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Association of deletion 9p, 46,XY gonadal dysgenesis and autistic spectrum disorder

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Deletions of distal chromosome 9p24 are often associated with 46,XY gonadal dysgenesis and, depending on the extent of the deletion, the monosomy 9p syndrome. We have previously noted that some cases of 46,XY gonadal dysgenesis carry a 9p deletion and exhibit behavioural problems consistent with autistic spectrum disorder. These cases had a small terminal deletion of 9p with limited or no somatic anomalies that are characteristic of the monosomy 9p syndrome. Here, we present a new case of 46,XY partial gonadal dysgenesis and autistic spectrum disorder associated with a *de novo* deletion of 9p24 that was detected by ultra-high resolution oligo microarray comparative genomic hybridization. The deletion included the candidate sex-determining genes in the region *DMRT1* and *DMRT3*. These data suggest that a gene responsible for autistic spectrum disorder is located within 9p24. It remains to be determined if the gonadal dysgenesis and autistic spectrum disorder are caused by a single gene or if they are caused by distinct genetic entities at 9p24.

Keywords: autistic spectrum disorder; gonadal dysgenesis; chromosome 9p deletion; sex determination; ultra-high resolution oligo microarray comparative genomic hybridization

Introduction

Deletions of the terminal portion of 9p are associated with monosomy 9p syndrome (also termed Afi syndrome), which is characterized by mental retardation and characteristic somatic anomalies [trigonocephaly and upward slanting palpebral fissures together with mental retardation, midface hypoplasia, hypertelorism, epicanthus, small palpebral fissures, flat nasal bridge, anteverted nares, low-set malformed posteriorly angulated ears, a long upper lip, microstomia and micrognathia (Alfi *et al.*, 1973; Huret *et al.*, 1988)]. In ~70% of individuals with a 46,XY male karyotype, there are various degrees of sex reversal ranging from a 46,XY female with complete gonadal dysgenesis to males with mild hypospadias (Hoo *et al.*, 1989; Bennett *et al.*, 1993). Christ *et al.* (1999) delimited the critical monosomy 9p syndrome region to a 4-6-Mb genomic region in 9p22–23 between the markers D9S1869 (telomeric) and D9S162 (centromeric). This data is consistent with the results of Veitia *et al.* (1998), who independently delimited the monosomy 9p critical region between the markers D9S144 and D9S168 and the sex-reversal critical region between D9S1813 and pter. These data indicated that the 9p-deletion syndrome and the sex-reversal locus are distinct entities and that some 46,XY females that do not present with somatic anomalies may harbour 9p deletions. Although the genes responsible for the monosomy 9p syndrome remain to be identified, there are two candidate genes that may be responsible for the gonadal phenotype. These are the *DMRT1* and *DRMTA3* (also known as *DMRT3*) genes on chromosome 9p. *DMRT1* and *DMRTA3* have been described in the consensus critical region by their homology with the DM domain genes *doublesex* (*dsx*) of *Drosophila* and *mab-3* of *Caenorhabditis*. The *dsx* controls the terminal switch of the pathway leading to sex fate choice in *Drosophila* and *mab-3* is necessary to confer male traits in *C. elegans* (Raymond *et al.*, 2000). Normal external genitalia and normal pubertal development have been described in 46,XX females with deletions of 9p (Vialard *et al.*, 2002; Ounap *et al.*, 2004) suggesting that the sex-determining gene(s) on 9p play a role only in the formation of the testis or that they play a role in gonadogenesis in both sexes but that testis formation is more sensitive to changes in gene dosage. The latter has been described for a number of genes involved in testis formation such as *SOX9* and *DAX1* (Bardoni *et al.*, 1994; Foster *et al.*, 1994).

Recently, we described two patients with small terminal deletions of 9p associated with 46,XY gonadal dysgenesis. Both of these patients did not exhibit the classical characteristics of the monosomy 9p syndrome. One patient had limited somatic anomalies (mild hypotonia and a left clubfoot), while the second patient, carrying a non-cytogenetically detectable microdeletion of 9p24, had no dysmorphic features (Veitia *et al.*, 1998). However, in both cases learning difficulties and disruptive behaviour were noted and both patients were diagnosed as having affective disorder as well as gonadal dysgenesis. Here, we describe a third case of a 46,XY individual with partial gonadal dysgenesis, a deletion of 9p24 and autistic spectrum disorder.

