Decreased Expression of Genes Associated with Memory and X-Linked Mental Retardation in Boys with Non-Syndromic Cryptorchidism and High Infertility Risk

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Key Words

Cryptorchidism · Gene expression · Infertility · Memory · Mental retardation · Mini-puberty

Abstract

An elevated odds ratio for low IQ has been found for cryptorchid boys. Furthermore, poor school performance has been observed in cryptorchid boys with impaired mini-puberty. Gene expression analysis, qPCR and immunohistology were performed on testicular biopsies from 7 boys who underwent orchiopexy and had testicular histology typical of a high risk of infertility (HIR). The results were compared with 12 biopsies from cryptorchid boys with a low risk for developing infertility. The following genes associated with mental retardation were identically expressed: *GDI1*, *OPHN1*, *PAK3*, *ARHGEF6*, *IL1RAPL*, *ACSL4*, *MECP2*, *RPS6KA3*, *ARX*, and *ATRX*. However, boys in the HIR group had low or no expression of *EGR4*, *FMR2* (*AFF2*) and *VCX3A*. In conclusion, impaired expression of genes known to encode proteins involved in signaling pathways that regulate cytoskeletal organization, synaptic vesicle transport and the establishment of connections between neuronal cells may contribute to reduced intellectual and cognitive functioning in infertile cryptorchid males.

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Undescended testes (cryptorchidism) is the most common genitourinary disorder of childhood and leads to 27,000 surgeries each year in the United States [Trussell and Lee, 2004]. The hallmark of successful treatment of cryptorchid boys is fertility. Whole-genome expression profiling shows that boys placed in the high infertility risk (HIR) group in accordance with their testicular histology have decreased or no expression of most of the genes essential for hypothalamic-pituitary-testicular axis function relative to boys with a low risk of infertility (LIR). In particular, *EGR4*, which is involved in regulating the secretion of luteinizing hormone, is virtually not expressed [Hadziselimovic et al., 2009]. Thus, *EGR4* appears to be a master gene for fertility development [Hadziselimovic et al., 2009]. Further important functions for *EGR4* have been identified; for example, *EGR4* is involved in receptor-modulated memory processes [Li et al., 2005]. The previously described impaired expression of *EGR4* would thus support Depue's [1988] observation of an elevated odds ratio for low IQ in boys with cryptorchidism. Here, we investigated the expression of genes associated with non-syndromic mental retardation and formation of long-term memory in cryptorchid boys with HIR versus LIR.

Table 1. Primers used for PCR

Genes	Forward primer	Reverse primer
<i>TFRC1</i>	TCGTCCCTGCATTTAAAGGCTTTC	CAACAGTGGGCTGGCAGAAAC
<i>GAPDH</i>	GATCATCAGCAATGCCTCCTG	GAGTCCTTCCACGATACCAAAG
<i>EGR4</i>	AGCAAGAGATGGGTTTATG	AGGAGTTGGAAGAAGAGC
<i>DLX2</i>	TTAGGTGCCTTTGCCGATGAC	ACAAACTCTGTGTCCAAGTCCAG
<i>DDX25</i>	AGAATGTCTCAGTGGGTTTGTG	GCTTCACATCAATTTGGCTTC
<i>MAGEA4</i>	GGCCAGTGCATCTAACAG	AAATAGAAACCCACTACTAAGAAC

Methods

Testicular Biopsies and Pooling of Patients

At our institution, it is routine practice to perform a testicular biopsy during surgery for undescended testes. We have found that testicular biopsies provide useful information about the quality of semen in the future and help to identify patients with atypical spermatogonia or carcinoma in situ. Cryptorchid testis is defined as a testis localized outside the scrotum and unable to be brought into a stable scrotal position. Nineteen rice-grain-sized testicular biopsies were analyzed, 7 from boys with impaired mini-puberty and HIR and 12 from boys with LIR. The age of the patients was comparable: HIR, mean 2.9 years (95% CI 0.5–5.84); LIR, mean 3.6 years (95% CI 2.78–4.41). Biopsies were fixed in 3% glutaraldehyde and embedded in Epon. Semi-thin 1- μ m-thick sections were examined by Zeiss Axioscope phase contrast and conventional light microscopy (Plan-Apochromat 63 $\times$ /1.40 oil). The methods for whole-genome analysis of testicular biopsies by Affymetrix microarrays were published in detail in our previous work [Hadziselimovic et al., 2009].

Statistical Analysis

The data analysis and gene filtering were performed using R/Bioconductor [Gentleman et al., 2004]. Signal condensation was performed using only the RMA from the Bioconductor Affy package. Differentially expressed genes were identified using the empirical Bayes method (F-test) implemented in the LIMMA package and adjusted with the False Discovery Rate (FDR) method [Wettenhall and Smyth, 2004]. We selected those probe sets with a log2 average contrast signal of at least 5, an adjusted p value <0.05 and an absolute log2-fold change of >0.585 (1.5-fold in linear space). Hierarchical clustering and visualization were performed in R.

Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was performed using a Corbett Research RG-6000 instrument. cDNAs were synthesized with the Reverse Transcriptase Core Kit (Eurogentec) using random primers (table 1). Real-time PCR runs were performed using the SYBR FAST Kit (Kapa Biosystems) with each gene-specific primer at 200 nM final concentration in a total volume of 17.5 μ l. Wave lengths of source and detection were set at 470 nm and 510 nm, respectively. Gain was set at 8.33. The PCR program was set as follows: 95°C, 60 s; 45 cycles 95°C, 3 s; 60°C, 10 s; 72°C, 4 s; followed by a melting curve analysis (65°C to 95°C, rising each step by 0.65°C) to attest amplification specificity. Threshold cycles (crossing point) were determined using Rotor-Gene software ver-

sion 6.1. Good PCR efficiency was checked by performing a dilution series of the cDNA.

Controls without reverse transcriptase were performed for each sample studied. Again, threshold cycles (crossing point) were determined using Rotor-Gene software version 6.1. Expression levels were normalized to *TFRC1* and *GAPDH* using a geometric mean of their level of expression. Those genes were selected because they showed minimum variation between individual samples (both on microarrays and by qPCR). Fold differences were calculated using the $\Delta\Delta C_t$ method (fig. 1).

Antibody Validation

For immunohistochemical analysis, the Epon was removed from the tissue sections. Sections were treated with 2% bovine serum albumin to reduce nonspecific binding and then incubated with the primary antibody overnight at 4°C. All samples were washed with PBS between incubations. We validated 8 antibodies, along with *EGR4*, as described previously [Hadziselimovic et al., 2009].

Ethical Considerations and Approval

In accordance with the Declaration of Helsinki, the Institutional Review Board and the Independent Ethics Committee of the Children's Clinic in Liestal approved all aspects of this study. Approval was provided for research involving the use of material (data records or biopsy specimens) that had been collected for non-research purposes.

Results

Total RNA and cRNA samples were of high quality, the signal intensity distributions were similar and were within the normal range. As expected, overall signal intensities visualized using a distance matrix appointed HIR and LIR to separate groups. Of all transcripts overlapping the 2 groups, 378 were differentially expressed in HIR and LIR testes after statistical filtering. This gene list comprised 8 elevated and 370 decreased transcripts in HIR testes. The 20 genes identified from Affymetrix microarrays that showed the greatest differential expression between HIR and LIR and the highest significant differ-

Fig. 1. Correlation of microarray data with data obtained by qRT-PCR on 4 genes that displayed significant expression changes, showing a high correlation in the obtained transcriptional profiles. The y-axis is the Z-normalized log2 expression (array) and 2-normalized QT value (qPCR). Numbers on the x-axis correspond to biopsy ID numbers. From Hadziselimovic et al. [2009].

Fig. 2. Immunohistochemical staining of EGR4 in testes of the LIR group showing EGR4 localized in the cytoplasm of Leydig and germ cells (arrows). Contrast staining with toluidine blue. Scale bar = 0.1 mm. From Hadziselimovic et al. [2009].

Fig. 3. Immunohistochemical validation of EGR4 in the HIR group. Atrophic Leydig cell cytoplasm was stained brown (arrows). We observed decreased EGR4 expression in degenerating spermatogonia. Contrast staining with toluidine blue. Scale bar = 0.1 mm. From Hadziselimovic et al. [2009].

Table 2. The 20 genes showing the greatest differential expression between HIR and LIR

Gene	Adjusted p value	Gene name
<i>TPTE</i>	8.70E-0.5	transmembrane phosphatase with tension homology
<i>ESX1</i>	8.63E-0.5	ESX homeobox 1
<i>MAGEC1</i>	8.24E-0.5	melanoma antigen family C1
<i>LOC722137</i>	8.24E-0.5	
<i>TKTL1</i>	8.24E-0.5	transketolase-like 1
<i>HEY1</i>	8.24E-0.5	hes-related family bHLH transcription factor with YRPW motif 1
<i>DDX4</i>	8.24E-0.5	DEAD box polypeptide 4
<i>STK31</i>	8.24E-0.5	serine/threonine kinase 31 (TDRD8)
<i>CPEB1</i>	8.24E-0.5	cytoplasmic polyadenylation element binding protein 1
<i>FOXG1</i>	1.21E-0.5	forkhead box G1
<i>ELAVL2</i>	0.00011	ELAV like neuron-specific RNA binding protein 2
<i>MAEL</i>	0.00017	maelstrom spermatogenic transposon silencer
<i>BEND4</i>	0.00017	BEN domain containing 4
<i>TTK</i>	0.00017	TTK protein kinase
<i>HMGA1</i>	0.00017	high mobility group AT-hook 1
<i>PIWIL2</i>	0.00017	piwi-like RNA-mediated gene silencing 2
<i>RGMA</i>	0.00018	repulsive guidance molecule family member a
<i>GTSF1</i>	0.00019	gametocyte specific factor 1
<i>FGFR3</i>	0.00019	fibroblast growth factor receptor 3
<i>BARD1</i>	0.00019	BRCA1 associated RING domain 1

ence are presented in table 2. The majority of the genes listed are dealing with spermatogenesis.

