Identification of small regions of overlap from copy number variable regions in patients with hypospadias

Carter H Scott
Ina E Amarillo

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs
Article

Identification of Small Regions of Overlap from Copy Number Variable Regions in Patients with Hypospadias

Carter H. Scott $^{1}$ and Ina E. Amarillo $^{2,*}$

$^{1}$ Washington University School of Medicine, St. Louis, MO 63110, USA; carter.scott@wustl.edu
$^{2}$ Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA
* Correspondence: ina.amarillo@wustl.edu

Abstract: Hypospadias is a common form of congenital atypical sex development that is often associated with other congenital comorbidities. Many genes have been associated with the condition, most commonly single sequence variations. Further investigations of recurrent and overlapping copy number variations (CNVs) have resulted in the identification of genes and chromosome regions associated with various conditions, including differences of sex development (DSD). In this retrospective study, we investigated the DECIPHER database, as well as an internal institutional database, to identify small recurrent CNVs among individuals with isolated and syndromic hypospadias. We further investigated these overlapping recurrent CNVs to identify 75 smallest regions of overlap (SROs) on 18 chromosomes. Some of the genes within these SROs may be considered potential candidate genes for the etiology of hypospadias and, occasionally, additional comorbid phenotypes. This study also investigates for the first time additional common phenotypes among individuals with hypospadias and overlapping CNVs. This study provides data that may aid genetic counseling and management of individuals with hypospadias, as well as improve understanding of its underlying genetic etiology and human genital development overall.

Keywords: hypospadias; DNA copy number variations; microarray analysis; small regions of overlap; CNV map; differences of sex development

1. Introduction

Hypospadias is a form of congenital atypical sex development that involves a urethral meatus located on the ventral aspect of the penis, scrotum, or perineum. It is very common, with an estimated incidence of one per 125 to one per 300 live male-assigned births, which has increased in frequency over the past few decades [1]. While most cases are distal to the neck of the glans, known as anterior hypospadias, the remaining cases have more proximal meatuses, known as middle (penile) or posterior (penoscrotal, scrotal, or perineal) hypospadias. Hypospadias is in contrast to epispadias, in which the urethral meatus is found on the dorsal penis.

Hypospadias can lead to both cosmetic and functional impairment, but only when severe. Infants with hypospadias, especially posterior cases, more frequently exhibit other congenital comorbidities compared to infants born without hypospadias [2]. In addition, people born with hypospadias are often found to have other urogenital abnormalities, especially cryptorchidism (undescended testis or testes) [2,3].

Despite decades of research, the etiology of most cases is unknown, likely a mix of environmental and hormonal factors with monogenic and/or multifactorial genetic features [2,4]. Specifically, it is likely due to a mix of hypoandrogenic states combined with pre-existing genetic susceptibility [5]. Environmental factors repeatedly associated with hypospadias include maternal hypertension and pre-eclampsia, placental insufficiency, maternal intrauterine exposure to diethylstilbestrol, being small for gestational age, and...
low birth weight [2,4]. About 7–25% of cases occur as part of familial clustering [6–8], and in pedigree and twin studies, heritability has been reported as anywhere from 35 to 77% [9], suggesting that genetic factors indeed play a strong role in causing the phenotype.

Over five decades of research have revealed many genes that are associated with hypospadias, most commonly involving sequence variations [2,10–18] (Table 1). Although several variants have been described as potentially causing hypospadias, researchers largely agree that very few cases of isolated hypospadias—especially milder ones—are caused by single sequence variations [2]. A limited but growing body of research suggests that epigenetic changes may contribute to differences of male sexual development, including hypospadias [19].

Table 1. Selected previously defined genes and regions in hypospadias.

| Genes   | Chromosome Locus (GRCh37/hg19) | Common Variation | SRO from Our Study (Gene) | Size (kb) | Reference |
|---------|-------------------------------|-----------------|---------------------------|----------|-----------|
| LHCGR   | 2p16.3                        | Sequence        | None                      | N/A/A    | Vezzoli et al. 2015 [9] |
| SRD5A2  | 2p23.1                        | Sequence        | None                      | N/A/A    | OMIM #607306 [10], HPA [11] |
| ZEB2    | 2q22.3                        | Sequence        | None                      | N/A/A    | van der Zanden et al. 2012 [2] |
| HOXA13  | 7p15.2                        | Sequence        | None                      | N/A/A    | OMIM #142959 [10] |
| SHH     | 7q36.3                        | Sequence        | SRO024                    | 473.21   | Carmichael et al. 2013 [12] |
| CYP17A1 | 10q24.32                      | Sequence        | None                      | N/A/A    | Singh et al. 2018 [13] |
| WT1     | 11p13                         | Sequence        | SRO036                    | 375.54   | van der Zanden et al. 2012 [2] |
| CYP11A1 | 15q24.1                       | Sequence        | None                      | N/A/A    | Lara-Velazquez et al. 2017 [14] |
| ADAT3   | 19p13.3                       | Sequence        | None                      | N/A/A    | Thomas et al. 2019 [15] |
| RBFOX2  | 22q12.3                       | Sequence        | SRO063                    | 83.35    | White et al. 2020 [16] |
| DGKK    | Xp11.22                       | Sequence        | None                      | N/A/A    | HPA [11] |
| MID1    | Xp22.2                        | Sequence        | None                      | N/A/A    | HPA [11] |
| AR      | Xq12                          | Sequence        | None                      | N/A/A    | OMIM #300633 [10] |
| MAML1   | Xq28                          | Sequence        | None                      | N/A/A    | OMIM #300120 [10]; HPA [11] |
| Regions |                               | CNV             | None                      | N/A/A    | OMIM #300856 [10] |

The advent of chromosomal microarray analysis (CMA) allows for the identification of copy number variations (CNVs) across the entire genome. Further investigations of recurrent and overlapping deleted and/or duplicated regions have resulted in the identification of genes and regions associated with various conditions, including differences of sex development (DSD) [20]. These documented CNVs are typically larger in size (≥1.5 Mb) [21]. In this retrospective study, we investigated the DECIPHER database (DatabasE of genomiC variation and Phenotype in Humans using Ensembl Resources) of deletions and duplications detected in patients with various clinical phenotypes [22], as well as an internal institutional database, to identify small recurrent CNVs among individuals with isolated or syndromic hypospadias. We further investigated these overlapping recurrent CNVs present in at least three patients to identify the smallest regions of overlap (SROs) between their CNVs, and the genes within these intervals, some of which can be considered potential candidate genes for the etiology of hypospadias. We then constructed a whole-genome CNV map from these SROs to illustrate the global genomic view of hypospadias. Furthermore, this study investigates for the first time additional common phenotypes among individuals with hypospadias and overlapping CNVs. This study expands genotype–phenotype correlation and provides data that may aid genetic counseling and management of individuals with hypospadias.
2. Results

The DECIPHER query returned 469 hypospadias cases, for which the number of reported copy number variants ranged from 0 to 7. About 76% (357/469) only had one reported variant, while the rest had at least two variants [22]. The majority were 46XY; however, we also found ten cases reported as 46XX, 22 reported as “other” or “unknown”, and one reported with sex chromosome aneuploidy [22].

