genes, especially located on Y chromosome, is critical for the regulation of spermatogenesis. In particular, fertility candidate genes such as deleted in azoospermia (*DAZ*) are believed to have important function in sperm production, since *DAZ* is frequently deleted in azoospermic and severy oligozoospermic men. The role of the *DAZ* gene is supported by its exclusive expression in the testis and by its deletion in about 10% of azoospermic and severely oligozoospermic patients. The distribution of *DAZ* transcripts in seminiferous epithelium of human testis is reported in the present study. The use of Adobe Photoshop and Scion Image softwares allowed for semi-quantitative analysis of *in situ* RT-PCR (ISRT-PCR) results. The intensity of ISRT-PCR product's fluorescence was different within individual seminiferous tubules. It was clearly shown by using the pseudocolour scale and transforming the intensity of the fluorescence into levels of greyscale images. The more intense fluorescence characterised single spermatogonia and those organized in small groups inside separate tubules. The most intense accumulation of *DAZ* mRNA was observed in spermatogonia.

Key words: *DAZ* gene - Transcript localization - Azoospermia - Oligozoospermia

Introduction

It has been presumed that 2-3% of men are considered to be infertile as a result of various defects in sperm production [4, 8, 18]. Spermatogenesis is a complex developmental process in which male germ cells progress trough mitotic proliferation, meiotic division and dramatic morphological changes to form mature sperm. Human spermatogenesis is regulated by a network of genes located on autosomes and on sex chromosomes, but especially on the Y chromosome. Molecular techniques employing Y chromosome-specific markers revealed that 5-10% of severely oligozoospermic or azoospermic men have cytogenetically invisible Yq microdeletions [9, 12, 13, 14, 16, 20, 22]. These fall into three classes mapping to discrete subintervals of the Yq region, termed AZFa, b and c (AZoospermia Factor) [16], and positional cloning strategies have led to the isolation of candidate genes from each subinterval. Mutations more frequently involve the *DAZ* gene in AZFc and could lead to both azoospermia and severe oligozoospermia. It is therefore difficult to find a clear relationship between genotype and phenotype. *DAZ* is present in multiple copies in AZFc, and this causes the gene to be difficult to analyze [6]. The critical role for *DAZ* in human male germ cell development is supported by the evidence that this gene is testis-specific, with transcriptions limited to germ cells [5, 7, 11], as well as by its deletion in about 10% of azoospermic and severely oligozoospermic subjects. The *DAZ* gene family encodes a protein with RNA-binding motif near its N-terminus [16]. RNA-binding proteins are critical in spermatogenesis, because many of the genes expressed in the sperm are regulated at the level of translation [17]. The function of the *DAZ* gene however, remains unclear, and the postulated RNA binding property of the gene product has not yet been demonstrated [4, 5].

The aim of our research was to determine distribution and concentration of *DAZ* transcripts in the seminiferous epithelium of human testis.

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Materials and methods

Material. Human testes were obtained from five fertile organ donors, fixed in 4% formaldehyde in phosphate buffered saline (PBS), dehydrated and embedded according to standard procedure [2, 3]. For the procedure approval was obtained from the institutional committee.

RT-PCR *in situ*. RT-PCR *in situ* was performed on paraffin sections of formalin-fixed tissue.

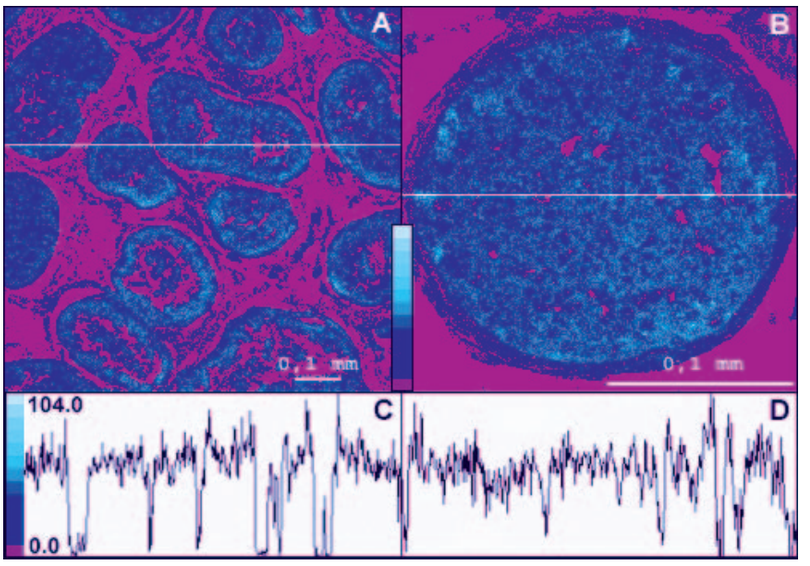

Fig. 1. Pseudocolour-transformed images of human seminiferous tubules. **A**. The results of ISRT-PCR. The *DAZ* gene products visible as green to yellow colour are present in all seminiferous tubules of human testis. **B.** Cross-section of single seminiferous tubule. Positively stained primary spermatocytes are located in the nearest neighbourhood of yellow spermatogonia. The cells visible as pale green do not express *DAZ*. **C, D.** The plots of the fluorescence intensity of cells measured along the lines shown in A and B, respectively.

