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ARTICLE

Characterization of deletions at 9p affecting the candidate regions for sex reversal and deletion 9p syndrome by MLPA

Michela Barbaro*[,1], Antonio Balsamo[2], Britt Marie Anderlid[1], Anne Grethe Myhre[3], Monia Gennari[2], Annalisa Nicoletti[2], Maria Carla Pittalis[4], Mikael Oscarson[1] and Anna Wedell[1]

[1]*Department of Molecular Medicine and Surgery, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden;* [2]*Division of Pediatric Endocrinology, Department of Pediatrics, Policlinico S. Orsola-Malpighi, University of Bologna, Bologna, Italy;* [3]*Department of Pediatrics, Rikshospitalet, Oslo, Norway;* [4]*Department of Obstetrics and Gynecology, Policlinico S. Orsola-Malpighi, University of Bologna, Bologna, Italy*

The distal region on the short arm of chromosome 9 is of special interest for scientists interested in sex development as well as in the clinical phenotype of patients with the 9p deletion syndrome, characterized by mental retardation, trigonocephaly and other dysmorphic features. Specific genes responsible for different aspects of the phenotype have not been identified. Distal 9p deletions have also been reported in patients with 46,XY sex reversal, with or without 9p deletion syndrome. Within this region the strongest candidates for the gonadal dysgenesis phenotype are the *DMRT* genes; however, the genetic mechanism is not clear yet. Multiple ligation-dependent probe amplification represents a useful technique to evaluate submicroscopic interstitial or distal deletions that would help the definition of the minimal sex reversal region on 9p and could lead to the identification of gene(s) responsible of the 46,XY gonadal disorders of sex development (DSD). We designed a synthetic probe set that targets genes within the 9p23-9p24.3 region and analyzed a group of XY patients with impaired gonadal development. We characterized a deletion distal to the *DMRT* genes in a patient with isolated 46,XY gonadal DSD and narrowed down the breakpoint in a patient with a 46,XY del(9)(p23) karyotype with gonadal DSD and mild symptoms of 9p deletion syndrome. The results are compared with other patients described in the literature, and new aspects of sex reversal and the 9p deletion syndrome candidate regions are discussed.
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Keywords: disorders of sex development (DSD); gonadal dysgenesis; sex reversal; multiple ligation-dependent probe amplification (MLPA); DMRT

Introduction

The 9p distal region has been extensively investigated to identify genes involved in sex development because of the observation of patients with the 9p deletion syndrome, including abnormal sex development. Patients with the 9p deletion syndrome present with mental retardation, craniofacial dysmorphic features (eg, trigonocephaly, long philtrum) and delayed motor development,[1] and in patients with XY chromosomes genital and/or gonadal disorders are quite frequent. Furthermore, in XY patients the disorders of sex development (DSD) have a wide spectrum of presentation. The external genital phenotype ranges from complete female to male with hypospadias.

*Correspondence: Dr M Barbaro, Department of Molecular Medicine and Surgery, Karolinska Institutet, Karolinska University Hospital, CMM L8:02, 17176 Stockholm, Sweden.
Tel: +46 8 51774643; Fax +46 8 51773620;
E-mail: Michela.barbaro@ki.se
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The gonadal phenotype ranges from complete gonadal dysgenesis to ovotestes and to cryptorchid and/or hypoplastic testis. The identification of 9p24 deletions in patients with XY gonadal dysgenesis but without typical 9p deletion syndrome features, and the detection of patients with interstitial 9p deletions, has allowed the identification of two distinct regions for 9p sex reversal and for the 9p deletion syndrome. The latter has been localized at 9p22.3-p23,[2–6] whereas the sex reversal region has been progressively narrowed down to the region 9p24.3, extending from the *DMRT* genes to the telomere (Figure 1a).[7–10] Within this region the strongest candidate genes for the gonadal dysgenesis phenotype are the *DMRT* genes that encode proteins with a DM domain. This is a zinc-finger-like DNA-binding motif that derives its name from the *Drosophila doublesex* (*dsx*) and the *Caenorhabditis elegans mab-3* genes where it was initially identified.[11] Both these genes are involved in downstream pathways of sex determination. There are three *DMRT* genes on 9p24, namely *DMRT1*, *DMRT3* and *DMRT2* (Figure 1).[12,13] All deletions reported so far in patients with XY gonadal dysgenesis include all three genes, except for one case where the deletion is telomeric of the *DMRT* genes in a potential regulatory region.[10] The molecular mechanism that leads to gonadal dysgenesis is not clear and could be caused by haploinsufficiency of one or more genes in the deleted region, by unmasking of a recessive mutation on the other chromosome or by more complicated mechanisms. The haploinsufficiency mechanism is the most likely because despite several attempts no mutations in *DMRT1*