Our CMA approach identified 75 small SROs (Figure 1) with a mean size of 136.04 kilobase pairs (kb) (range, 0.21–574.57 kb), and a mean of 8.9 individuals with overlapping CNVs (range, 3–28) (Figure 2; Table 2 and S1). Thirty-one of the 75 SROs (41%) included at least one CNV of a patient from our center. While most SROs included a mixture of deletions and duplications, 7 SROs exclusively included deletions, while 8 included only duplications. SROs were found across the genome, with the highest frequencies of SROs found on chromosomes 22 (17 SROs) and 1 (9 SROs); no SROs were identified on chromosomes 3, 14, 19, 20, 21, or the Y chromosome (Figure 3).

Of the loci given for the nine hypospadias phenotype entries in OMIM®, we found four SROs within the locus for susceptibility to X-linked hypospadias (HYSP4, Xp11.22) [11]. There was only one known gene within these SROs (HUWE1), which was identified as a candidate gene. We also identified SROs overlapping with the following genes with established associations with hypospadias: RBFOX2, SHH, and WT1.

Several studies have used other methods to identify CNVs potentially involved in hypospadias, although they typically use smaller sample sizes and do not discriminate based on CNV size. Such studies have associated duplications of 17q12 [23], as well as duplications of Ypter-Yq11.223 and loss of the remainder of chromosome Y (mosaic dicentric Y) [21], but neither overlapped with our SROs. Similarly, we did not find any SROs overlapping potential hypospadias susceptibility loci identified on chromosomes 2, 7, 9, and 10 in two studies using familial linkage analysis [24,25]. However, our findings support one study which described clinically significant CNVs on chromosomes 2, 5, 12, 16, and X [26]: we identified 3 SROs within their CNV at 16p11.

Figure 1. Distribution of SRO size.
Figure 2. SRO Distribution across the Genome. Chromosomal locations for each SRO were used to construct a BED (Browser Extensible Data) file which was imported into Chromosome Analysis Suite (ChAS; Affymetrix, Inc./Thermo Fisher Scientific, Santa Clara, CA, USA) as a track. Green stars indicate SROs or clusters of SROs composed strictly of duplications, red stars indicate deletions, and blue stars indicate SROs that featured both types of CNVs.

Figure 3. SRO frequency across chromosomes.
Table 2. Candidate genes in hypospadias.