In order to detect the *DAZ* mRNA, the sections (3 μm) were prepared according to the procedure described by Bagasra and coworkers [1]. Reverse transcripton *in situ* was performed using Expand Reverse Transcriptase (Roche Molecular Biochemicals, Mannheim, Germany) with the primer specific for the *DAZ* gene (reverse primer: 5'-ATGCAAAGCTCAATACTGA-3', complementary to nucleotides 36319-36337, PubMed AC00021). The 460 bp fragment of cDNA of the *DAZ* gene transcript was amplified by *in situ* PCR, using the following primers: sense 5'-TGTTACCAGAAGGCAAAATC-3' (nucleotides 1413-1432, PubMed AC00021) and antisense 5'-GCAACTGACATCCAGTGATG-3' (complementary to nucleotides 4613-4632, PubMed AC00021). Fluorescence-labelled nucleotides Cy^{TM}3-dUTP (Amersham Biosciences, Little Chalfont Buckinghamshire, UK) were used in order to identify the amplified product. *DAZ* mRNA distribution was examined using Carl Zeiss LSM 510 confocal microscope.

Computer generation of pseudocolours. The use of computer-assisted image transformation allowed for semi-quantitative analysis of ISRT-PCR results. Images generated in .lsm true colour file format from Zeiss LSM Image Browser Version 3,1,0,99 for Carl Zeiss Laser Scanning System LSM 510 (Carl Zeiss GmbH) were exported to Microsoft Windows® Bitmap File Format and saved. In Adobe Photoshop® 5.5 CEEA (Adobe Systems Inc.1989-1999), the colour mode was changed to greyscale format (8-bit per channel; 256 grey levels) and stored as a Windows® 8 Bitmap File Format. Using Scion Image® (Release beta 4.0.2) for Windows software (Scion Corporation© 2000), the greyscale was changed into 32-colour table mode and stored as a pseudocolours.

Results

The use of Adobe Photoshop and Scion Image softwares allowed for semi-quantitative analysis of RT-PCR *in situ* results. The results of the research based on the ISRT-PCR technique demonstrated that active *DAZ* gene products were present in all studied seminiferous tubules of human testis (Fig. 1A). The intensity of ISRT-PCR product fluorescence was different within individual seminiferous tubules, as clearly shown by applying the pseudocolour scale and expressing the intensity of the fluorescence as levels of greyscale images. The more intense fluorescence characterised single spermatogonia and those organized in small groups inside separate tubules. The intensity reached 104th greyscale level (Fig. 1C, D). A weaker fluorescence was detected in primary spermatocytes, mostly located in the nearest neighbourhood of positively stained spermatogonia (Fig. 1B). The fluorescence intensity of primary spermatocytes ranged from 73rd to 84th greyscale levels. A very weak fluorescence (around 50th grey level) was noted for the other cells of seminiferous tubules, *i.e.* secondary spermatocytes and spermatids. This signal, however, was stronger than that found in cells of the basement membrane, for which the intensity was only 10 to 20 grey levels above the background.

In case of control reactions in which the reverse transcription step was omitted, the fluorescence product of the PCR reaction was not observed.

Discussion

The precise biological significance of the *DAZ* gene is still unknown. Recent studies demonstrated that deletion of the gene cause male infertility, but not all the mutations of the *DAZ* gene lead to azoospermia or oligozoospermia. Some authors suggest that the role of the *DAZ* in regulation of spermatogenesis is, if any, rather marginal.

The research on the expression and mutations of the *DAZ* and its homologues in humans and other mammals suggests that protein products of these genes can mainly affect development of germinal cells [15].

The aim of the present study was to semi-quantitatively analyse the expression of the *DAZ* gene in semi-

niferous tubules of the human testis. The results of the study clearly showed the strongest fluorescence which can correspond to high level of the *DAZ* gene expression in spermatogonia and primary spermatocytes. Weak fluorescent signal was also observed in secondary spermatocytes and spermatids and in interstitial cells.

The results of the study did not depend on the thickness of the analysed specimens, since confocal microscope was used to visualise the fluorescence-labelled products of ISRT-PCR.

The obtained data partly correspond to the results of other authors, based on the hybridization techniques and to our previous study which showed the presence of active *DAZ* gene only in spermatogonia and primary spermatocytes [19, 21]. The observed difference seems to be due to various fixation methods applied as well as to a choice of new primers. The fluorescence product of ISRT-PCR observed by us also in secondary spermatocytes and spermatids can be visualized due to high sensitivity of direct detection method based on fluorescence labeled nucleotides. It made also possible to show the presence of *DAZ* mRNA in human spermatozoa [19].

Localization of active *DAZ* gene mainly in spermatogonia and primary spermatocytes suggests a possible role of this gene in the first steps of spermatogenesis. *DAZ* gene can be especially important for regulation of the number of spermatogonia stem cells and their differentiation. This hypothesis corresponds to the theory of self-renewing mechanism of stem cells in germline [10] as well as to the result of Reijo *et al.* [15] suggesting the role of DAZ family proteins in male germ cell development.

Since the *DAZ* gene is present in multiple copies in AZFc, it is difficult to analyze and find a clear relationship between genotype and phenotype in case of mutations causing azoospermia and oligozoospermia. Further research on the role of *DAZ* can have important clinical consequences in the diagnostic and therapeutic treatment of the infertile patients, above all when they are candidates for assisted reproduction techniques.

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