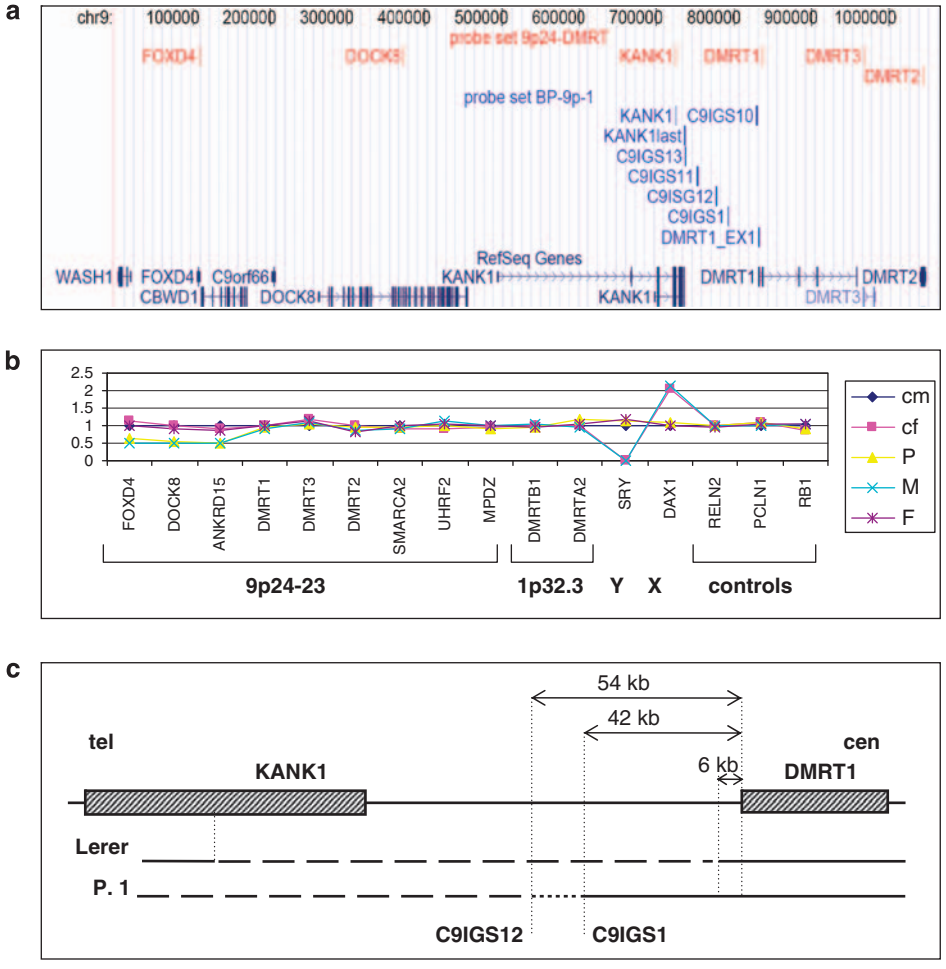

Figure 1 MLPA results for patient 1. (**a**) Representation from the UCSC genome browser of the 9p24.3 locus. Some of the probes included in the 9p24-DMRT probe sets and probes included in the BP-9p-1 set are represented by red and blue vertical lines, respectively. (**b**) Graph showing results of the MLPA analysis with the 9p24-DMRT probe set for patient 1 (P) and her mother (M) and father (F), when normalized to the male control (cm). As expected the female control (cf) shows a double intensity for the DAX1 probe on chromosome X. The patient and her mother show a deletion encompassing probes FOXD4, DOCK and KANK1, whereas her father has a pattern similar to the controls. Probe names are indicated along the *x*-axis and ratio values along the *y*-axis. (**c**) Schematic representation and comparison of the deletions at 9p24.3 carried by the subjects described by Lerer *et al*[24] and patient 1 (P.1). Dashed lines represent the deleted regions, and dotted lines correspond to the breakpoint region.

or *DMRT2* have been identified.[10,12,13] The *DMRT3* gene was only recently described and no mutation screening has been reported so far. Furthermore, gene dosage imbalances of other genes are known to affect male gonadal development, involving for example *SF1, NR0B1 (DAX1), WT1, WNT4* and *SOX9*. It is not clear if haploinsufficiency of one or a combination of the *DMRT* genes is responsible for the phenotype. *Dmrt1*$^{-/-}$ mice have been reported and they presented with hypoplastic testis but not with sex reversal.[14] These results confirm that the *DMRT1* gene is involved in sex development also in mammals and differences in the severity of the phenotype between murine and human abnormal sex development are not surprising. However, as 9p24.3 deletions have shown a noncomplete penetrance in patients, we still do not know if a single *DMRT1* haploinsufficiency leads to gonadal dysgenesis or if other factors can modulate the phenotype.

The identification and fine mapping of submicroscopic distal or interstitial deletions on 9p24.3 would help to define the minimal sex reversal region and could lead to the identification of gene(s) responsible of the 46,XY gonadal DSD. Until now 9p deletions were mostly detected by conventional karyotyping, and were caused by both distal deletions and unbalanced chromosome translocations. Screening for submicroscopic deletions has been performed by FISH or microsatellite analysis, but both these techniques have limited resolution. Lately array-CGH (comparative genomic hybridization) has been introduced in clinical practice thus improving the resolution.

Multiple ligation-dependent probe amplification (MLPA) represents an easy and reliable technique for detection of copy number variations and does not require parental samples for the evaluation of the results. We developed a probe set for MLPA analysis to detect deletions and duplications of genes on 9p24 in the region surrounding the suggested candidate genes for gonadal dysgenesis. Two additional probe pairs were designed within the *DMRTB1* and *DMRTA2* genes, which are located on chromosome band 1p32.3. These *DMRT* genes were chosen because they both show high expression in the adult human testis and have weak or absent expression in the adult ovary.[15] Moreover, duplication of 1p31-p32.3 has been reported in some cases of 46,XY DSD.[16,17] Interestingly, the related *dmrta2* in the Tilapia fish maps in a quantitative trait locus region for sex determination.[18]

Here, we report the results obtained from the analysis of 24 patients with 46,XY testicular DSD.