We found that the following genes, known to be involved in non-syndromic mental retardation, were equally expressed in the HIR versus LIR group of cryptorchid boys: *GDII*, *OPHN1*, *PAK3*, *ARHGEF6*, *IL1RAPL*, and *ACSL4*. Furthermore, no differences in expression between the 2 groups of cryptorchid boys were observed for the genes *MECP2*, *RPS6KA3*, *ARX*, and *ATRX*, which are known to be involved in syndromic and nonspecific forms of mental retardation. In contrast, compared to the LIR group, the HIR group had low or no expression of *EGR4*, *FMR2* (*AFF2*) and *VCX3A* (HIR vs. LIR; median values, log2: *EGR4* 2.7 vs. 5.65, $p < 0.0006$; *FMR2* 4.71 vs. 6.03, $p < 0.003$; and *VCX3A* 5.9 vs. 7.46, $p < 0.01$).

To analyze tissue and organ gene expression, we included samples from patients diagnosed with undescended testes [Hadziselimovic et al., 2009] or from different human organs [Lardenois et al., 2010].

The tissue and organ gene expression profiles of the genes that we analyzed are known to vary considerably. *GDII*, *OPHN1*, *PAK3*, *ARHGEF6*, *ACSL4*, *MECP2*, *RPS6KA3*, *ARX*, and *ATRX* are ubiquitously expressed in most human organs, while *EGR4* is exclusively expressed in testis and brain tissue. *VCX3A* is expressed in brain, testis and salivary gland tissue, while *FMR2* expression is specific to the testes. Finally, *IL1RAPL* is expressed only in the brain. In the current study, immunohistological analysis revealed lower *EGR4* expression in the spermatogonia and Leydig cells of the HIR group compared to those of the LIR group (figs. 2, 3).

Discussion

Mental retardation is a symptom of many clinical conditions and affects around 3% of the population [Nawarra et al., 2002]. Although the genetic causes of X-linked mental retardation are heterogeneous and complex, 8 genes involved in nonspecific X-linked mental retardation have been identified: *FMR2*, *GDII*, *OPHN1*, *PAK3*, *ARHGEF6*, *IL1RAPL*, *TM4SF2*, and *FACL4*. Four other genes, *MECP2*, *RSK2*, *ARX*, and *ATRX*, are involved in syndromic and nonspecific forms of mental retardation [Nawarra et al., 2002]. We did not observe any differences between the HIR and LIR groups in the expression of most of the genes involved in mental retardation, except for *FMR2*. However, we found that the genes *EGR4* and *VCX3A* were virtually not expressed in the HIR group. Early growth response (EGR) transcription factors (*EGR1* to *EGR4*) are synaptic activity-inducible immediate early genes that regulate some of the aspects of synaptic plasticity that are related to learning and memory. Long-term synaptic plasticity responses are long-lasting changes in the strength of synaptic connections, and they are believed to represent the cellular correlate of learning and memory [Lynch, 2004]. Previous studies have demonstrated that *VCX-A* is involved in nonspecific X-linked mental retardation [Fukami et al., 2000; Van Esch et al., 2005]. *VCX-A* is a non-canonical cap-binding protein that binds to capped RNA, but not to cap structures lacking bound RNA. Its cap association is enhanced by hDCP2, which further augments the ability of *VCX-A* to inhibit decapping. Thus, *VCX-A* can regulate mRNA stability and is an example of a tissue-specific decapping regulator [Jiao et al., 2006].

Nonspecific X-linked mental retardation is a clinically homogeneous but genetically heterogeneous entity. Apart from mental retardation, affected men in families segre-

gating nonspecific X-linked mental retardation have no consistent phenotypic expression that distinguishes them from unaffected men or affected men in other families with X-linked mental retardation [Gecz et al., 1996]. Loss of *FMR2* causes Fragile X E (FRAXE) site-associated intellectual disability [Melko et al., 2013].

In human organs, *EGR4* is exclusively expressed in the testes and brain, while *VCX3A*, in addition to strong expression in the brain and testes, is also upregulated in the salivary gland. In humans and mice, the gene expression profiles of the brain, cerebellum and testes are more similar to each other than in other tissues [Guo et al., 2005]. Therefore, our finding of decreased expression of 3 genes in testicular biopsies, known to be involved in formation of long-term memory, adds additional support to the ob-

servation of an elevated odds ratio for low IQ in cryptorchid boys. The Stanford-Binet test of intelligence was administered at age 4. IQ was considered low if <70 [Depue, 1988]. In addition, it may explain poor school performance in infertile cryptorchid men with impaired *EGR4* expression [Hadziselimovic and Herzog, 1990].

In conclusion, we observed impaired expression of 3 memory genes known to encode proteins involved in signaling pathways that regulate cytoskeletal organization, synaptic vesicle transport and the establishment of connections between neuronal cells. This impaired expression may contribute to reduced intellectual and cognitive function in cryptorchid men. Thus, our findings may provide new insights into potential molecular mechanisms involved in the development of these functions.

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