Materials and Methods

Patient description

This individual of French origin was born prematurely at 32 weeks of gestation with ambiguous external genitalia. The external genitalia consisted of a genital bud and striated non-fused labioscrotal folds and perineal hypospadias. The basal plasma gonadotropin levels were evaluated at birth (J1). Serum testosterone was 1.8 ng/ml and rose to 3.3 ng/ml following stimulation by human chorionic gonadotrophin (hCG). Serum 17-OH progesterone was 2.9 ng/ml and rose to 3.6 following hCG stimulation. Dihydrotestosterone levels were 0.6 ng/ml rising to 0.9 ng/ml following stimulation. Family history was unremarkable although the mother reported premature ovarian failure. At the age of two months, laparatomy revealed a left streak gonad and a right dysgenetic gonad, a left hemiuterus (13 cm) and fallopian tube. The left gonadal histology revealed undifferentiated parenchymal tissue, with no follicular structures visible. The dense stroma contained poorly defined cord-like structures with an absence of germ cells. The right gonad consisted of a streak of fibrous tissue (superior pole) and poorly differentiated testis (inferior pole). Histology revealed both numerous poorly defined cord-like structures (superior pole) and seminiferous tubule-like structures (inferior pole) with some rare Sertoli cells. The patient was diagnosed as 46,XY partial gonadal dysgenesis and was raised as a male. At 18 months of age, behavioural problems were noted: at crèche he displayed considerable agitation and stereotypic verbal and gestural movements. There was an absence of interaction with other children and adults. He also showed a fascination for lights, water and noise.

At 3 years of age, relational difficulties were reported as well as an inability to follow simple tasks. The patient was noted as stubborn, overactive, impulsive and had temper tantrums when frustrated. He showed delays in motor acquisitions and language development. He was insensitive to pain and had poor eye contact. At 11 years 7 months, height was 142.7 cm, weight 33 kg and bone aged 11–12 years. Serum FSH levels were 15.6 U, LH 1.3 U/l and testosterone 0.7 ng/ml. At this time molecular biology studies were conducted. An analysis of the *SRY* gene by direct sequencing revealed an open-reading frame identical to that of a normal male. Since we have previously noted two patients with deletions of chromosome 9p, 46,XY gonadal dysgenesis and affective disorder, we decided to further investigate the karyotype and perform molecular analyses of distal 9p.

Molecular analyses

DNA was extracted from peripheral blood lymphocytes using standard techniques. The *SRY* gene was sequenced as described elsewhere (Veitia *et al.*, 1998). Using the PCR primers indicated in Table 1, we sequenced the open-reading frame of both the *DMRT1* and *DMRT3* genes. The conditions of amplification were: for *DMRT1* exon 1, incubation at 95°C for 5 min followed by 40 cycles of 95°C 1 min, 68°C 1 min and 72°C 30 s. For *DMRT1* exons 2 and 4, incubation at 95°C for 5 min followed by 40 cycles of 95°C 1 min, 57°C 30 s and 72°C 1 min. For *DMRT1* exon 3, incubation at 95°C for 5 min followed by 40 cycles of 95°C 1 min, 62°C 1.30 min and 72°C 30 s. For *DMRT1* exon 5, incubation at 95°C for 5 min followed by 40 cycles of 95°C 1 min, 50°C 30 s and 72°C 30 s. For *DMRT3* exon 1, the PCR conditions were incubation at 95°C for 5 min followed by 40 cycles of 95°C 1 min and 62°C 1.30 min with no extension time. For *DMRT3* exon 2, two amplicons were used to amplify the entire exon for direct sequencing (Table 1). Both primer pairs of each amplicon were used with the same conditions of incubation conditions at 95°C for 5 min followed by 35 cycles of 95°C 1 min and 60°C 1 min with no extension time.

Table 1: PCR primer pairs used for amplification of the *ANKRD15*, *DMRT1* and *DMRT3* genes

Gene	Forward primer	Reverse primer
ANKRD15		
ANKRD15exon1	ttcaaaaccaccaggcattt	cctctgaggaacagataaaagca
ANKRD15exon4	gagttttggtggctgggata	ctggccctagaatcctgatg
ANKRD15exon6	cagaacccaggaccacatct	tcctttatcctctgcgtgct
DMRT1		
Exon 1	ggcagacctcgccactccag	aaggctgaacccgggctccc
Exon 2	tctgtgttttggcaaagctg	ctgcttctgtggctgcaa
Exon 3	gcaggtcttgggtaggaagg	catgtggctttcacacaacc
Exon 4	caaggtgtcgggaacatagg	ctctctcaaccccaaatcca
Exon 5	ggagagcgtcactttctttgtt	ccatgcagatggtagtcacg
DMRT3		
Exon 1	cggagcacacacgaccac	gtcctcccaagtggagctg
Exon 2–5′ region	aacttccgcagaacctgaga	agatgtggcctctcctcaga
Exon 2–3′ region	tgcatttgctcttccaaaaa	agagtcggcagaaaacctca