Subjects and methods

Samples selected for this study were collected from patients that had been referred for 46,XY gonadal DSD to the Clinical Genetic Unit of Karolinska University Hospital, Stockholm, Sweden, or to the Molecular Genetic Laboratory of the Pediatric Unit at the S. Orsola-Malpighi Hospital, Bologna, Italy. The 12 patients (including one pair of siblings) from Sweden had 46,XY gonadal dysgenesis and female external genitalia with no signs of virilization. Abnormal gonadal development was diagnosed by the presence of female internal genitalia, very high levels of FSH and gonad biopsies, when available. The 10 Italian patients formed a more heterogeneous group of 46,XY patients with abnormal gonadal development without a genetic diagnosis; the external genitalia ranged from completely female to ambiguous. Informed consents were collected. The study was approved by the regional ethics committee at the Karolinska Institutet. DNA was obtained from blood samples, EBV-immortalized cell lines or gonadal fibroblasts, when available. *SRY* gene mutations were excluded in all cases, as well gene dosage imbalances for *SF1*, *SOX9*, *DAX1* and *WNT4* genes.[19] None of these 22 patients have 9p deletion syndrome. We also included a patient with a 46,XY karyotype and a deletion of approximately 700 kb at the 9p telomere previously detected by tiling BAC array-CGH (data not shown) and a patient with a 46,XY del(9)(p23) karyotype, both with gonadal dysgenesis.

Controls

Blood samples were collected anonymously from five fertile males and five healthy postpubertal females. Lymphocytes were isolated and EBV immortalized. DNA was extracted from the cell lines by phenol/chloroform extraction. Control DNA samples were also obtained from Karolinska University Hospital blood donors.

Cases histories

Patient 1 has been previously described as patient RB by Calvari *et al*.[10] She has a 46,XY karyotype, and isolated complete gonadal dysgenesis with consequent female external genitalia.

Patient 2 was delivered by cesarean section at term due to fetal stress (Apgar 8–9), after an uncomplicated pregnancy. Birth weight and length were 3750 g and 49 cm, respectively. The newborn presented normal female external genitalia. At 6 months of age she was referred to a pediatric neuropsychiatric unit because of mild developmental delay. She sat independently at 11 months, started walking at 20 months and spoke her first words at 24 months of age. She showed persisting developmental delay and hypotonia. Mild facial dysmorphism was present with methopic prominence, brachycephaly, flat nasal bridge, epicanthic folds, long philtrum and posteriorly rotated ears but no trigonocephaly. In addition, she had bifid uvula, high and narrow palate, webbed neck and a broad chest. Genetic evaluation revealed a 46,XY karyotype, and at the age of 6.8 years, she was further investigated by a pediatric endocrinologist. She presented female external genitalia, with mild hypoplasia of minor and major labia and no

signs of virilization. Height was at the third percentile and the dysmorphic features as described above. A pelvic ultrasound examination showed the presence of a uterus with bilaterally visible gonadal tissue. A GnRH test (50 μg i.v.) showed pathological results with basal FSH and LH values of 44.3 mU/ml (reference values for prepubertal males 1.6 ± 1.05 and prepubertal females 3.1 ± 1.8 mU/ml) and 2.5 mU/ml (reference values for prepubertal males 0.82 ± 0.64 and prepubertal females 0.65 ± 0.55 mU/ml), and peak values of 130.2 and 37.3 mU/ml, respectively. An hCG stimulation test (2000 UI i.m. for 3 days) was performed. Blood samples were analyzed after 3, 5 and 6 days. Basal and peak values for estradiol were 66.1 and 117.6 pmol/l (basal reference values 12.5 ± 8.4 pmol/l), for testosterone 1.0 and 0.7 nmol/l (basal and after stimulation normal values 0.6 ± 0.02 and $>$3.1 nmol/l), for D4-androstenedione 1.6 and 2.2 nmol/l (basal normal values 1.17 ± 0.66 nmol/l), for dehydroepiandrosterone 3.5 and 1.2 nmol/l, and for 17-OHP 1.2 and 2.4 nmol/l (normal values 1.2 ± 0.82 nmol/l). Further cytogenetic studies on peripheral lymphocytes showed a 46,XY,del(9)(p23) karyotype in all 42 metaphases examined. The same result was achieved on 100 metaphases from gonadal fibroblast obtained after gonad biopsy. Histological examination of multiple biopsies from the left gonad showed the presence of fibrovascular tissue with Müllerian glandular–tubular structures. No normally differentiated testicular structure was seen, confirming the diagnosis of complete XY gonadal dysgenesis. No histological information is available for the right gonad. Prophylactic bilateral gonadectomy was performed. The patient is raised as a female and she will be treated with estrogen substitutive therapy as she approaches pubertal age. A conventional karyotype at 550 bands excluded balanced translocations in the parents.

