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DELETION MAPPING OF CRITICAL REGION FOR HYPOSPADIAS, PENOSCROTAL TRANSPOSITION AND IMPERFORATE ANUS ON HUMAN CHROMOSOME 13

Nilda M. Garcia, MD[2], **Jocelyn Allgood, RN**[1], **Lane J. Santos**[5], **D. Lonergan**[1], **J.R. Batanian**[6], **Mark Henkemeyer, PhD**[4], **Oliver Bartsch, MD, PhD**[7], **Roger A. Schultz, PhD**[3], **Andrew R. Zinn, MD, PhD**[5], and **Linda A. Baker, MD**[1]

1*Departments of Urology, University of Texas Southwestern Medical Center, Dallas, TX, USA*

2*Department of Surgery, University of Texas Southwestern Medical Center, Dallas, TX, USA*

3*Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX, USA*

4*Center for Developmental Biology, University of Texas Southwestern Medical Center, Dallas, TX, USA*

5*The McDermott Center for Human Growth & Development, University of Texas Southwestern Medical Center, Dallas, TX, USA*

6*Department of Pediatrics, Pediatrics Research Institute, University of Saint-Louis, MO, USA*

7*Institut für Klinische Genetik, Universitätsklinikum, Technische Universität, University Hospital Carl Gustav Carus, Dresden, Germany.*

Abstract

Background—The 13q-deletion syndrome causes human congenital birth defects due to the loss of regions of one long arm of human chromosome 13. A distal critical region for severe genitourinary and anorectal birth defects in the region of 13q32.2-34 has been suggested; we sought to narrow this critical region.

Methods—From patients with karyotypes revealing haploinsufficiency for distal chromosome 13q and their parents, peripheral blood was obtained and lymphocytes were immortalized for DNA isolation. Genetic and molecular cytogenetic methods were used to map deletions. Patient and parental samples were genotyped with a panel of 20 microsatellite markers spanning 13q31.3 qter and deletions identified by loss of heterozygosity. Deletions were also mapped using a panel of 35 BAC clones from the same region as probes for fluorescence *in-situ* hybridization on patient lymphoblastoid metaphase preparations. The data were synthesized and a deletion map defining the critical region was generated.

Results—Eight patients with known deletions around 13q32qter and their parents were analyzed, and categorized into three groups: three patients with anorectal and genitourinary anomalies

Corresponding Author: Linda A. Baker, MD, Director of Pediatric Urology Research, Aassociate Professor of Urology, University of Texas Southwestern Medical Center at Dallas, 5325 Harry Hines Boulevard, U9110 Dallas, TX, USA 75390-9110, Tel: 214-648-2278, Fax: 214-648-8786, Linda.baker@utsouthwestern.

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(hypospadias, penoscrotal transposition), four male patients without anorectal and genitourinary anomalies, and one XY patient with ambiguous genitalia without anorectal anomalies. We mapped the critical region for anorectal and genitourinary anomalies to a ~9.5-Mb interval of 13q33.3-q34 delineated by markers D13S280-D13S285; this spans ~8% of the chromosome and contains 20 annotated genes

Conclusion—The critical region of chromosome 13q mediating genitourinary/anorectal anomalies has been mapped, and will be narrowed by additional patients and further mapping. Identification of the gene(s) mediating these syndromic genitourinary defects should further our knowledge of molecular mediators of non-syndromic hypospadias, penoscrotal transposition and anorectal malformations.

Keywords

Chromosome; 13q; Hypospadias; Penoscrotal Transposition; Anorectal malformation; Deletion; Haploinsufficiency

Introduction

The molecular mechanisms and factors that govern embryological morphological events such as penoscrotal positioning, urethral tubularization, cloacal septation, and closure of the perineum and scrotum are not fully understood. In order to identify new molecular mediators of these developmental processes in humans, we investigated the molecular basis of genital and anorectal abnormalities in patients with the chromosome 13q-deletion syndrome. This syndrome [1], or loss of regions of one long arm of human chromosome 13, causes major human congenital birth defects, including severe genitourinary and anorectal malformations (Figures 1 and 2). Bartsch and colleagues [2, 3] described deletions of 13q (q33.2-qter) associated with penoscrotal inversion, hypospadias, reduced anogenital distance, imperforate anus, facial anomalies and developmental delay. They suggested the presence of a critical gene(s) in 13q32.2-q34 mediating these genitourinary and anorectal anomalies[1]. Additional phenotypes associated with distal 13q deletion in the literature include ambiguous genitalia in males[4-6], and absent or bicornuate uterus, imperforate anus with vaginal fistula, or cloaca in females[7-9]. Thus, haploinsufficiency of one or more distal 13q genes appears to cause these phenotypes.