For the parental origin of the 9p deletion was explored using a series of markers insertion/deletion (in/del) polymorphisms located within the minimal critical region on 9p that have been described elsewhere (Ergun-Longmire *et al.*, 2005). Each in/del polymorphism was amplified using a PCR reaction mixture containing 20 ng of genomic DNA, all 4 dNTPs (each at 200 μM), 1.5 mM $MgCl_2$, 1 unit of *Taq* polymerase (Eurobio), and each oligonucleotide at 0.25 μM. After heating for 10 min at 95°C, the PCR reaction was performed for 35 cycles at 95°C for 30 s, 56°C for 30 s and 72°C for 30 s. The PCR products were separated by electrophoresis on 4% agarose gel with 0.1 g/ml ethidium bromide.

Ultra-high resolution oaCGH

Isothermal oligo design, array fabrication, DNA labelling, oaCGH experiments, data normalization and $\log_2$(Cy3/Cy5) ratio calculations were performed by NimbleGen (NimbleGen Systems, Inc., Madison, WI, USA). The arrays were constructed by maskless array synthesis technology (NimbleGen Systems, Inc.), with up to 385 000 oligonucleotides being synthesized by photolithography on an array by previously described methods (Singh-Gasson *et al.*, 1999; Nuwaysir *et al.*, 2002; Selzer *et al.*, 2005).

Results and Discussion

A small terminal deletion of chromosome 9p was observed by ultra-high resolution comparative genomic hybridization and subsequent high resolution cytogenetic analysis (Fig. 1). The karyotype of the patient was 46,XY,del(9p24). The karyotype of the patient's father and mother was normal. In order to determine the parent origin of the chromosome 9p with a distal deletion, we screened for loss-of-heterozygosity using a series of in/del polymorphisms within the deleted region. The in/del marker rs3028563 proved informative. This marker is located within the open reading frame of the *DOCK8* gene on 9p24 (Fig. 1). The patient inherited his intact chromosome 9p from his mother and inherited his deleted chromosome 9p from his father. In a previous case, a chromosome 9p deletion associated with 46,XY complete gonadal dysgenesis, learning difficulties and affective disorder, the deleted chromosome 9 was of maternal origin (Veitia *et al.*, 1998, patient 2). In a second patient (Veitia *et al.*, 1998, patient 1) with affective disorder but normal IQ, the rearranged chromosome 9p with a deletion was of paternal origin. Other studies have shown that the inheritance of a deleted chromosome 9 can be of either paternal or maternal origin suggesting that genetic imprinting is not involved in the development of the phenotype (Christ *et al.*, 1999). The *SRY* gene sequence of the patient was identical to that of a normal male. Taking these cases together (Veitia *et al.*, 1998), the minimal region associated with gonadal dysgenesis and autistic spectrum disorder extents from *DMRT3* to the 9p telomere.

Mental retardation has been described in a 45,Xt(Y;9) girl who presented with normal female external genitalia, uterus and a right streak gonad and a left dysplastic ovary (de Ravel *et al.*, 2004). Mental retardation, together with characteristic monosomy 9p syndrome features were described in a female patient with a deletion of distal 9p (46,XY, del(9)(p22); Livadas *et al.*, 2003). A child with craniosynostosis, partial absence of the corpus callosum, developmental delay, precocious puberty and deletion of chromosome 9(p12p13.3) has also been reported (Eshel *et al.*, 2002). In addition, a locus associated with obsessive-compulsive disorder has been mapped to 9p24 (Willour *et al.*, 2004). Recently, three patients with terminal deletions