MLPA

Synthetic probes for MLPA were designed as previously described.[20,21] Probes targeting specific genes were designed within or at least partially covering the coding region. In addition, three control probes hybridizing to *RELN, CLDN16* and *RB1* genes located at chromosomes 7q22.1, 3q28 and 13q14.2, respectively, were included together with a 'pilot' probe pair to filter noise (Tables 1 and 2). The MLPA reactions were carried out, starting from 100 ng of genomic DNA, and using the in-house designed probe set and the reagents from the EK1 reagent kit (MRC-Holland, Amsterdam, the Netherlands), according to the manufacturer's recommendations. PCR products were separated using capillary electrophoresis system on an ABI 3100 genetic analyzer (Applied Biosystems). Trace data were analyzed using the GeneMapper version 3.7 software, and the integrated peak areas were exported to an Excel 2003 spreadsheet (Microsoft) for further calculations. For each sample, the peak areas were first normalized to the average peak area of the three control probes, followed by normalization to the average peak area of the control

Table 1 9p24-DMRT probe set used for MLPA analysis

Probe name MIX 9p24	*Size*	*5′ Half probe*[a]	*3′ Half probe*[b]	*UCSC position*[c]	*Chromsome band*
GABRA4 (pilot)	84	CAGCCTGTTGTCATAACCATCG	AGCAAACTGTCCAGGATGCG	46689618–46689659	4p12
RELN (control)	87	CAGCATTACGGAATGAAGGTCA	CCACAAGAAGTGGCTTCACAACC	102919656–102919700	7q22.1
FOXD4	90	GGAGAGGAGGAAGATGAAGACGAGG	AGGAGGCGGCGAGCCAGCAGTTC	107982–108029	9p24.3
DMRT2	93	GGAAACAGAATAATTTCGAGCGCA	AAGCTGTGTACCAGAGGCAAGTCAGAG	1043741–1043791	9p24.3
DOCK8	96	GCTACCACACGTATGGCCGCACATCA	GCTGCTGCTGTGAGTTCAAAGCTGCTGC	372543–372596	9p24.3
KANK1	99	CTGCAAACACCATCCCCAGTGTGTACAA	AGTGCATGAGTGGGTTCATTGTCAAGGCC	724847–724903	9p24.3
DAX1	102	GCAGCCTCAGCGGGCCTGTTGAAGACGCTG	CGCTTCGTCAAGTACTTGCCCTGCTTCCAG	30236574–30236633	Xp21.2
CLDN16 (control)	105	GACACAAGGGTGTAAAATGCACG	TTTCAGGGTGTGTTTGCATATGATTTAATCAA TCAGTATG	191610505–191610567	3q28
DMRT1	108	CAGTGCGGCCGAGCTGCTTGTCAAAAGAGAGAA	CAATGGCAGTAACCCGTGCCTCATGACTGAGTG	837025–837090	9p24.3
DMRT3	111	CTCAAGGGCCACAAGCGTTACTGCCGCTTCA	AGGACTGCACCTGCGAGAAGTGCATCCTCA TCATCGAG	967122–967190	9p24.3
DMRTB1	114	CTGCGAGAAGTGCTACCTGATCTCCGAGC GCCAGA	AGATCATGGCCGCGCAGAAGGTGCTCAAGAC GCAGGC	53697822–53697893	1p33
DMRTA2	117	CACGTTTTCTCCCCTCCCAGAGGCCAAGTTGCA GAAGT	TTGACCTGTTTCCTAAGACGCTGCTGCAGG CAGGCCG	50657939–50658013	1p32.3
SRY	120	GAATGCGAAACTCAGAGATCAGCAAGC AGCTGGGA	TACCAGTGGAAAATGCTTACTGAAGCCGAA AAATGGCCATTCG	2715318–2715394	Yp11.31
MPDZ	123	GTGATCCAGTACTTGATCTGCATATGTCTCTGGAG GAACT	ATATACCCAGAATCTCCTGCAAAGACAGGATG AGAATACAC	13166317–13166397	9p23
SMARCA2	126	GAAAGGATTCGTAATCATAAGTACCGGAGCCTAGG CGACCTGGAG	AAGGATGTCATGCTTCTCTGTCACAACGCTC AGACGTTC	2172141–2172224	9p24.3
RB1 (control)	129	GTCACCAATACCTCACATTCCTCGAAGCCCTT ACAAGTTTCCT	AGTTCACCCTTACGGATTCCTGGAGGGAACAT CTATATTTCACC	47937353–47937439	13q14.2
UHRF2	132	GTACGAGAGAATGTACTATTGTCCCTTCTAATCA TTATGGACCCATTC	CTGGTATTCCTGTTGGATCAACTTGGAGATTT AGAGTTCAGG	6472011–6472100	9p24.1

[a]The 5′ half probes are preceded by the universal tag sequence GGGTTCCCTAAGGGTTGGA.
[b]The 3′ half probes are followed by the universal tag sequence TCTAGATTGGATCTTGCTGGCAC and are phosphorylated at the 5′-end.
[c]UCSC assembly: May 2006; numbers indicate the coordinate of the 5′ half probe first base and the 3′ half probe last base.