| SRO from Our Study | Chromosome Locus (GRCh37/hg19) | CNV Type | Size (kb) | Genes | Most Common Comorbid Phenotype ** |
|--------------------|--------------------------------|----------|-----------|-------|----------------------------------|
| SRO001             | 1p36.33                        | Del/Dup  | 68.05     | ATAD3A, ATAD3B, ATAD3C, ANKR35, GNRHR2, ITGA10, LIX1L, PEX11B, RBM8A * |                          |
| SRO002             | 1q21.1                         | Del/Dup  | 81.26     | ANKR35, NUDT17, PIAS3 * |                          |
| SRO003             | 1q21.1                         | Del/Dup  | 29.44     |                      |                          |
| SRO004             | 1q21.1                         | Del/Dup  | 103.47    | POLR3C, RNF115, BCL9, CHD1L, LINC00624, OR13Z1P, OR13Z2P, OR13Z3P |                          |
| SRO005             | 1q21.1-21.2                    | Del/Dup  | 341.53    |                      |                          |
| SRO006             | 1q31.1                         | Del      | 251.69    | LINC01036          | Ventricular septal defect |
| SRO007             | 1q44                           | Del/Dup  | 8.65      | AKT3 *             |                          |
| SRO008             | 1q44                           | Del/Dup  | 15.32     | SMYD3              |                          |
| SRO009             | 1q44                           | Del/Dup  | 178.41    |                     |                          |
| SRO010             | 2q37.3                         | Del/Dup  | 3.55      | MTERFD2, SNED1 *   | Hypotonia               |
| SRO011             | 4q35.2                         | Del/Dup  | 481.63    | ADAM20F3, ZFP42    | Feeding difficulties in infancy |
| SRO012             | 4q35.2                         | Del/Dup  | 9.73      |                     | Feeding difficulties in infancy |
| SRO013             | 4q35.2                         | Del/Dup  | 94.01     | RNU6-173P, TRIM2L  |                          |
| SRO014             | 4q35.2                         | Del/Dup  | 3.61      |                     |                          |
| SRO015             | 4q35.2                         | Del/Dup  | 38.47     |                     |                          |
| SRO016             | 4p16.1                         | Del/Dup  | 148.71    | MIR4798            |                          |
| SRO017             | 4p16.1                         | Del/Dup  | 68.85     | SORCS2             | Hypotelorism            |
| SRO018             | 5q35.3                         | Dup      | 237.41    |                     |                          |
| SRO019             | 6q27                           | Del/Dup  | 3.77      | C6orf23 (lncRNA)   | Hypoplastic nail        |
| SRO020             | 6q27                           | Del/Dup  | 235.235   | FAM120B, PDCD2, PSMB1, TBP * |                          |
| SRO021             | 7q22.1                         | Del/Dup  | 133.74    | CUX1 *             |                          |
| SRO022             | 7q36.2                         | Del/Dup  | 12.67     |                     |                          |
| SRO023             | 7q36.2                         | Del/Dup  | 22.22     | DPP6               | Congenital heart defect |
| SRO024             | 7q36.3                         | Del/Dup  | 473.21    | SHH                |                          |
| SRO025             | 7p15.2                         | Del      | 112.98    | SNX10              |                          |
| SRO026             | 8p22                           | Del/Dup  | 88.73     | MSR1               |                          |
| SRO027             | 9q34.3                         | Del      | 204.21    | NACC2, C9orf69, LHX3, QSOX2 |                  |
| SRO028             | 9p22.2-22.2                    | Del      | 337.25    | ADAMTS1L 1 *, MIR3152, RN7SKP258 |                  |
| SRO029             | 9p24.1                         | Del      | 159.76    | ERMP1, KIAA1432   | Cryptorchidism           |
| SRO030             | 9p24.1                         | Del/Dup  | 333.12    | PTPRD *            | Anteverted narese        |
| SRO031             | 10p14                          | Del/Dup  | 155.6     | LINC00707          |                          |
| SRO032             | 11q23.3                        | Del/Dup  | 54.78     | GRIK4              | Anteverted narese        |
| SRO033             | 11q25                          | Del/Dup  | 378.38    | B3GAT1, GLB1L2    |                          |
| SRO034             | 11q25                          | Del/Dup  | 186.74    |                     | Abnormality of the pinna |
| SRO035             | 11p14.1                        | Del      | 4.62      | BDNF *             |                          |
| SRO036             | 11p13                          | Del/Dup  | 375.54    | CCDC73, EIF3M *, HNRNPA3P9, WT1, WT1-AS |                  |
| SRO037             | 11p13                          | Del/Dup  | 112.5     | EHF *              | Short stature           |
| SRO from Our Study | Chromosome Locus (GRCh37/hg19) | CNV Type | Size (kb) | Genes | Most Common Comorbid Phenotype ** |
|-------------------|--------------------------------|----------|-----------|-------|----------------------------------|
| SRO038            | 11p15.5                        | Del/Dup  | 323.55    | ATHL1, B4GALNT4, BETH1, CICP23, IFITM1, IFITM2, IFITM3, IFITM5, LINCO1001, NLRP6, ODF3, OR4F2P, PSMD13 *, RIC8A, RNU6-447P, SGCBI1C1, SIRT3, DDX51, EP400 *, EP300NL, FBRSL1, GALNT9, MUC8, NOC4L, SNORA49 | Long philtrum |
| SRO039            | 12q24.33                       | Del/Dup  | 574.57    | DDX51, EP400 *, EP400NL, FBRSL1, GALNT9, MUC8, NOC4L, SNORA49 | Micrognathia |
| SRO040            | 13q34                          | Del      | 17.78     | CYTP1 *, ELMO2P1, GOLGA81, NIPA1, NIPA2 *, TUBGCP5, WHAMMP3, ABC11, ABC26, ATP2A1, ATXN4L, CD19, LAT, MIR4517, MIR4721, NFATC2IP, RABEP2, SH2B1 *, SPNS1, TUFM, SPN, ALDOA, ASPHD1, C16orf54, C16orf92, CDIPT *, CDIPT-AS1, DOC2A, FAM57B, GDDP3, HIRIP3, INO80E, KCTD13, KIF22, MAPK3 *, MAZ *, MVP, PAG1, PPP4C, PRRT2, QPRT, RN7SKP127, SEZ6L2, SPN, TAOK2 *, TBX6, TMEM219, YPEL3, ZG16 | Micropenis |
| SRO041            | 14q11.2                        | Del/Dup  | 490.07    | DDX51, EP400 *, EP400NL, FBRSL1, GALNT9, MUC8, NOC4L, SNORA49 | Micrognathia |
| SRO042            | 16p13.11                       | Dup      | 38.27     | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1 | Micropenis |
| SRO043            | 16p11.2                        | Dup      | 214.84    | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1, GRIN2A * | Short neck |
| SRO044            | 16p11.2                        | Del/Dup  | 21.6      | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1, GRIN2A * | Low-set ears |
| SRO045            | 16p11.2                        | Del/Dup  | 516.64    | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1, GRIN2A * | Low-set ears |
| SRO046            | 16q13.3                        | Del/Dup  | 121.78    | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1 | Micrognathia |
| SRO047            | 16q24.2                        | Del      | 99.1      | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1, GRIN2A * | Micrognathia |
| SRO048            | 16p13.2                        | Del      | 57.33     | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1, GRIN2A * | Micrognathia |
| SRO049            | 17p13.3                        | Del/Dup  | 251.23    | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1, GRIN2A * | Micrognathia |
| SRO050            | 17q23.2                        | Del/Dup  | 314.38    | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1, GRIN2A * | Micrognathia |
| SRO051            | 17p13.1                        | Del/Dup  | 4.52      | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1, GRIN2A * | Micrognathia |
| SRO052            | 18p11.31                       | Del/Dup  | 429.94    | RBFOX1 *, RNU6-457P, ZC3H18 *, ZFPM1, GRIN2A * | Micrognathia |
| SRO053            | 22q11.1                        | Del/Dup  | 2.19      | CLTCL1, KRT18P62, P4KA | Microcephaly |
| SRO054            | 22q11.21                       | Del/Dup  | 77.94     | CLTCL1, KRT18P62, P4KA | Preauricular skin tag |
| SRO055            | 22q11.21                       | Del/Dup  | 8.18      | CLTCL1, KRT18P62, P4KA | Hearing impairment |
| SRO056            | 22q11.21                       | Del/Dup  | 29.74     | CLTCL1, KRT18P62, P4KA | Cleft palate |
| SRO057            | 22q11.21                       | Del/Dup  | 13.9      | CRKL *, RN7SL389P, PPIL2, YPEL1 | Hearing impairment |
| SRO058            | 22q11.22                       | Del/Dup  | 34.4      | CRKL *, RN7SL389P, PPIL2, YPEL1 | Cleft palate |
| SRO059            | 22q11.22                       | Del/Dup  | 85.87     | CRKL *, RN7SL389P, PPIL2, YPEL1 | Cleft palate |
| SRO060            | 22q11.22                       | Del/Dup  | 28.54     | CRKL *, RN7SL389P, PPIL2, YPEL1 | Cleft palate |
| SRO061            | 22q11.22                       | Del/Dup  | 9.29      | CRKL *, RN7SL389P, PPIL2, YPEL1 | Cleft palate |
Table 2. Cont.