Each of these birth defects, when independently assessed, is incompletely understood at the embryological and molecular level. In fact, despite the frequency of these individual birth defects in the general population, the etiology is unknown in the majority of human cases. Several studies conclude that the incidence of hypospadias is increasing [10] possibly due to environmental exposures to ‘endocrine disruptors’ [11-13]. Hypospadias is the second most common human birth defect, with an incidence of 1 in 125 male births [14]. Given the increasing incidence and significant morbidity associated with these birth defects, the importance of understanding embryonic and molecular mechanisms of normal and abnormal development has emerged as a key issue.

Although cytogenetic karyotyping is standard testing for children with multiple congenital birth defects, the accuracy and resolution of classic cytogenetic karyotyping is limited to changes >5 Mb. To improve resolution, we mapped distal 13q deletions using molecular markers and examined associated phenotypes in order to refine the critical region(s) for urogenital and anorectal anomalies and identify candidate gene(s) for these birth defects.

Materials and Methods

Patient recruitment and phenotype analysis

Using University of Texas Southwestern Institutional Review Board approved methods, patients were ascertained with karyotypes revealing haploinsufficiency for distal chromosome 13q (distal 13q deletion) via medical record review and search of cytogenetics databases. Patients and their family members were recruited through local sources and international collaborators. Detailed phenotypic data were collected from interviews with parents and physicians as well as from medical records. When available, local subjects were clinically evaluated by the senior author, an experienced pediatric urologist, documenting phenotype.

Genotype mapping

Peripheral blood samples were collected from probands, their available parents, and any other affected family members. From proband blood, Epstein-Barr virus-immortalized lymphoblastoid cell lines were generated by standard methods. Genomic DNA was extracted from all remaining family members' blood samples. 13q deletions were mapped to high resolution by testing for loss of heterozygosity (LOH) of 20 polymorphic microsatellite markers from the ABI PRISM® Linkage Mapping Set version 2.5 (Applied Biosystems, Foster City, CA, USA), supplemented by custom markers chosen from the genome database (www.gdb.org). One marker was specifically designed to amplify a polymorphic CA dinucleotide repeat within the ephrin-B2 (EFNB2) gene. For samples without parental DNAs or where key markers were not informative, fluorescence *in-situ* hybridization (FISH) was performed on proband lymphoblastoid metaphase preparations using probes from our panel of 35 BAC clones from the same chromosomal region (BACPAC Resource Center, Children's Hospital Oakland Research Institute, Oakland, CA, USA; (http://bacpac.chori.org/).

Determination of critical region by genotype-phenotype correlation

The data were synthesized and a deletion map defining the critical region was generated. Critical regions for urogenital and anorectal phenotypes were defined by the minimal overlap among deletions in patients with urogenital and/or anorectal phenotypes. With the possible exception of hypospadias, which is relatively common, 13q-patients with a genitourinary or anorectal anomaly should be deleted for the critical region for that phenotype. However, every patient who is deleted for the critical region will not necessarily have the associated phenotype, because haploinsufficiency of the culprit gene may show incomplete penetrance. Thus, we defined the critical region by comparing the deletions among only those patients with the phenotype in question.

Generation of the candidate gene list from annotated databases

A refined list of genes in the critical region was generated from the most current version of the UCSC Genome Browser. Functional genomic databases (e.g. GeneCards) and the PubMed literature database were searched to evaluate current knowledge about these genes regarding embryonic expression and known or suspected function in developmental signaling pathways.

Results

Phenotypic analysis of 13q-patients

Ten probands (9M:1F determined by sex chromosomal complement) with known deletions around 13q32-qter and 24 family members (parents, siblings, and grandparents) were recruited (Table 1). Of these, eight were included in this analysis, excluding one female and one male whose 13q deletions proved to be proximal to our region of interest. Phenotypic analysis is displayed in Table 2. Of these eight probands, six were newly ascertained for this study; one

sample (#7) was obtained from the NIGMS Camden Repository (ccr.coriell.org/nigms), and one previously reported sample (#6) [2] was a kind gift obtained from Dr Oliver Bartsch. These eight probands could be classified into three phenotypic groups: (1) three male patients with developmental delay and without anorectal and genitourinary anomalies (#1-3); (2) four patients with anorectal malformation or anorectal malformation + genitourinary anomalies (hypospadias, penoscrotal transposition) (#4-7); and (3) one XY patient with ambiguous genitalia without anorectal anomalies (#8).