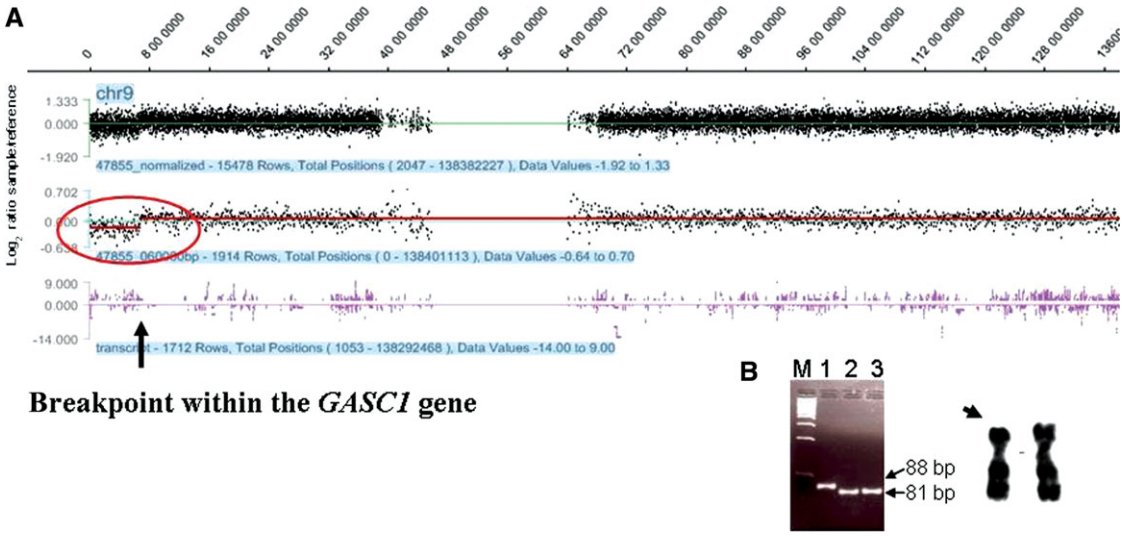

Figure 1: **(A)** Ultra-high resolution oaCGH using the Nimblegen whole genome CGH array (NimbleGen Systems, Inc.) The high-capacity microarray contains 385 000 probes with a median probe spacing of 6 kb. Array data was analysed using the SignalMap Software V1.8 (NimbleGen Systems, Inc.). The genome profile of the patient versus normal male reference genomic DNA is shown for chromosome 9. The X-axis coordinates indicate the relative position of the oligo probes ordered by genomic map position on chromosome 9. The Y-axis shows the $\log_2$ ratio shift. The top panel shows the normalized signal whole-chromosome profile using qspline normalization. The middle panel shows the window averaging of signals in 60 kb segments. Segmented data are shown in red and the deleted region is indicated by the red circle. The lower panel indicates the genomic position of transcripts on chromosome 9p. Raw data are shown in black. **(B)** Partial karyotype of patient after RHG banding showing a terminal deletion of 9p24 (indicated by arrow, right). Agarose gel electrophoresis of in/del marker rs3028563 (left) . The patient did not inherit the 88 bp fragment of DNA from his father indicating that the deletion was of paternal origin. The paternity was confirmed using microsatellite markers (data not shown). M, 100 bp molecular weight ladder; 1, amplification from father's DNA; 2, amplification of patient's DNA; 3, amplification of DNA from the mother

of 9p24 have been described (Ounap *et al.*, 2004). One of these individuals (patient 3) was a 46,XX female with severe mental retardation, clumsy gait, aggressiveness and autistic behaviour. These data and those of the patient described here suggest that there is a gene associated with autistic spectrum disorders located on distal 9p24.

The gonadal phenotype may be caused by either haploinsufficency of one or more of the tightly-linked DM-domain containing genes on 9p24 or by unmasking a recessive mutation within the deleted region. *DMRT1* forms part of a 220 kb cluster of DM-domain containing genes on chromosome 9p comprising *DMRT1–DMRTA3–DMRT2* (Brunner *et al.*, 2001; Ottolenghi *et al.*, 2002). *DMRT1* is an excellent candidate for the gonadal dysgenesis, although point mutations in the gene have not yet been identified associated with this phenotype (Calvari *et al.*, 2000; Ottolenghi and McElreavey, 2000). In many vertebrate species, *DMRT1* expression is limited to the developing gonads and expression is upregulated specifically in the developing testis (De Grandi *et al.*, 2000; Kettlewell *et al.*, 2000; Moniot *et al*., 2000). The chicken *DMRT1* orthologue maps to the Z chromosome suggesting again a role in sex determination. Mice lacking *Dmrt1* do not show complete sex reversal. Instead, the testes are small and show signs of defects in somatic and germ cells after birth. *DMRTA3* has a restricted expression pattern in vertebrate embryos and may also contribute to both the gonadal and behavioural phenotypes. In both chicken and mouse embryos, *DMRTA3* is expressed primarily in the developing forebrain, neural tube and nasal placodes (Smith *et al.*, 2002). *DMRTA3* is also expressed in human brain tissue (Ottolenghi *et al.*, 2000a,b). Expression has also been detected in human testis but not ovarian tissue and may contribute to the more severe gonadal phenotype seen in the human cases of monosomy 9p whne compared with the *Dmrt1* loss-of-function mutation in the mouse (Ottolenghi *et al.*, 2002; Smith *et al.*, 2002). *DMRT2* expression data from fish suggest that it may function during early somitogenesis (Meng *et al.*, 1999). Direct sequencing of the *DMRT1* and *DMRT3* amplicons revealed that the sequence of both genes was identical to that of a normal male control. This result is consistent with the hypothesis that haploinsufficency is responsible for the phenotype.