| SRO from Our Study | Chromosome Locus (GRCh37/hg19) | CNV Type | Size (kb) | Genes | Most Common Comorbid Phenotype ** |
|--------------------|-------------------------------|----------|-----------|-------|----------------------------------|
| SRO062             | 22q11.23                      | Del/Dup  | 1.74      | BCR * | Small for gestational age         |
|                    |                               |          |           |       |                                  |
| SRO063             | 22q12.3                       | Del/Dup  | 83.35     | RBFOX2|                                  |
|                    |                               |          |           |       | C22orf46, CCDC134,               |
|                    |                               |          |           |       | CENPM, CYP2D6, CYP2D7P,          |
|                    |                               |          |           |       | FAM109B, HMGN2P10,               |
|                    |                               |          |           |       | LINC00634, MEI1,                 |
|                    |                               |          |           |       | MIR33A, MIR3781, NAGA,           |
|                    |                               |          |           |       | NDUFA6, NDUFA6-AS1,              |
|                    |                               |          |           |       | NHP2L1, OLA1P1,                  |
|                    |                               |          |           |       | RNU6-476P, RNU6ATAC22P,          |
|                    |                               |          |           |       | SEPT3, SHISA8, SLC25A5P1,        |
|                    |                               |          |           |       | SMRT1, SREBF2 *, TCF20 *, TNFRSF13C, WBP2NL, RN7SKP80, RNU6-513P, RRP7A, RRP7B, SERHL, SERHL2 |
| SRO064             | 22q13.2                       | Dup      | 502.25    |       |                                  |
|                    |                               |          |           |       |                                  |
| SRO065             | 22q13.2                       | Dup      | 69.15     | MAPK11, PLXNB2 * | Small for gestational age |
|                    |                               |          |           |       |                                  |
| SRO066             | 22q13.33                      | Del/Dup  | 8.67      | NCAH2 |                                  |
|                    |                               |          |           |       |                                  |
| SRO067             | 22q13.33                      | Del/Dup  | 3.06      |       |                                  |
|                    |                               |          |           |       |                                  |
| SRO068             | 22q13.33                      | Del/Dup  | 2.01      |       |                                  |
|                    |                               |          |           |       |                                  |
| SRO069             | 22q13.33                      | Del/Dup  | 8.77      | SHANK3 * |                                  |
|                    |                               |          |           |       |                                  |
| SRO070             | Xp11.22                       | Del/Dup  | 2.7       |       |                                  |
|                    |                               |          |           |       |                                  |
| SRO071             | Xp11.22                       | Dup      | 0.21      |       |                                  |
|                    |                               |          |           |       |                                  |
| SRO072             | Xp11.22                       | Del/Dup  | 7.86      |       |                                  |
|                    |                               |          |           |       |                                  |
| SRO073             | Xp11.22                       | Dup      | 1.58      | HUWE1 * |                                  |
|                    |                               |          |           |       |                                  |
| SRO074             | Xp22.31                       | Del/Dup  | 150.48    | MIR651 |                                  |
|                    |                               |          |           |       |                                  |
| SRO075             | Xp22.31                       | Del/Dup  | 16.6      |       |                                  |

* Candidate gene. ** This column includes the most common comorbid phenotype other than neurodevelopmental abnormality. For a complete list of commonly comorbid phenotypes, please see Supplementary Table S4.

2.1. Candidate Genes

These SROs involved a total of 242 genes; 20 genes had an HI score ≤10%, and 36 had a pLI score ≥0.9 [22]. A total of 43 genes met either of the criteria. Of these 43 genes, 40 (93%) had never been identified as being associated with hypospadias and were thus identified as candidate genes (Table 2). DECIPHER does not provide triplosensitivity data, but we expanded our analysis to include this data. Of the candidate genes, they either had no evidence or record of triplosensitivity. Although the Xp11.22 region (SRO070-073) is documented to be triplosensitive, the candidate gene HUWE1 within SRO073 is not [27]. Of the 40 candidate genes, 30 (75%) had low tissue specificity; the only gene with specificity for male tissues was EHF, which is tissue enhanced in ductus deferens, seminal vesicle, and salivary gland [28]. Thirteen of the 40 candidates (33%) had a “high” protein expression score in at least one of the male tissues for which protein expression scores are available (epididymis, prostate, seminal vesicle, testis) [28] (Table S2).

Through literature review, we identified 80 genes previously published in association with hypospadias as either being causative, a risk factor, or associated with isolated hypospadias in screening studies, as well as genes proposed as candidates in gene expression studies or given their role in genital and gonadal development (Table S3). The majority of these are sequence variations. Additionally, more than 600 genes were listed in OMIM® as having at least one patient with hypospadias as a reported phenotype [29].

We also identified 14 recurrent copy number regions with sizes ranging from 0.21 kb to 186.74 kb. These regions are gene deserts, or regions with no protein-coding genes.
2.2. Common Comorbidities

Of the 469 DECIPHER cases with hypospadias, only two had no documented comorbidities (“isolated hypospadias”), and both only had one reported CNV. Of note, one of these CNVs is the basis for SRO041, which is 30.67 kb narrower than the CNV; this SRO contained 2 candidate genes, CYFIP1 and NIPA2. The remaining 467 DECIPHER cases had anywhere from 2 to several dozen comorbid phenotypes affecting nearly all organ systems [22]. The most commonly reported comorbidity present in at least three cases with CNVs overlapping an SRO was neurodevelopmental abnormality, including intellectual disability and neurodevelopmental delays such as global developmental delay or delayed speech and language development (found in 24 SROs). Other abnormalities of the genitourinary system were found, including micropenis (3 SROs), cryptorchidism (1), and hydronephrosis (1). Comorbidities found in multiple (2 to 4) SROs include short stature, small size for gestational age, inguinal hernia, feeding difficulties in infancy, microcephaly, hypertelorism, low-set ears, hearing impairment, abnormality of the pinna, anteverted nares, micrognathia, and cleft palate. For other comorbidities and their proportions, please reference Table S4.

3. Discussion

Human genital development is complex, controlled by an intricate network of factors regulating endocrine function, organ development, and sex determination and differentiation. The fragility of this network is demonstrated by the high incidence of DSD worldwide, estimated at 1:4500 to 1:5000 live births [30]. Patients with isolated hypospadias, even when severe, are typically not referred for genetic testing. When initial hormonal analyses are unremarkable, the anomaly is often termed isolated anatomical atypia [31,32]. Therefore, the causes underlying most cases have not been found. Indeed, there were only two patients with isolated hypospadias in the entire DECIPHER database. In this study, chromosome microarray-based technology (CMA) identified many copy number variations recurrently found in individuals with hypospadias, and sometimes with other phenotypes as well. Although some genes have been implicated, the genomic etiology of hypospadias remains poorly understood. Many genomic variants of individuals with hypospadias are exceedingly rare, making genotype-phenotype correlations uncertain and thus clinical interpretation challenging. While a small number of CNVs have been identified in individuals with hypospadias, they have either focused on large (>1.5 Mb) CNVs or studied few (<30) participants [21,26]. In fact, few CMA studies have investigated small (<1 Mb) CNVs in any human genetic disorder [33]. Thus, the contribution of CNVs, especially those with few genes, to the etiology of hypospadias remains largely unexplored.

The use of large, multi-institutional databases allows for the identification of individuals that share both phenotypic features and gene variants, which can improve certainty in gene pathogenicity and may allow for clarification of the role of novel genes in development, physiology, and disease. The largest of such databases to date is DECIPHER, which compiles genotype and phenotype information of more than 35,000 individuals from over 250 centers around the world [22].