Deletion mapping of 13q critical region for urogenital and anorectal phenotypes-Candidate Gene List

LOH and FISH results (Fig. 3) were concordant in cases where both analyses were performed. Due to limited quantities of DNA for proband #6, the proximal limit of the deletion in the proband was obtained from the literature. The deletion in this patient is unlikely to be smaller than the deletion in proband #4 or #8, and thus will probably not narrow the critical region. Data synthesis to date has yielded a deletion map defining the 13q critical region mediating genitourinary/anorectal anomalies to an ~11-Mb interval distal to marker D13S280 (Fig. 4). Five of the eight subjects have either anorectal or urogenital abnormalities, and three have both. The minimal overlapping deletion for both phenotypes (#4-anorectal malformation; #8-ambiguous genitalia/male pseudohermaphrodite) is proximally delimited by marker D13S280 in 13q33.1, making the critical region D13S280-D13S285, an interval of 9.5 Mb. This assumption is based on the deletion noted on patient #3, but a possible lack of penetrance of urogenital/anorectal malformations should be considered in this patient. This would then make the critical region D13S280-qtel, an interval of 11 Mb. It may be possible to narrow the anorectal anomaly critical region further by genotyping additional microsatellite markers or by FISH studies in #4, as D13S158 and D13S274 were uninformative. The breakpoint in #8 is already mapped to an ~400-kb interval between markers D13S280 and D13S158. As shown in Fig. 4, anorectal malformation is associated with both maternal and paternal 13q deletions, suggesting that this phenotype does not involve an imprinted locus. At present, four out of four deletions associated with urogenital abnormalities are paternal, but the numbers are too small to know whether this phenotype involves a maternally imprinted gene. From this deletion map, 20 annotated genes are identified in the interval D13S280-D13S285, based on the UC Santa Cruz Genome Browser annotation (May 2004 Freeze) (Table 3).

Discussion

Human chromosomal alterations can range from those detectable at the karyotypic level to point mutations. This translates to a broad range of phenotypic variability, especially when one considers the vast number of mechanisms that regulate gene expression. While this phenotypic variability complicates the analysis, cases with chromosomal or gene alterations are ‘experiments by nature’ that have led to elucidation of the genetic basis of many congenital birth defects.

Originally designated as one of the D-group autosomes, human chromosome 13 is depicted in Fig. 4 with its Giemsa banding nomenclature. In 1969, Allderdice and colleagues [1] described the ‘Chromosome 13q-Syndrome’. A list of the many phenotypes described in this syndrome is presented in Table 4; the exact phenotypes expressed are related to the chromosomal segment (s) deleted. Investigations of monosomy 13 patients with retinoblastoma or holoprosencephaly resulted in the positional cloning of the retinoblastoma gene (Rb) at 13q14 [15] and the ZIC2 gene [16] at 13q32, respectively. From a review of 20 patients with 13q deletions, Brown et al. [7] concluded that proximal deletions (q13-q31) are associated with mental retardation without major deformities except for retinoblastoma, while deletions involving band 13q32 are usually associated with numerous major malformations. They reported that deletions from q33-qter typically manifest severe mental retardation with more minor abnormalities; however, they did

not include genitourinary/anorectal anomalies as a major malformation. Unfortunately, one problem with karyotype/phenotype correlations is interpreting banding patterns of deleted chromosomes. As reported by Brown et al., "it is particularly difficult to decide, in the case of distal 13q deletions involving loss of the Giemsa-dark band q33, whether a remaining Giemsa-light band represents part of q32 or q34 or both" [7]. These authors recommended molecular markers to define accurately the exact deletions in the distal 13q region.

Two reports by Bartsch and colleagues [2, 3] of the clinical syndrome of penoscrotal inversion, hypospadias, reduced anogenital distance, imperforate anus, facial anomalies and developmental delay in distal chromosome 13q deletions (Online Mendelian Inheritance in Man # 602553) led these authors to suggest a critical region 13q33.2-qter mediating these defects. As seen in Figs 1 and 2, the external genital phenotype of the male child with a distal deletion in chromosome 13q is variable in severity[3, 17]. Some case reports have indicated ambiguous genitalia in XY cases of 13q deletion syndrome[4-6];however, it is not clear by what criteria this term was used, thus adding to some phenotypic confusion in the literature. Nevertheless, the male phenotype is severe and some die in utero or soon after birth[18]. In females, absent or bicornuate uterus, imperforate anus with vaginal fistula, and cloaca have been reported[7-9].