Table 2 BP-9p-1 and BP-9p-2 probe sets used for MLPA analysis

Probe name	*Size*	*5′ Half probe*[a]	*3′ Half probe*[b]	*UCSC position*[c]	*Chromsome band*
MIX BP-9p-1					
GABRA4 (pilot)	84	CAGCCTGTTGTCATAACCATCG	AGCAAACTGTCCAGGATGCG	46689618–46689659	4p12
RELN (control)	87	CAGCATTACGGAATGAAGGTCA	CCACAAGAAGTGGCTTCACAACC	102919656–102919700	7q22.1
C9IGS1	93	GTGCTGTGGCCTCATCCCAGGAAGTA	GCAAGTCAACTGTATCCAATGAGGG	791605–791654	9p24.3
KANK1last	96	GCTCCGTTTTGTACAGTCACAGGGAA	TTCTGATCTGAAGGGGCACCTTCTGTTC	735482–735535	
KANK1	99	CTGCAAACACCATCCCCAGTGTGTACAA	AGTGCATGAGTGGGTTCATTGTCAAGGCC	724847–724903	9p24.3
DMRT1ex1	102	CCTAGGGGCACCATGCCCAACGACGAGGCATT	CAGCAAGCCCTCTACACCGTCGGAAGCC	831827–831886	9p24.3
CLDN16 (control)	105	GACACAAGGGTGTAAAATGCACG	TTTCAGGGTGTGTTTGCATATGATTTAATCAA TCAGTATG	191610505–191610567	3q28
C9IGS12	108	CAGTGATTTAATCCATAATAAGGGAGAGTCT	GCTCAGTTTTTAGGGAATTTCAATGAAGTTGATTC	776889–776954	9p24.3
C9IGS11	111	CCTGTTGAAAGCCACTCAATGGCTTCAGCTCGGT	CTGGCATGATGAAGCTTCCTCCGTAATCTGGGTTC	752281–752349	9p24.3
C9IGS10	117	GGAGAGAAAGGAGACAACAAAGCCAGGCTACT TGAAATATC	CAGTACAAATTTGTTCATTCTTGGGGCCTTCCTG	828029–828103	9p24.3
C9IGS13	123	GCAGCAACAGACCAGCTCTCCCAGGACCACAAG GATGACTTT	CCTCTGCATGAACTGTGAAAGTGAGGTCACAGC CACCTG	737115–737195	
RB1ex23 (control)	129	GTCACCAATACCTCACATTCCTCGAAGCCCTTA CAAGTTTCCT	AGTTCACCCTTACGGATTCCTGGAGGGAACATC TATATTTCACC	47937353–47937439	13q14.2
MIX BP-9p-2					
GABRA4 (pilot)	84	CAGCCTGTTGTCATAACCATCG	AGCAAACTGTCCAGGATGCG	46689618–46689659	4p12
RELN	87	CAGCATTACGGAATGAAGGTCA	CCACAAGAAGTGGCTTCACAACC	102919656–102919700	7q22.1
PTPRD	90	CAGCGAGTCTGTCCGATCTGAAAT	TTCAGCTGGAACACTTTCAGAGCC	8723900–8723947	9p24.1
DAX1	102	GCAGCCTCAGCGGGCCTGTTGAAGACGCTG	CGCTTCGTCAAGTACTTGCCCTGCTTCCAG	30236574–30236633	Xp21.2
CLDN16 (control)	105	GACACAAGGGTGTAAAATGCACG	TTTCAGGGTGTGTTTGCATATGATTTAATCAAT CAGTATG	191610505–191610567	3q28
C9IGS11	108	GGTGAAACACTGAATACTTTCTTCCACATA TCAGGAC	AACAATGGAGATGTGAGGTCTCACCACTG	11354381–11354445	9p23
TYRP1	111	GCTCAATTCCCAAGACAGTGTGCCACT GTTGAGGCTT	TGAGAAGTGGTATGTGTTGCCCAGACCTGTCC	12684066–12684134	9p23
JMJD2C	114	GCCGAGGTGGAAAGTCCTCTGAACCCCAGCTG TAAGAT	AATGACCTTCAGACCCTCCATGGAGGAGTTCCGG	6782998–6783069	9p24.1
SRY	120	GAATGCGAAACTCAGAGATCAGCAAGCAGC TGGGA	TACCAGTGGAAAATGCTTACTGAAGCCGAAAA ATGGCCATTCG	2715318–2715394	Yp11.31
MPDZ	123	GTGATCCAGTACTTGATCTGCATATGTCTCTG GAGGAACT	ATATACCCAGAATCTCCTGCAAAGACAGGATGA GAATACAC	13166317–13166397	9p23
C9IGS12	126	GGATTTATCTCTTCTTCAAGTCTGTTGGCCTTTTT GAACATGGACGT	AAATGTCCTTCCTCAGATTTGGGAAGATTGGGT CACG	11547281–11547363	9p23
RB1ex23 (control)	129	GTCACCAATACCTCACATTCCTCGAAGCCCTTA CAAGTTTCCT	AGTTCACCCTTACGGATTCCTGGAGGGAACATC TATATTTCACC	47937353–47937439	13q14.2
UHRF2	132	GTACGAGAGAATGTACTATTGTCCCTTCTAATCAT TATGGACCCATTC	CTGGTATTCCTGTTGGATCAACTTGGAGATTTA GAGTTCAGG	6472011–6472100	9p24.1

[a]The 5′ half probes are preceded by the universal tag sequence GGGTTCCCTAAGGGTTGGA.
[b]The 3′ half probes are followed by the universal tag sequence TCTAGATTGGATCTTGCTGGCAC and are phosphorylated at the 5′-end.
[c]UCSC assembly: May 2006; numbers indicate the coordinate of the 5′ half probe first base and the 3′ half probe last base.

samples included in the run. The analysis was considered acceptable if the ratio for the internal control was between 0.8 and 1.2. Threshold values for deletion and duplication were set at 0.75 and 1.25, respectively.