There are several genes on chromosome 9p that may be responsible for the behavioural phenotype (Ottolenghi *et al.*, 2000a,b). These include the genes *DOCK8*, *COBW*, *ANKRD15* and *FOXD4*. Forkhead genes are key regulators of embryogenesis and tumourigenesis (Kaufmann and Knochel, 1996). These genes are members of a large family of transcription factors with highly conserved 100-amino acid DNA-binding forkhead domains (Kaufmann and Knochel, 1996). In 1994, Pierrou *et al*. identified a 318-bp partial human cDNA sequence (U13 223) with high homology to the forkhead domain and named it *FREAC5* for forkhead-related activator 5. This gene was recently renamed *FOXD4* (Kaestner *et al.* 2000). The *FOXD4* gene is located ~100 kb from the 9p telomere and it is predicted to encode a single-exon ORF of 408 amino acids with a conserved amino termini, including a highly conserved forkhead domain. Although mutations in forkhead genes are a cause of specific human diseases, including a speech and language disorder (*FOXP2*; Lai *et al.* 2001) the *FOXD4* gene is expressed at high levels in heart and skeletal muscle and at low levels in other tissues including brain tissue (Pierrou *et al.* 1994).

DOCK8 is located ~400 kb distal to the *DMRT1* gene. In mammalian cells, the DOCK family of proteins have roles in regulating cytoskeletal reorganization (Ruusala *et al.*, 2004). *DOCK8* may be involved in the reorganization of the actin filament system and is expressed in placenta, lung, kidney and pancreas, with low levels in heart and brain tissue. The kidney ankyrin repeat-containing protein (*ANKRD15*) gene is located ~100 kb distal to the *DMRT* gene cluster (Ottolenghi *et al.*, 2000a,b, 2002). Northern blot analysis revealed expression in all tissues examined except peripheral blood leukocytes. Highest expression was found in heart, prostate and ovary (Nagase *et al.*, 1996). Recently, a deletion polymorphism in the general population that encompasses the *ANKRD15* gene has been reported (Iafrate *et al.*, 2004; Conrad *et al.*, 2006). We explored the possibility that the patient carried this deletion by amplifying

segments of the *ANKRD15* gene. The amplification of *ANKRD15* exons 1, 4 and 6 revealed PCR fragments of the expected size indicating that the patient did not carry the deletion polymorphism (data not shown).

Cobalamin synthetase W (*COBW*) is an intronless gene isolated from *Pseudomonas denitrificans*, which encodes a 354-amino acid cobalamin (vitamin B12)-synthesis protein (Crouzet *et al.*, 1991). In *Pseudomonas*, the gene functions in the synthesis of vitamin B12 but it is unknown whether the human orthologues have maintained this function. The early onset type of cobalamin deficiency is characterized by feeding difficulties, failure to thrive, hypotonia, seizures, microcephaly and developmental delay. Mental retardation is a common finding (Biancheri *et al.*, 2001).

Each of these genes is a candidate for the autistic spectrum disorder phenotype. It is unknown if this phenotype is caused by haploinsufficiency or by the unmasking of a recessive allele. It is also unclear if a mutation involving a single gene is responsible for 46,XY gonadal dysgenesis and autistic spectrum disorder or if they are genetically distinct entities. It would be of interest to systematically screen this region of 9p for microdeletions in patients presenting with either 46,XY gonadal dysgenesis and/or autistic spectrum disorders. This could be easily achieved using the high resolution microarray approaches or by fluorescent *in situ* hybridization on metaphase spreads with informative BACs corresponding to the region.

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