In this study, we focused on the male urogenital/anorectal manifestations of the 13q deletion syndrome. Hypospadias as an isolated defect is clearly a common human disorder with mild to severe phenotypic variability. Fortunately, proximal hypospadias accounts for about 15% of cases. In addition, proximal hypospadias cases can be associated with additional malformations, including penoscrotal transposition and anorectal malformations. The literature reports that up to 2.2%-10.5% of patients with anorectal malformations also have hypospadias [19-21]. Penoscrotal transposition, wherein the scrotum is partially or completely above the penis (see Figs 1 and 2), is a severe anomaly most commonly associated with severe hypospadias. This birth defect is strongly associated with deletions of chromosome 13q. Forty-six PubMed citations discuss penoscrotal transposition and, of these, six of 11 with chromosomal anomalies had 13q distal deletions [2, 17, 22-25]. Penoscrotal transposition is associated with imperforate anus in about a third of cases [26]. Anorectal malformations also demonstrate great phenotypic variability, affecting 1 in 1500 to 1 in 5000 live births, with an even gender distribution [27-30]. Although a male patient with this constellation of birth defects is a rare case, molecular based investigations may lead to the elucidation of key developmental molecular mechanisms not yet understood. This suggests that an alteration of human chromosome 13 could account for a significant proportion of patients with anorectal malformations and hypospadias/ penoscrotal transposition.

Animal models of hypospadias and anorectal malformations have been genetically engineered and can be created by drug exposure. Hypospadias has been noted in mouse models mutant for Shh[31, 32], Bmp's and Fgf's[33], Hoxa13[34-36], Hoxd13[37], and in retinoic acid-treated animals[38, 39]. Several genes have been implicated in anorectal malformations in mutant mouse models, namely Sonic hedgehog (Shh) [40-43], Gli [44, 45] and ephrin-B2(Efnb2) [46]. An ephrin-B2 homozygous null mutation is embryonic lethal due to defects in angiogenesis, and no heterozygous phenotype was reported [47]; however, our group generated a partial loss of function mutation that interferes with ephrin signaling, ephrin-B2 $^{lacZ/+}$, which revealed hypospadias in heterozygous males at 40% penetrance [46]. The striking phenotype of male imperforate anus (see Fig. 6) and female persistent cloaca was seen in the homozygous ephrin-B2 $^{lacZ/lacZ}$ mice at 100% penetrance [46]. The observations of this mutant mouse phenotype and the fact that the human ephrin-B2 gene (EFNB2) is located at 13q33.3 has prompted us to investigate the role of EFNB2 in human hypospadias and in the 13q deletion syndrome.

In our study of eight genetically male probands with distal 13q deletion syndrome, five patients manifest urogenital/anorectal malformations and three do not. Our molecular mapping of their 13q deletions reveals that the minimal critical region for anorectal and urogenital anomalies is delimited by microsatellite markers D13S280-D13S285, approximately 9.5-11 Mb, when considering possible lack of penetrance in patient 3. This corresponds to 13q33.1-q34/tel, 3.5-5 Mb smaller than the critical region previously defined cytogenetically[2]. The critical region contains 20 annotated genes, including EFNB2. Based on our literature review, this gene appears to be the top candidate causal gene. Both urogenital and anorectal anomalies are seen with either maternal or paternal deletions, implying that imprinting does not play a role in these phenotypes. Given these urogenital and anorectal anomalies show incomplete penetrance in 13q-patients, a larger sample of male patients will need to be studied to further refine the critical region and confirm or refute the role of EFNB2 in the urogenital/anorectal phenotype of the 13q-deletion syndrome. The recent introduction of molecular karyotyping via microarray comparative genomic hybridization may further validate this work with much greater resolution.

Conclusions

The critical region of chromosome 13q mediating genitourinary/anorectal anomalies has been further refined to a an ~9.5 Mb interval containing 20 annotated genes. Haploinsufficiency of 13q shows incomplete penetrance for anorectal and genitourinary anomalies. Recruitment and mapping of additional 13q-cases will further narrow the critical region. Identification of the gene(s) mediating these syndromic genitourinary defects will likely further our knowledge of molecular mediators of non-syndromic hypospadias, penoscrotal transposition and anorectal malformations.