Results

MLPA analysis

The performance of the 9p24-DMRT probe set (Table 1) was tested using DNA samples from 10 normal controls (5 males and 5 females). All probes presented an SD $\leq$ 10% thus proving to be reliable and sufficiently consistent. A total of 22 patients were analyzed. Deletions were confirmed in the two cases with the 9p24 deletions already identified by other methods, but no other deletions or duplications were detected.

In patient 1, a deletion of approximately 700 kb at 9p24.3 had been identified using a 33K tiling BAC array-CGH (unpublished data). No other rearrangements have been identified in the patient. The MLPA analysis confirmed the deletion of three subtelomeric genes *FOXD4, DOCK8* and *KANK1* (previously known as *ANKRD15*), whereas the probe located within the *DMRT1* gene did not show a deletion. Analysis of parental DNA showed that the mother is the healthy carrier of the deletion (Figure 1). After this finding we received more information about the patients and came to know that this patient had been previously studied by Calvari *et al*.[10] The deletion breakpoint had been mapped in a region of 30 kb upstream the *DMRT1* gene. To further narrow down the breakpoint and in particular to evaluate if the *DMRT1* promoter was deleted we designed an additional MLPA probe set BP-9p-1 (Table 2). We determined that the deletion extends until probe C9IGS12, located 54 kb upstream of the *DMRT1* gene, whereas the region detected by the probe C9IGS1 is still present in two copies, indicating that at least 42 kb of the *DMRT1* promoter is still present.

In patient 2, the deletion extended from the probe pair located within the *UHRF2* gene to the most telomeric probe pair within *FOXD4* (Figure 2b). All probes within this

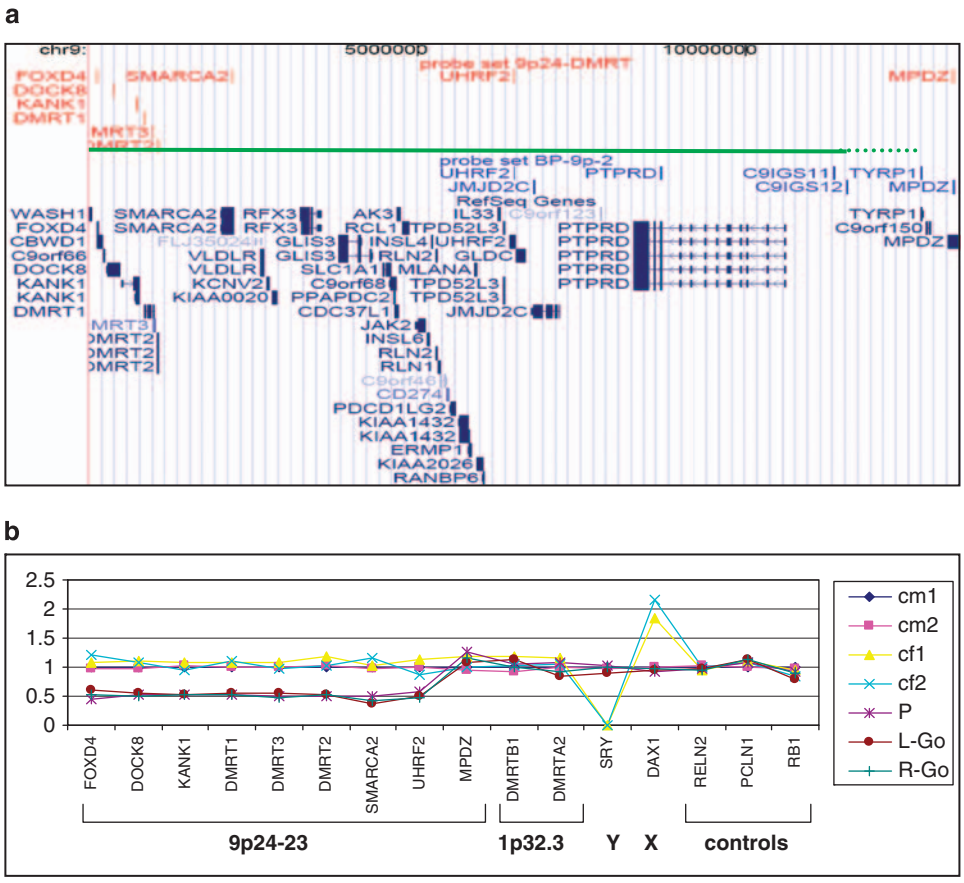

Figure 2 MLPA results for patient 2. (**a**) Representation from the UCSC genome browser of the 9p24-p23 region. The location of probes included in the 9p24-DMRT and BP-9p-2 probe sets is represented by red and blue vertical lines, respectively. The green line represents the deleted region with the breakpoint region indicated by dotted line. (**b**) Graph showing the results for patient 2 when normalized to male controls (cm) using the 9p24-DMRT set. As expected the female controls (cf) shows a double intensity for the DAX1 probe on chromosome X. DNA obtained from a blood sample (P) and fibroblasts from left and right gonads (L-Go and R-Go) show a deletion extending from the telomeric probe pair FOXD4 to the probe pair UHRF2, whereas the MPDZ probe pair has a ratio value corresponding to two copies of the gene as well as the probe pairs on 1p32.3.

region had a ratio below the threshold that was set to 0.75, with a median value of 0.51 (range 0.34–0.54), near the theoretical value of 0.5. The probe located in the *MPDZ* gene had a ratio consistent with two copies. Genomic DNA derived from the fibroblasts obtained from bilateral gonad biopsies was analyzed and the genetic alteration was confirmed in the gonadal tissue (Figure 2b). We designed the BP-9p-2 probe set (Table 2) to further narrow down the breakpoint region by MLPA. The distal deletion extended for at least 11.5 Mb, until the probe C9IGS12, whereas the more centromeric probe TYRP1, located within the first coding exon of the *TYRP1* gene, had a ratio value consistent with two copies of the gene.