Use of these large-scale databases can allow for the identification of small CNVs recurrent among database participants with certain phenotypes. Historically, CNVs reported in association with syndromes have been large, containing many genes. However, the advent of publicly available, high-resolution CNV maps (with CNVs as small as 50 bp) now allows for comparison of overlapping CNVs of various sizes to identify SROs, or the smallest common CNV associated with a specific phenotype [34,35]. More recently, SROs as small as 5.2 kb have been identified upstream of SOX9 in patients with DSD [20]. These data, then, provide an opportunity to construct maps of these SROs across the genome. Rather than the large, nonspecific CNVs previously reported with various genetic syndromes, these small CNVs sometimes contain one or few genes, intragenic regions, and regulatory regions. Importantly, these maps may assist with shrinking the genomic “gap” in understanding human genetic disorders, especially phenotypes with complex etiologies such as hypospadias.
Our findings illuminate for the first time many potential candidate genes not previously thought to play a role in hypospadias etiology, as well as many chromosomal structural imbalances that may serve as risk factors for this and other phenotypes. Additionally, these results demonstrate the powerful potential of chromosome microarray-based analysis in the discovery of genetic factors contributing to diseases with complex etiologies.

3.1. Hypospadias and Sex Chromosomes

It might be assumed that all cases of hypospadias are exhibited by Y chromosome-bearing patients (e.g., XY, XXY). However, DECIPHER had several cases reported as 46XX and even more reported as “other” or “unknown.” Given that hypospadias may occur in the spectrum of DSD, it is imperative that sex chromosomes are reported and analyzed when performing research on this and other conditions related to DSD [36]. Since the major classification of DSD is based on sex chromosomes, it may be helpful to include the reason why sex chromosomes are listed as “other” or “unknown.”

The Y chromosome has long been speculated to be associated with hypospadias; some of the earliest research on the genetics underlying the condition described several cases with a variety of translocations and deletions on this chromosome [37]. However, the lack of SROs found on the Y chromosome support more recent research that has found no CNVs on this chromosome, including SRY, in patients with isolated hypospadias [38,39]. The lack of SROs on this chromosome and chromosome 2 are also in agreement with a paper that found no recurrent and overlapping CNVs in patients with DSD on either of these chromosomes [33].

3.2. SRO Relationships to Previously Described Regions and Genes

Our study found very few SROs that overlapped with previously described regions or genes. This is in agreement with the fact that existing studies have largely associated cases of hypospadias with sequence variations, rather than CNVs [2] (Table S3). However, this may change given the recent increase in CMA utilization, use of gene-targeted CMA, increased data sharing through DECIPHER, or increased genetic evaluation of patients with hypospadias.

3.3. Candidate Genes

One study used DECIPHER to identify recurrent CNVs in patients with isolated (“non-syndromic”) congenital genitourinary anomalies [40]. That study identified RBFOX2 as a candidate gene and used mouse models to explore its role in upper and lower genitourinary tract development. Our study used a similar approach to identify SROs; however, it explored the whole genome and utilized the CNVs of patients with both syndromic and non-syndromic hypospadias. Through these methods, we identified many regions and genes that have never been reported as being associated or potentially associated with hypospadias.

Certain candidates have high tissue specificity, both within and outside of the male urogenital system [12] (Table S2). Some SROs containing these candidates had common comorbidities related to the extra-urogenital tissues in which they are enhanced. For example, DLAGAPI (SRO052) and GRIN2A (SRO048) are tissue enriched in the brain, and the majority of cases within these SROs had neurodevelopmental abnormalities. Additionally, PTPRD is tissue enhanced in the brain, and 7 of 11 cases in the SRO (SRO030) were also reported to have intellectual disability, and 4 cases had microcephaly.

Many candidate genes with tissue specificity did not have recurrent phenotypes in this study population, such as RBM8A (SRO002), which is tissue enriched in blood. However, more in-depth investigation of these tissues in individuals with relevant CNVs may identify common comorbidities. Additionally, investigation of cases with hypospadias and variants in genes enriched in multiple tissues (e.g., ADAMTS11, BDNF) may potentially lead to the identification of new syndromes, or the expansion of phenotypes for existing syndromes.

Of the 40 candidate genes, it would seem prudent to most strongly consider those candidate genes with high specificity and expression in “male tissues” (e.g., EHF, EP400, RBM8A, SNED1, YWHAE, ZC3H18). However, that should not discount those genes
expressed at low levels in male tissues, which may demonstrate important temporal expression. Such candidate genes for which the SRO is composed mostly of duplications (e.g., DBN1) may suggest that overexpression disrupts typical urogenital development, and those with mostly deletions (e.g., ADAMTSL1, BDNF, COL4A1) may suggest haploinsufficiency.

For candidate genes, further investigation of tissues in which they are highly expressed—and potentially associated phenotypes—may allow for the identification of common comorbidities (or, potentially, novel syndromes) associated with certain CNVs. For example, DLGAP1 and GRIN2A are enriched in brain tissue, and 12 of the 13 cases with relevant CNVs feature neurodevelopmental abnormalities. Although cases with CNVs overlapping PTPRD (enhanced in parathyroid tissue) were not explicitly reported to have parathyroid-related abnormalities, further investigation may uncover relevant comorbidities. The same could be said for cases with CNVs affecting RBM8A (enriched in blood) and hematologic disorders. Additionally, data regarding co-expression, physical interactions, predicted co-expression, genetic interactions, co-localization, and shared protein domains may provide important clues for further functional study [41].

3.4. Syndromes

SROs were found within loci known to be relevant to syndromes that have previously been associated with hypospadias. Identification of SROs within these loci—and especially candidate genes—may provide insight into the specific genetic etiology of hypospadias for that syndrome. For example, Hunter–McAlpine Syndrome is associated with hypospadias, as well as other phenotypes such as intellectual disability, microcephaly, and short stature. The syndrome is known to be caused by a partial duplication of 5q35-qter [42], although the specific region of this duplication underlying hypospadias has never been proposed. SRO018 is within this locus and is composed of only cases with duplications; this SRO contains candidate genes DBN1 and NSD1, which may contribute to hypospadias etiology in these cases. SROs with candidate genes were found in the established risk loci for other syndromes known to be associated with hypospadias, such as Silver–Russell Syndrome (PSMD13); 13q Deletion Syndrome (COL4A1); and 22q Deletion/Duplication Syndromes, which includes DiGeorge Syndrome (CRKL).

In addition, several SROs were located within known susceptibility regions for genetic syndromes for which hypospadias is not a commonly associated phenotype. These findings suggest that hypospadias may be investigated further as a potential syndromic feature, and that individuals with relevant CNVs and hypospadias may need to be evaluated further for comorbidities associated with these syndromes.

For example, SRO041 overlaps with the newly established Burnside–Butler Syndrome, which is associated with various developmental and psychiatric disorders [43]. Notably, three of four cases included in this SRO have delayed speech and language development. This SRO contains two candidate genes, CYFIP1 and NIPA2, both of which are highly expressed in ductus deferens, testis, epididymis, seminal vesicles, and prostate [28]. Both genes have been implicated in Burnside–Butler Syndrome, although hypospadias has not yet been identified as a comorbidity. Additional examples of such syndromes and SROs can be found in Table S5.