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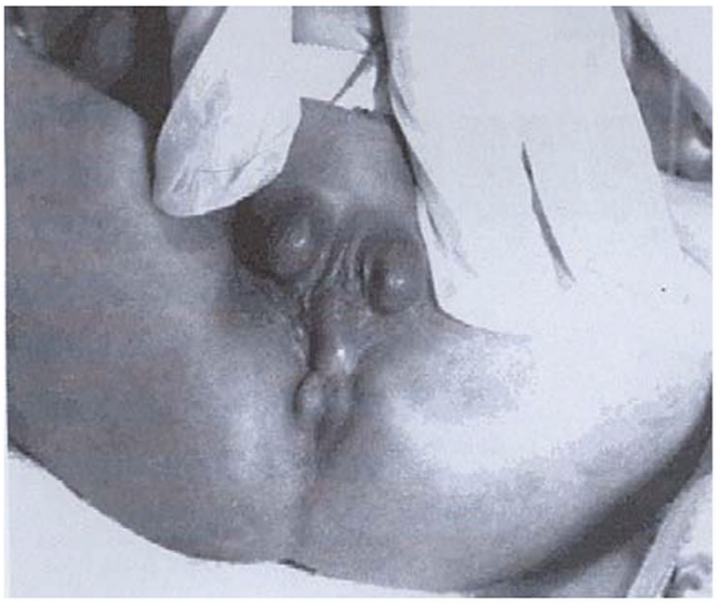

Figure 1.
The perineum of a 46, XY, del (13)(q31.1) male with complete penoscrotal transposition, perineal hypospadias and imperforate anus (Walsh et al., 2001[17]).

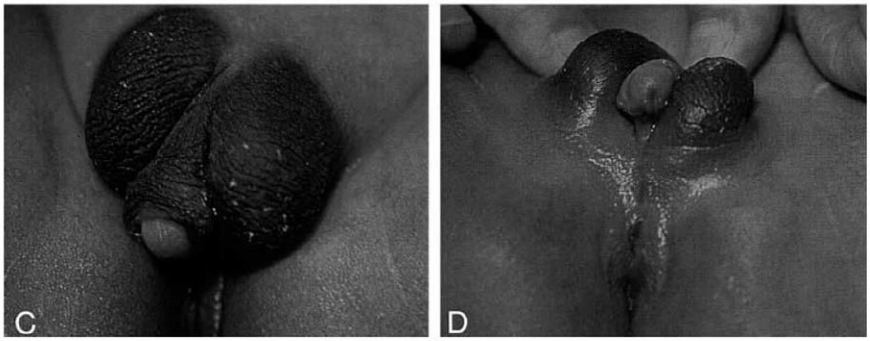

Figure 2.
The external genitalia and the perineum of a male with mosaicism of ring chromosome 13 and monosomy 13q del q33.2-qter (Kuhnle et al., 2000 [3]).

