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Joint effects of genetic variants and residential proximity to pesticide applications on hypospadias risk

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Abstract

Background—We examined risks associated with joint exposure to gene variants and pesticides.

Methods—Analyses included 189 cases and 390 male controls born from 1991-2003 in California's San Joaquin Valley. We used logistic regression to examine risks associated with joint exposures to gene variants and pesticides that our previous work identified as associated with hypospadias. Genetic variables were based on variants in *DGKK*; genes involved in sex steroid synthesis/metabolism; and genes involved in genital tubercle development. Pesticide exposure was based on residential proximity to commercial agricultural pesticide applications.

Results—Odds ratios (ORs) were highest among babies with joint exposures, who had 2- to 4-fold increased risks; e.g., the OR was 3.7 (95% CI 0.8-16.5) among subjects with the risk-associated *DGKK* haplotype *and* pesticide exposure; 1.5 (0.7-3.1) among subjects with the haplotype and no pesticide exposure; and 0.9 (0.5-1.6) among subjects without the haplotype but with pesticide exposure, relative to subjects with neither. However, results did not provide statistical evidence that these risks were significantly greater than expected on an additive scale, relative to risks associated with one exposure at a time.

Conclusions—We observed elevated risks associated with joint exposures to selected pesticides and genetic variants but no statistical evidence for interaction.

Keywords

pesticides; genes; hypospadias; birth defects; urogenital

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Introduction

Hypospadias, which occurs when the urethral opening occurs on the ventral side of the penis, affects around 5 per 1,000 newborn males (Carmichael and others, 2012b). Given the necessity of dihydro-testosterone to normal urethral closure in males, as well as experimental evidence indicating that estrogenic or anti-androgenic substances can disrupt this process (Gray and others, 2004; Noriega and others, 2005; Ostby and others, 1999), it is suspected that endocrine-disrupting environmental exposures may affect hypospadias risk. Pesticides, some of which are endocrine disruptors, have received the most attention. Most previous studies have been based on occupation as a proxy for exposure, and results have suggested modest to no association (Carmichael and others, 2012b), although a recent study of occupational exposures suggested stronger risks (Kalfa and others, 2015). We previously reported increased risk with residential proximity to a few – but not most – of many different pesticides that were studied (Carmichael and others, 2013b). Hypospadias is also highly heritable (Schnack and others, 2008), but the specific genes that contribute to risk are in large part unknown. Strong candidates include *DGKK*, which was discovered through a genome-wide association study (van der Zanden and others, 2010), as well as genes associated with genital tubercle development (the anlage for the penis) and sex steroid synthesis and metabolism (Carmichael and others, 2012b). We previously reported an association of hypospadias risk with variants in *DGKK* and a variety of genes that contribute to these processes (Carmichael and others, 2013a; Carmichael and others, 2012a; Carmichael and others, 2014). The pathway by which *DGKK* affects hypospadias risk is unknown.

Ultimately, it is expected that a combination of genetic and environmental factors will underlie risk of hypospadias. Thus, the objective of the current analysis was to examine the joint effects of genetic variants and residential proximity to specific agricultural pesticide applications on hypospadias risk. Our hypothesis was that risks would be highest among women who had pesticide exposures and whose babies had genetic variants that we previously identified as being associated with hypospadias risk. The analysis is based on our previous findings for the individual effects of these exposures in a large population-based cohort of California births (Carmichael and others, 2013a; Carmichael and others, 2012a; Carmichael and others, 2014; Carmichael and others, 2013b).

Methods

The study population included male infants born from 1991-2003 to mothers who were residents of eight California Central Valley counties (Fresno, Kern, Kings, Madera, Merced, San Joaquin, Stanislaus, and Tulare counties) and who were included in our previous studies of pesticides and genes and hypospadias (Carmichael and others, 2013a; Carmichael and others, 2012a; Carmichael and others, 2014; Carmichael and others, 2013b). California Birth Defects Monitoring Program staff ascertained cases by reviewing medical records at hospitals and genetic centers (Croen and others, 1991). Cases with known syndromes or single gene disorders, based on information available from medical records, were excluded. Cases were classified by hypospadias severity, which was based on the reported anatomical

position of the urethral opening. Mild cases were those for which the meatus was limited to the coronal or glanular penis (British Pediatric Association [BPA] codes 752.605, 752.625), moderate cases were those for which the meatus was on the penile shaft, and severe cases were those for which the meatus was at the peno-scrotal junction or perineal area (BPA codes 752.606, 752.607, 752.626, 752.627) (Carmichael and others, 2003). Cases for which the anatomical position was described as "not otherwise specified" (BPA codes 752.600, 752.620) were ineligible, as were cases with a known single gene disorder or chromosomal abnormality. Male controls were randomly selected from all males who did not have major congenital malformations and were delivered in the same counties and years as the cases, to give an approximately 2:1 ratio of controls to cases. Registry data are routinely linked with vital statistics, from which we derived descriptive information (e.g., maternal race-ethnicity).