3.5. Common Comorbidities

For almost 50 years, hypospadias has been associated with an increased risk of other congenital comorbidities, especially cryptorchidism [37]. One study suggests that as high as 29.4% of cases have additional comorbidities—often urogenital, but also extra-urogenital [3]. Genitourinary comorbidities were indeed common in our population, found as a recurrent phenotype in 5 SROs, although other common comorbidities affected a variety of organ systems. Another study found that individuals with hypospadias are more likely to be diagnosed with intellectual disability, autism spectrum disorder, and other behavioral or emotional disorders [44]. Although they did not provide a conclusive explanation for these findings, the increased risk persisted after adjusting for known genetic syndromes, suggesting
a complex model of heritability that may be confounded by environmental, psychosocial, and endocrine factors. However, this potentially suggests that there may be unknown genetic syndromes involving both hypospadias and neurodevelopmental abnormalities. Our study found similar results, as 24 of the 75 SROs (32%) had neurodevelopmental abnormality as a phenotype present in at least three cases contributing to the SRO.

One paper found that 28% of the patients they studied—those with both hypospadias and neurodevelopmental delay—also had cardiovascular abnormalities [45]. They identified various syndromes associated with similar constellations of comorbidities, citing several pathogenically implicated genes as modulators of transcription and epigenetic regulation that may serve related functions in development. Indeed, our methods identified three SROs featuring neurodevelopmental delay and cardiovascular abnormalities as shared comorbidities. SRO008 contains SMYD3, a transcriptional regulator, and SRO030 contains candidate gene PTPRD, which plays an important role in memory, learning, and synaptic plasticity [29]. SRO023 contains DPP6, which is associated with hereditary ventricular fibrillation, as well as autosomal dominant (AD) microcephaly and neurodevelopmental delay which was replicated on knockdown of DPP6 in mice [29]. A CNV analysis of patients with AD microcephaly identified two small de novo deletions in the gene [46]; which is supported by SRO023, which is composed of 13 deletions and only 3 duplications. Such findings suggest a potential regulatory role for DPP6 in cardiac, neurological, and genital development.

3.6. Recommendations

Our findings provide further support for genomic influences in the etiology of hypospadias. Hypospadias is thought to arise during external genitalia development, between gestational weeks 8–12, due to incomplete fusion of the labioscrotal folds [38]. The roles of these genes in the development of hypospadias will be elucidated by studying spatial and temporal expression during this development of the urethra and nearby structures. These findings, in concert with existing genetic and environmental studies, the DECIPHER database, and animal models [47], will facilitate the functional studies needed to understand the roles of these genes in the genetic etiology of hypospadias, as well as in the broader context of urogenital development.

In addition, these findings call for more frequent genomic analysis in patients found to have hypospadias, which may further elucidate candidate genes for this condition as well as novel genes involved in human genital development. Ideally, this analysis should include both sequencing and genome-wide CNV analyses. Additionally, the specific category of hypospadias (e.g., coronal, penoscrotal) should be reported in public databases to improve genotype-phenotype correlation. Although increased hypospadias severity has been found to be associated with increased likelihood of identifying likely pathogenic variants, patients with even mild hypospadias can benefit from genetic testing, which may provide early diagnoses, reveal undiagnosed syndromes, or identify candidate genes or variants [48]. Indeed, these results echo recommendations from panels of international experts for the appropriate diagnostic approach to DSD. These recommendations highlight the importance of increased genetic testing and results sharing in centralized databases such as DECIPHER to promote functional studies that allow for the identification of novel implicated genes and variants [49].

As our understanding of comorbidities associated with different structural abnormalities unfolds, CMA may allow for earlier screening and detection. For example, it has been suggested that WT1 testing is not indicated in isolated hypospadias without cryptorchidism, but there have been several reports of patients with such phenotype who later develop Wilms tumors [31]. Recent research suggests that although genetic evaluation of patients with proximal (severe) hypospadias is not the typical standard of care, when evaluated, many are found to harbor clinically relevant genetic variations, supporting increased genetic evaluation of these individuals [26,32]. In addition, as the understanding of the genetic etiology of this phenotype improves, genetic counseling for families may better
inform estimates of recurrence risk for subsequent pregnancies. Improved understanding of comorbidities may also allow for the provision of anticipatory guidance to parents, and thus earlier detection and treatment.

4. Materials and Methods

4.1. CNV Data Sources

DECIPHER was queried to identify all cases with “hypospadias” as a listed phenotype [22], which included those with the HPO term HP:0000047 “hypospadias” as well as HPO terms further defining hypospadias as penile, penoscrotal, glandular, perineal, mid-shaft, scrotal, or coronal. All cases were included, regardless of karyotype. CMA data were also obtained from 32 patients with hypospadias referred to our center since 2008. CMA data for patients from our center were acquired following the methods previously described in “Integrated Small Copy Number Variations and Epigenome Maps of Disorders of Sex Development” [33]. CMA data from these 32 cases had not yet been submitted to DECIPHER.

To construct the whole-genome CNV map described in this study, we used the karyotype map function of DECIPHER to identify chromosome regions with three or more recurrent and overlapping deletions or duplications. From such regions, the smallest region of overlap (SRO) is defined (Figures S1 and S2). We then integrated the CNV data obtained from our center. Only SROs less than 1 Mb in size were included in this analysis.

Other genomic information collected for each SRO includes size, base-pair coordinates, chromosome locus, and gene content. We analyzed all genes within the SROs using DECIPHER’s predictive tools such as haploinsufficiency index (HI) (scores of ≤10% indicate a high likelihood of exhibiting haploinsufficiency) [50] and pLI (score of ≥0.9 indicates very low tolerance to loss of function mutations) [28]. In accordance with prior studies using these cutoff values [28,50], genes that met either of these criteria and had never been previously described in association with hypospadias were deemed candidate genes. In addition to genotype information, the spectrum of phenotypes in patients with CNVs included in the SRO was also analyzed. These SROs were cross-referenced with loci listed for the nine hypospadias phenotype entries in OMIM® as well as previously described CNVs associated with hypospadias [11].

4.2. Limitations

Our analysis of potential common phenotypes was limited by how cases were entered in DECIPHER and our genetic database, including which phenotypes were reported. For example, depending on when participants’ phenotypes were last updated, or their age of death, age-related phenotypes (e.g., malignancy, short stature, intellectual disability, behavioral/emotional disorders) may not have yet manifested. In addition, distal hypospadias may not even be clinically recognized, much less reported in DECIPHER, so there is likely under-reporting of the phenotype in this database.