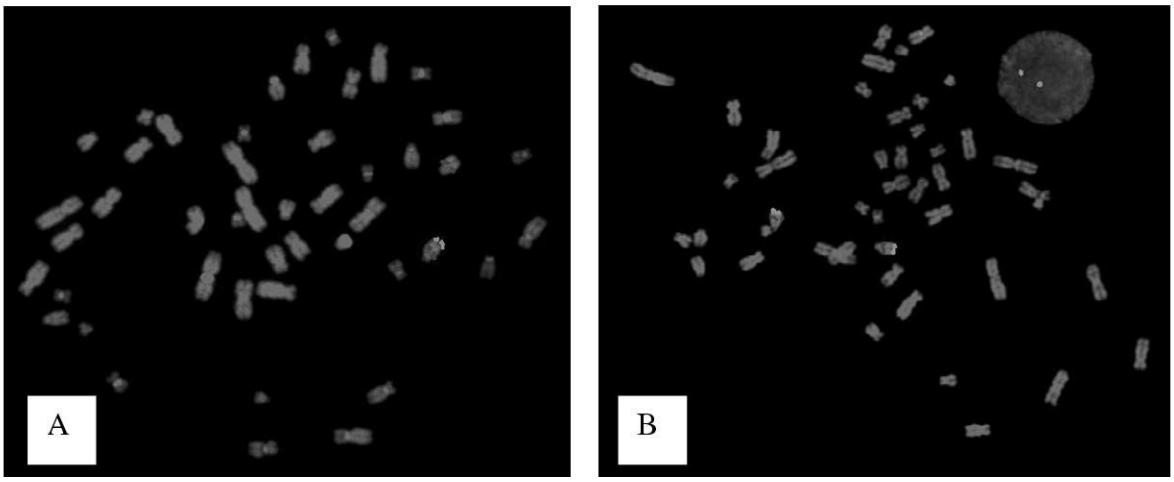

Figure 3.
FISH on lymphoblastoid metaphase preps. Green probe is BAC AL138689.21, containing the ephrin-B2 gene (EFNB2). Red probe is retinoblastoma gene (RB) control. (A) Proband #7-EFNB2 is deleted. (B) Proband #3-EFNB2 is not deleted.

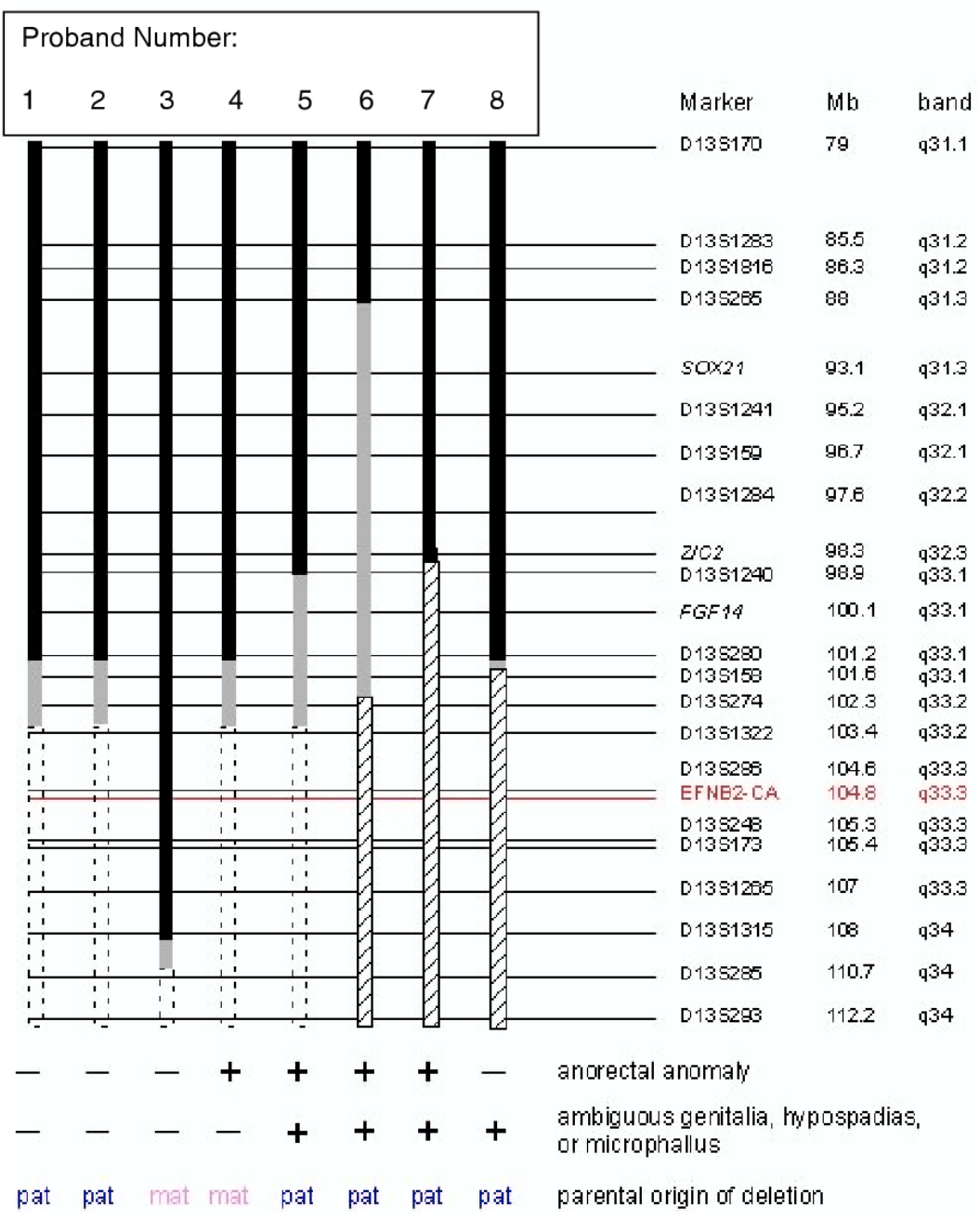

Figure 4.