As some of the target genes in the 9p24-DMRT probe set are listed in the database of genomic variants[22] (http://projects.tcag.ca/variation/), we also analyzed 68 healthy controls (36 males and 32 females) to evaluate the frequency of such polymorphisms in the Swedish population. No copy number variations were detected.

Discussion

The candidate region for sex reversal on 9p has been narrowed down to the 9p24.3 region. Even if it is not clear which gene(s) and genetic mechanisms are involved in the impairment of gonadal development, the major candidate genes are *DMRT1* and *DMRT3*. Mice with a targeted deletion of *Dmrt1* have shown postnatal testis defects, thus proving the function of *DMRT1* in mammalian sexual differentiation.[14] As the *dmrt1* deficiency was not sufficient to cause sex reversal, the concomitant haploinsufficiency of the conserved downstream *DMRT3* gene may be necessary for a more severe disruption of gonadal development. *Dmrt2* knockout mice have been recently described.[23] They show embryonic somite patterning defects and no sex development impairments, thus making *DMRT2* haploinsufficiency less likely as the cause of gonadal dysgenesis.[23] As 9p24.3 is located in the subtelomeric region of 9p, small deletions can easily escape detection by karyotyping using conventional banding

techniques. We therefore developed an MLPA probe set covering this region to screen for submicroscopic deletions, or duplications, in patients with 46,XY testicular dysgenesis. Probes were designed within the three *DMRT* genes, in three more telomeric genes *FOXD4*, *DOCK8* and KANK1 and three centromeric genes *SMARCA2, UHRF2* and *MPDZ* outside the candidate gonadal dysgenesis region (Figures 1a and 2a). The MLPA analysis with the 9p24-DMRT probe set readily detected the two already identified deletions on 9p24, proving to be a reliable method. Unfortunately, no deletions were identified in other patients with 46,XY DSD neither for genes at 9p24 nor for the *DMRT* genes on the 1p32.3 region.

In patient 1, who has an isolated 46,XY gonadal DSD, the small distal deletion that does not include the *DMRT* genes gives rise to many hypotheses and considerations in favor and against *DMRT* haploinsufficiency as the cause of gonadal dysgenesis. A study from 2005[24] reported a deletion that extends from within the *KANK1* gene to approximately 6.5 kb upstream the *DMRT1* gene (Figure 1c), which segregates in a large pedigree of patients affected by familial cerebral palsy. Among the carriers there are three males that are fathers, indicating that a deletion of this region does not affect male sex development or fertility. Thus, if DMRT1 expression is required for testis development, 6.5 kb of the *DMRT1* promoter is sufficient for its function. In patient 1 more than 40 kb of the promoter region is still present, which suggests that DMRT1 expression is retained and that the GD phenotype is not due to *DMRT1* haploinsufficiency. The gonadal phenotype could instead be caused by haploinsufficiency of one of the more telomeric genes. Another possibility, which we believe is more likely, is that in patient 1 the distal deletion locates the *DMRT1* gene in proximity of the telomere and this could affect the expression of *DMRT1* even if the regulatory region is intact.[25]

Although additional patients with 9p24 deletions and 46,XY gonadal dysgenesis have been identified,[26–30] at the same time patients with 9p24 deletions and normal male external genitalia and/or very mild gonadal abnormalities have also been described.[6,31,32]

We believe that a DMRT defect is involved in the gonadal dysgenesis phenotype, however a more complicated mechanism should be hypothesized, to explain the variable penetrance. One possibility could be that the *DMRT* haploinsufficiency together with other genetic defects would lead to gonadal dysgenesis through a dosage threshold effect. Interestingly, five copy number variants (CNVs) affecting *DMRT1* are listed in the Database of Genomic Variants.[33–36] The data are however not sufficient to consider a *DMRT1* deletion as a CNV polymorphism. Other genes important for gonadal development that act in a dosage manner are listed in the database, for example, *NR0B1* (*DAX1*), *WT1* and *SOX9*. This indicates also that the information contained in the Database of Genomic Variants should be carefully interpreted when evaluating regions involved in sex development; CNVs for autosomal genes may cause a sex development defect only in subjects with XY chromosomes and be carried by healthy fertile XX subjects, or vice versa.

The different type of genetic rearrangements leading to 9p deletions that could affect gene expression in a different manner, and the apparent non-full penetrance of the phenotype shown by the patients make it very difficult to understand the genetic and molecular mechanism leading to DSD. Furthermore, for some XY patients genital ambiguities are reported without any information about the gonadal development, which is fundamental to distinguish between a defect of gonadal development or a defect only of external genitalia development, which are caused by different mechanisms.

In patient 2, the telomeric deletion, already identified by conventional karyotyping, extends for 11.5 Mb and the breakpoint is located within a 1.1 Mb region between the probe C9IGS12 and the probe TYRP1, located in the first exon of the *TYRP1* gene.