When using public databases, different array technologies used by reporting institutions may lead to differences in the reported size of CNVs that are actually identical [51]. For that reason, the coordinates of our SROs should not be considered absolute or definitive loci.

Although HPA lists tissue specificity and expression levels in several “male tissues,” they do not list values for tissues specifically associated with urethral or penile development; therefore, these values should only be regarded as an estimate.

SROs that contain no genes may hold important regulatory regions. We identified three such SROs within what OMIM® describes as a susceptibility locus for X-linked hypospadias [11], which were all located less than 3.5 Mb downstream of DGKK, the gene most commonly associated with hypospadias [2,52,53]. These regions may contain key regulatory elements for DGKK not previously understood in relation to hypospadias pathogenesis, such as those recently found for SOX9 while using a similar method to identify SROs [54]. However, analyzing these regions is beyond the scope of this paper and should be considered an area for future study.
Finally, many candidate genes have been proposed to be associated with this phenotype, but such studies often report small numbers of cases and controls. Often, they are not able to be replicated in future studies, although this could be due to differences in study populations. Additionally, this study does not address the non-genetic factors contributing to hypospadias such as environmental, psychosocial, and endocrine factors. These studies, as well as our results, should not be interpreted as asserting causation, but rather as preliminary data for future areas of functional research to further understand the genetic etiology of hypospadias.

5. Conclusions

This study used chromosome microarray-based technology (CMA) to identify genomic structural variations recurrently found in individuals with hypospadias, a common congenital atypical sex development. These findings propose many of these loci and the candidate genes within them as novel potential underlying causes of hypospadias and, occasionally, additional comorbid phenotypes. However, given that the role of non-genetic factors remains to be explored, these candidate genes should be investigated with functional studies that will further determine their potential relevance to hypospadias. Recurrent losses and gains of DNA were detected on nearly all chromosomes, suggesting genome-wide CMA as an ideal assessment tool for this population. Increased genetic screening of individuals with hypospadias, as well as reporting these data to collaborative databases, will improve detection of common comorbidities as well as additional candidate genes. These results provide insight into many novel risk loci and genes for further investigation to improve understanding of the genetic etiology of this common, yet poorly understood condition, as well as human genital development overall.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23084246/s1.

Author Contributions: C.H.S. and I.E.A.: conceptualization, validation, formal analysis; C.H.S.: software, investigation, resources, data curation, writing—original draft preparation, visualization; I.E.A.: methodology, review of manuscript, supervision, project administration. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and data collection from our center was approved by the Institutional Review Board of Washington University School of Medicine in St. Louis (IRB# 201701044).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Publicly available datasets were analyzed in this study. This data can be found here: https://decipher.sanger.ac.uk accessed on 26 May 2020. Data from our center presented in this study are available on request from the corresponding author.