Deletion mapping of 13q-patients. Solid black bars indicate 13q sequences that are not deleted; dashed open boxes denote deletions; gray bars indicate regions of uncertainty; hatched bars indicate deletions due to unbalanced translocations. Loci names and physical and cytogenetic map locations of microsatellite markers and selected FISH probes (italics) according to the UC Santa Cruz Genome Browser (genome.ucsc.edu, July 2003 assembly) are indicated. EFNB2-CA marker is indicated in red. Presence (+) or absence (-) of phenotypes and parental origin (pat = paternal; mat = maternal) of deletions are listed.

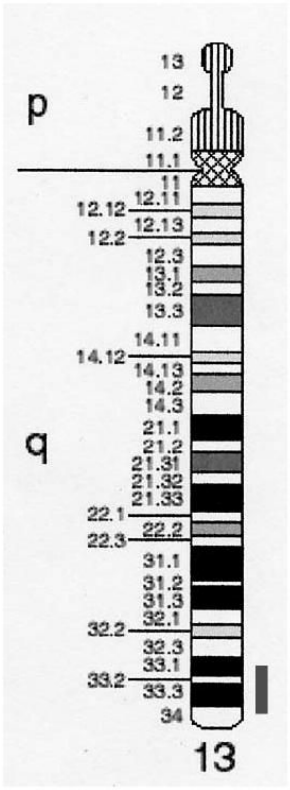

Figure 5.
Idiogram of chromosome 13. Red bar indicates critical region.

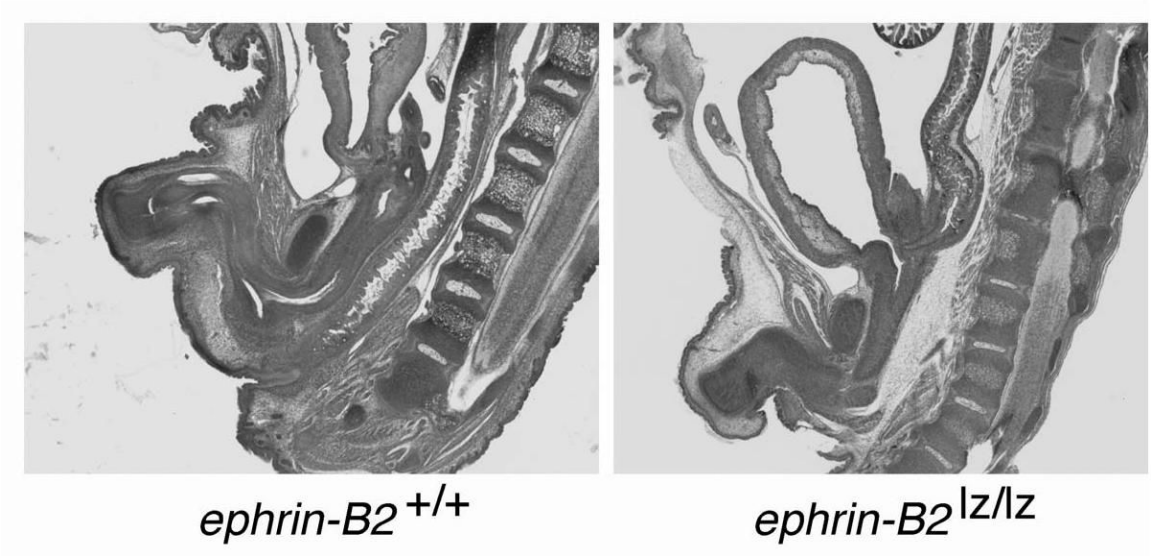

Figure 6.

Left panel reveals a sagittal H&E-stained section of an embryonic day 18 wild-type male mouse with normal genitourinary and anorectal development. Right panel contrasts the embryonic day 18 ephrin-B2 $^{lacZ/lacZ}$ male littermate with malformed urethra, high imperforate anus and abnormal colonic connection to the base of the bladder (rectovesical fistula). From Dravis et al. 2004 [46].