A recent study has clearly divided the patients with 9p deletions in two groups. One group includes patients with the consensus phenotype of 9p deletion syndrome (trigonocephaly, small palpebral fissures, flat nasal bridge with anteverted nostrils, long philtrum and micro/retrognathia), whereas the other group includes patients that, although they carry 9p deletions, do not meet all the criteria of the consensus phenotype, in particular they lack trigonocephaly. Patient 2 does not present with trigonocephaly, thus she belongs to the latter group; however, she has metopic prominence and other dysmorphic features. In fact, the deletion breakpoint is telomeric to the critical region for 9p deletion syndrome recently described by Swinkels *et al*,[6] which has been obtained by comparing the genetic rearrangements in the two groups of patients. We noticed that some patients had distal deletions whereas others had interstitial deletions. The breakpoint in patient 2 is located in the region between the most distal and the most centromeric breakpoints carried by patients without trigonocephaly described by Swinkels *et al* (Figure 3); however, it is not stated if these patients had other cranial dysmorphologies and/or metopic prominence. We have carefully searched the literature to identify patients with clear molecular characterization and phenotype to compare with patient 2. We identified two patients with a mild 9p deletion syndrome presenting with prominent forehead and a 9p deletion breakpoint distal to the Swinkels' critical region [32,37] (Figure 3). Furthermore, a patient with a complex rearrangement with a distal 9p24 deletion but a duplication on 9p12-p23 is described to have XY gonadal DSD but no dysmorphic features.[7] We believe that there is a region, distal to Swinkels' critical region, that should still be carefully taken into account when genotype–pheno-

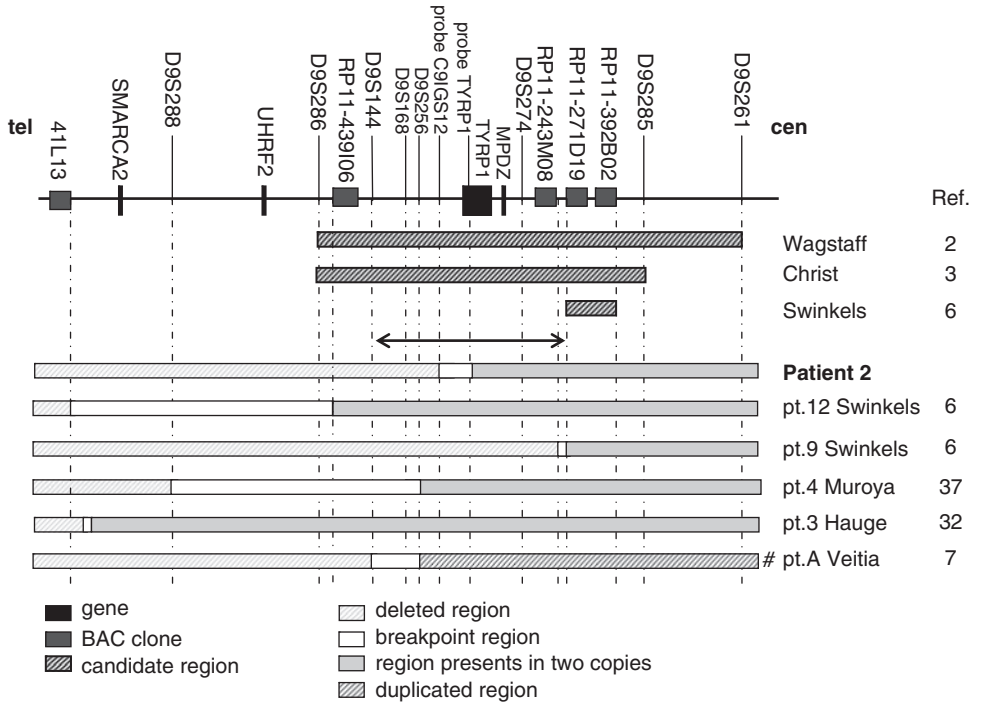

Figure 3 Critical region for the 9p deletion syndrome. Molecular characterization of critical 9p deletion regions and of different patients is compared. All patients have a mild form of the 9p deletion syndrome except the one labeled with a # symbol that has isolated 46,XY gonadal DSD. Black arrows indicate the region that should still be taken into account for a genotype–phenotype correlation of dysmorphic features in patients with 9p deletions. The chromosomal region is not drawn to scale; only selected genes are represented as black boxes.

type correlation for 9p deletion syndrome is evaluated (Figure 3). The mild cranial dysmorphism in patients with deletion distal to the critical region could be caused either by misregulation of the candidate gene for trigonocephaly or by the deletion of other gene(s) involved in craniofacial development.

Correlation between genotype and phenotype has proven to be difficult in patients with 9p deletion. As the region is near the telomere and the loss of material can be due to several types of rearrangements, it is fundamental to compare patients with and without a phenotypical characteristic and to have a highly refined molecular characterization. Furthermore, determination of a minimal critical region using only patients with distal deletion should be avoided as positional effects are difficult to evaluate and the results could be completely different when patients with interstitial deletions are included and compared, as shown by the different conclusions in Hauge *et al*[32] compared to Swinkels *et al*.[6]

To conclude, the MLPA-based analysis of all patients with 46,XY gonadal dysgenesis or other 46,XY testicular DSD, and even patients with 9p deletion syndrome, would lead to the identification and rapid characterization of more patients with rearrangements on 9p24. The accurate genetic characterization of a sufficient number of patients together with a detailed clinical description, as well as a morphological and molecular examination of gonadal tissue, would represent the starting point for identifying the molecular mechanisms that lead to each characteristic of the phenotype.

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