Acknowledgments: This study makes use of data generated by the DECIPHER community. A full list of centers that contributed to the generation of the data is available from https://decipher.sanger.ac.uk accessed on 26 May 2020 and via email from decipher@sanger.ac.uk. Funding for the DECIPHER project was provided by Wellcome. Those who carried out the original data collection and analysis bear no responsibility for later data interpretation or analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Samtani, R.; Bajpai, M.; Ghosh, P.; Saraswathy, K. Hypospadias Risk among North-Indian Children. Ann. Health Health Sci. 2014, 1, 61. [CrossRef]
2. Van der Zanden, L.F.M.; van Rooij, I.A.L.M.; Feitz, W.F.J.; Franke, B.; Knoers, N.V.A.M.; Roeleveld, N. Aetiology of Hypospadias: A Systematic Review of Genes and Environment. Hum. Reprod. Update 2012, 18, 260–283. [CrossRef] [PubMed]
3. Akin, Y.; Erkan, O.; Telatar, B.; Tarhan, F.; Comert, S. Hypospadias in Istanbul: Incidence and Risk Factors. Pediatr. Int. Off. J. Jpn. Pediatr. Soc. 2011, 53, 754–760. [CrossRef] [PubMed]
28. Lek, M.; Karczewski, K.J.; Minikel, E.V.; Samocha, K.E.; Banks, E.; Fennell, T.; O’Donnell-Luria, A.H.; Ware, J.S.; Hill, A.J.; Cummings, B.B.; et al. Analysis of Protein-Coding Genetic Variation in 60,706 Humans. *Nature* **2016**, *536*, 285–291. [CrossRef] [PubMed]
29. McKinsey-Nathans Institute of Genetic Medicine. *Online Mendelian Inheritance in Man, OMIM®*; Johns Hopkins University: Baltimore, MD, USA, 2020.
30. Walia, R.; Single, M.; Vaiheki, K.; Kumar, S.; Bhansali, A. Disorders of Sex Development: A Study of 194 Cases. *Endocr. Connect.* **2018**, *7*, 364–371. [CrossRef] [PubMed]
31. Dabrowski, E.; Armstrong, A.E.; Leeth, E.; Johnson, E.; Cheng, E.; Gosiengfiao, Y.; Finlayson, C. Proximal Hypospadias and a Novel WTI Variant: When Should Genetic Testing Be Considered? *Pediatrics* **2018**, *141*, S491–S495. [CrossRef]
32. Johnson, E.K.; Jacobson, D.L.; Finlayson, C.; Yerkes, E.B.; Goetsch, A.L.; Leeth, E.A.; Cheng, E.Y. Proximal Hypospadias: Isolated Genital Condition or Marker of More? *J. Urol.* **2020**, *204*, 345–352. [CrossRef] [PubMed]
33. Amarillo, I.E.; Nievera, I.; Hagan, A.; Huchthagower, V.; Heeley, J.; Holland, A.; Koenig, J.; Austin, P.; Wang, T. Integrated Small Copy Number Variations and Epigenome Maps of Disorders of Sex Development. *Hum. Genome Var.* **2016**, *3*, 16012. [CrossRef]
34. Zarrei, M.; MacDonald, J.R.; Merico, D.; Scherer, S.W. A Copy Number Variation Map of the Human Genome. *Nat. Rev. Genet.* **2015**, *16*, 172–183. [CrossRef]
35. Uddin, M.; Thiruvahindrapuram, B.; Walker, S.; Wang, Z.; Hu, P.; Lamoureux, S.; Wei, J.; MacDonald, J.R.; Pellecchia, G.; Lu, C.; et al. A High-Resolution Copy-Number Variation Resource for Clinical and Population Genetistics. *Genet. Med.* **2015**, *17*, 747–752. [CrossRef] [PubMed]
36. Theisen, J.G.; Amarillo, I.E. Creating Affirmative and Inclusive Practices When Providing Genetic and Genomic Diagnostic and Research Services to Gender-Expansive and Transgender Patients. *J. Appl. Lab. Med. Mol. Med.* **2020**, *6*, 142–154. [CrossRef] [PubMed]
37. Chen, Y.C.; Woolley, P. v Genetics Studies on Hypospadias in Males. *J. Med. Genet.* **1971**, *8*, 153–159. [CrossRef] [PubMed]
38. Tateno, T.; Sasagawa, I.; Ashida, J.; Nakada, T.; Ogata, T. Absence of Y-Chromosome Microdeletions in Patients with Isolated Hypospadias. *Fertil. Steril.* **2000**, *74*, 399–400. [CrossRef]
39. Kon, M.; Saito, K.; Mitsui, T.; Miyado, M.; Igarashi, M.; Moriya, K.; Nonomura, K.; Shinohara, N.; Ogata, T.; Fukami, M. Copy Number Variations of the Azoospermia Factor Region and SRY Are Not Associated with the Risk of Hypospadias. *Sex. Dev.* **2016**, *10*, 12–15. [CrossRef]
40. O’Neill, M.A.; White, J.T.; Lamb, D.J. Gene Dosage Changes in RBFOX-2 Can Affect Upper and Lower Tract Genitourinary Development. *J. Endocr. Soc.* **2020**, *4*, OR02-01. [CrossRef]
41. Ward-Pearle, D.; Donaldson, S.L.; Comes, O.; Zuberi, K.; Badrawi, R.; Chao, P.; Franz, M.; Grouios, C.; Kazi, F.; Lopes, C.T.; et al. The GeneMANIA Prediction Server: Biological Network Integration for Gene Prioritization and Predicting Gene Function. *Nucleic Acids Res.* **2010**, *38*, W214–W220. [CrossRef]
42. Jamsheer, A.; Sowińska, A.; Simon, D.; Jamsheer-Bratkowska, M.; Trzeciak, T.; Latos-Bieleriska, A. Bilateral Radial Agenesis with Absent Thumbs, Complex Heart Defect, Short Stature, and Facial Dysmorphism in a Patient with Pure Distal Microduplication of 5q35.2–5q35.3. *BMC Med. Genet.* **2013**, *14*, 13. [CrossRef]
43. Rafi, S.K.; Butler, M.G. The 15q11.2 BP1-BP2 Microdeletion (Burnside–Butler) Syndrome: In Silico Analyses of the Four Coding Genes Reveal Functional Associations with Neurodevelopmental Disorders. *Int. J. Mol. Sci.* **2020**, *21*, 3296. [CrossRef]
44. Butwicka, A.; Lichtenstein, P.; Landén, M.; Nordenvall, A.S.; Nordenström, A.; Nordenskjöld, A.; Frisén, L. Hypospadias and Increased Risk for Neurodevelopmental Disorders. *J. Child Psychol. Psychiatry Allied Discip.* **2015**, *56*, 155–161. [CrossRef] [PubMed]
45. Gazdagh, G.E.; Wang, C.; McGowan, R.; Tobias, E.S.; Ahmed, S.F. DDD Study Cardiac Disorders and Structural Brain Abnormalities Are Commonly Associated with Hypospadias in Children with Neurodevelopmental Disorders. *Clin. Dysmorphol.* **2019**, *28*, 114–119. [CrossRef] [PubMed]
46. Liao, C.; Fu, F.; Li, R.; Yang, W.; Liao, H.; Yan, J.; Li, J.; Li, S.; Yang, X.; Li, D. Loss-of-Function Variation in the DPP6 Gene Is Associated with Autosomal Dominant Microcephaly and Mental Retardation. *Eur. J. Med. Genet.* **2013**, *56*, 484–489. [CrossRef] [PubMed]
47. Lin, C.; Werner, R.; Ma, L.; Miner, J.H. Requirement for Basement Membrane Laminin A5 during Urethral and External Genital Development. *Mech. Dev.* **2016**, *141*, 62–69. [CrossRef] [PubMed]
48. Ea, V.; Bergougnoux, N.; Philibert, P.; Servant-Fauconnet, N.; Faure, A.; Breaud, J.; Gaspary, L.; Sultan, C.; Paris, F.; Kalfa, N. How Far Should We Explore Hypospadias? Next-Generation Sequencing Applied to a Large Set of Hypospadiac Patients. *Urol. Eur. 2021*, *79*, 507–515. [CrossRef]
49. Van Bever, Y.; Brüggewirth, H.T.; Wollenbuttel, K.P.; Dessens, A.B.; Groenenberg, I.A.L.; Knapen, M.E.C.M.; de Baere, E.; Cools, M.; van Ravenswaaij-Arts, C.M.A.; Sikkema-Raddatz, B.; et al. Under-Reported Aspects of Diagnosis and Treatment Addressed in the Dutch-Flemish Guideline for Comprehensive Diagnostics in Disorders/Differences of Sex Development. *J. Med. Genet.* **2020**, *57*, 581–589. [CrossRef]
50. Huang, N.; Lee, I.; Marcotte, E.M.; Hurles, M.E. Characterising and Predicting Haploinsufficiency in the Human Genome. *PLoS Genet.* **2010**, *6*, e1001154. [CrossRef] [PubMed]
51. Haraksingh, R.R.; Abyzov, A.; Gerstein, M.; Urban, A.E.; Snyder, M. Genome-Wide Mapping of Copy Number Variation in Humans: Comparative Analysis of High Resolution Array Platforms. *PLoS ONE* **2011**, *6*, e27859. [CrossRef] [PubMed]
52. Geller, F.; Feenstra, B.; Carstensen, L.; Pers, T.H.; van Rooij, I.A.L.M.; Körberg, I.B.; Choudhry, S.; Karjalainen, J.M.; Schnack, T.H.; Hollegaard, M.V.; et al. Genome-Wide Association Analyses Identify Variants in Developmental Genes Associated with Hypospadias. Nat. Genet. 2014, 46, 957–963. [CrossRef]

53. Van der Zanden, L.F.M.; van Rooij, I.A.L.M.; Feitz, W.F.J.; Knight, J.; Donders, A.R.T.; Renkema, K.Y.; Bongers, E.M.H.F.; Vermeulen, S.H.H.M.; Kienemey, L.A.L.M.; Veltman, J.A.; et al. Common Variants in DGKK Are Strongly Associated with Risk of Hypospadias. Nat. Genet. 2011, 43, 48–50. [CrossRef]

54. Sreenivasan, R.; Gordon, C.T.; Benko, S.; de Iongh, R.; Bagheri-Fam, S.; Lyonnet, S.; Harley, V. Altered SOX9 Genital Tubercle Enhancer Region in Hypospadias. J. Steroid Biochem. Mol. Biol. 2017, 170, 28–38. [CrossRef]