Table 1

Sex and racial distribution of the 13q-deletion study participants

ETHNICITY/GENDER	13q Deletion probands	13Q Family members	TOTAL
Caucasian (MALE)	3	5	8
(FEMALE)	1	8	9
African-American (MALE)			
(FEMALE)			
Hispanic (MALE)	5	4	9
(FEMALE)		7	7
Asian (MALE)			
(FEMALE)			
Other (MALE)			
(FEMALE)			
Unknown	1		1
TOTAL	10	24	34

Table 2

Phenotypic characterization of eight XY patients with known 13q32-34 deletions

Patient no.	Group	Sex	Karyotype	Phenotype
1	1	M	ring 13q	Bilateral VUR, dysplastic upper tracts, meningomyelocoele, microcephaly and developmental delay
2	1	M	46,XY del 13q33.2	No genitourinary anomalies, microcephaly
3	1	M	46,XY del 13q33.2	Right ureteral duplication, no genital or anal anomalies
4	2	M	46,XY del 13q33.2	Anorectal malformation
5	2	M	46,XY del13q33	Bilateral cleft lip and palate, anteriorly displaced anus, hypospadias
6	2	M	45,XY,-13,-22,+der(13:22)(q32.2;p11)	Perineal hypospadias, penoscrotal transposition, bifid scrotum, bilaterally descended testes, low anteriorly displaced imperforate anus
7	2	M	46,XY,-13,+der(13)t(6;13)(13pter>13q32::6q27>6qter)mat	Bifid scrotum, displaced anus, micropenis
8	3	Intersex, raised female	46,XY,der(13)t(6;13)(p25;q33)	Ambiguous genitalia (Male pseudohermaphrodite), no anal anomaly

Table 3

The 20 annotated genes in the interval D13S280-13qter, based on the UC Santa Cruz Genome Browser annotation (May 2004 Freeze)

Candidate gene name	Description	Comment
SLC10A2	Ileal sodium/bile acid cotransporter	Mutations cause 1° bile acid malabsorption
DAOA	D-amino acid oxidase activator	
EFNB2	pEhrin B2	Cell-cell signaling; neural and vascular development; hypospadias and anorectal malformation in mice[46]
FLJ10154	Hypothetical protein	Unknown function
LIG4	DNA ligase 4	Functions in immunoglobulin gene rearrangement
C13orf6	Novel protein	Esterase motif
TNFSF13B	TNF ligand superfamily	B cell cytokine
KIAA0865	Novel protein	Expressed in brain
IRS2	Insulin receptor substrate 2	Implicated in type II diabetes
COL4A1	alpha 1 type IV collagen preproprotein	Structural component of kidney glomerulus basement membrane
COL4A2	alpha 2 type IV collagen preproprotein	Structural component of kidney glomerulus basement membrane
RAB20	RAS oncogene family member	Plays a role in apical endocytosis/recycling
FLJ10769	Hypothetical protein	Unknown function
FLJ12118	Hypothetical protein	cysteinyl-tRNA synthase motif
ING1	Inhibitor of growth family member 1	Tumor suppressor; interacts with p53
LOC283487	Hypothetical protein	Unknown function
ANKRD10	Ankryn repeat domain 10	
ARHGEF7	Rho guanine nucleotide exchange factor 7 isoform	Induces cellular membrane ruffling
MGC35169	Hypothetical protein	Actin crosslinking domain
SOX1	SRY-box 1 gene	Transcription factor; mouse mutation causes epilepsy and abnormal brain development

Table 4

Common phenotypes observed in the human chromosome 13q-deletion syndrome

Phenotype	Frequency
Psychomotor retardation	94%
Hypertelorism, microcephaly	94%
Holoprosencephaly - ZIC2 gene at 13q32	
Agenesis of the corpus callosum	
Prominent nasofrontal bones	66%
Ear abnormalities	79%
Microphthalmia/coloboma of the iris	25%
Retinoblastoma - RB1 gene at 13q14.1-q14.2	18%
High arched or cleft palate	
Congenital heart disease (atrial septal defect, ventricular septal defect, tetralogy of Fallot, patent ductus arteriosus, aortic coarctation)	55%
Factor VII and X clotting deficiency	
Hypoplasia or aplasia of the thumbs/toes, syndactyly, brachydactyly	27%
Duodenal atresia, intestinal malrotation, Hirschsprung's disease	
Renal hypoplasia, hydronephrosis	
Biseptate or absent uterus, cloaca	
Imperforate or anteriorly displaced anus*	16%
Genital ambiguity*	
Hypospadias, penoscrotal transposition, bifid scrotum*	38%
Neural tube defects	

*Genitourinary/anorectal phenotypes. When known, causal genes, their chromosomal location, and the frequency of the anomaly are noted 48.48