Cases and controls were linked with archived bloodspots that were collected for newborn screening, which served as the source of DNA for genotyping. TagSNPs with minor allele frequencies (MAF) >10% were selected for study using the Genome Variation Server (http://gvs.gs.washington.edu/GVS/) and assayed using a custom multiplex Illumina GoldenGate assay. We used genotype information on 102 ancestry informative markers (AIMs) to create variables that reflect proportion Native American, African, and European ancestry for each study subject (Carmichael and others, 2014; Choudhry and others, 2010; Gamboa-Melendez and others, 2012; Risch and others, 2009; Via and others, 2010). Given that the three proportions sum to one, we only incorporated two (Native American and African) into our analyses to adjust for potential population stratification.

Genetic variables were defined based on the following: 1) for *DGKK*, we classified subjects as having a high-risk haplotype observed in our previous work, or not (block 2 haplotype #6, TTGCCGTA) (Carmichael and others, 2012a); 2) for genes associated with sex steroid synthesis and metabolism, we classified subjects as having risk-associated SNPs in 0, 1 or 2 or more genes in this set (based on $p<0.01$) because the frequency of having an at-risk variant in any of these genes was so high (72% among controls); and 3) for genes associated with genital tubercle development, we classified subjects as having any versus no risk-associated SNP for any of the genes in this set (i.e., $p<0.01$), based on previous analyses (Carmichael and others, 2013a). Sex steroid-related genes with risk-associated variants included *HSD17B3, HSD3B1, SRD5A2, STARD3* and *STS*. Genital tubercle-related genes with risk-associated variants included *BMP7, FGF10, GLI1* and *GLI2*.

We defined early pregnancy exposure as agricultural pesticide applications within a 500 m radius of maternal residence, using detailed data on applications and land use (details are provided elsewhere) (Carmichael and others, 2013b). The time window evaluated was 1-14 weeks of embryonic age (i.e., 1-98 days post-conception), which approximately encompasses the time of genital tubercle and urethral development and closure (Shoenwolf and others, 2009), as well as several weeks preceding it, given the potential physiologic persistence of some pesticides. Last menstrual period was derived primarily from birth certificates. To estimate pesticide applications, we obtained Pesticide Use Reporting records from the California Department of Pesticide Regulation describing agricultural pesticide applications and spatially referenced them to public land survey sections and land-use survey field polygons from the California Department of Water Resources. We then used the

California Environmental Health Tracking Program Pesticide Linkage Tool to assign exposure to specific pesticides and physicochemical groupings of pesticides. In addition, pesticides were noted as being endocrine disruptors (European-Commission, November 10, 2000; Keith, 1997); having reproductive or developmental toxicity based on the California Proposition 65 list (California-Office-of-Environmental-Health-Hazard-Assessment, 2012); or as being a reproductive or development toxicant based on having a US EPA-determined Reference Dose (RfD) from a toxicological study with a reproductive or developmental endpoint (EPA, 2012).

Pesticide exposure was defined based on any versus no exposure to any specific pesticide or pesticide group that was associated with overall hypospadias risk based on our previous analyses (i.e., had a CI excluding 1.0 in our previous analyses of pesticides that included all hypospadias cases) (Carmichael and others, 2013b). Associated pesticides included monochlorophenoxy acid or ester herbicides; the insecticides aldicarb, dimethoate, phorate, and petroleum oils; and the adjuvant polyoxyethylene sorbitol.

There were 196 cases and 420 controls born in Central Valley counties from 1991-2003 who had pesticide exposure assessment and genetic data. Among these subjects, 189 cases and 390 controls had data on the ancestral informative marker variables and maternal race-ethnicity. We incorporated the maximum number of subjects available into each comparison, which varied depending on the specific gene set being evaluated.

Logistic regression was used to generate odds ratios and 95% confidence intervals for the association of each combination of pesticide and genetic variables with hypospadias. All models also included variables reflecting genetic markers for ancestry (i.e., AIMs, see above) and maternal self-reported race-ethnicity (non-Hispanic white, Hispanic, other). In addition, we estimated the relative excess risk due to interaction (RERI) and its 95% CI to reflect additive interaction, which makes sense biologically and has more power than testing interaction on the multiplicative scale (Andersson and others, 2005; Correa and others, 2012). This research was approved by the California Committee for the Protection of Human Subjects.

Results

Mothers of cases were more likely than controls to be non-Hispanic white, have greater than high school education, and to have had no previous live births, and less likely to be <25 years old (Table 1). Case infants were more likely than control infants to have birthweight ≤2500 gm and be delivered at <37 weeks gestation. Among the 189 cases, 62 were mild, 75 were moderate, and 52 were severe. Frequencies of each of the genetic- and pesticide-related variables among cases and controls are shown in Table 2.

Odds ratios were highest for infants who had risk-associated genetic variants *and* whose mothers had residential pesticide exposure, versus subjects with neither (Table 3). For example, the odds ratio was 3.7 (95% CI 0.8, 16.5) among subjects with the risk-associated *DGKK* haplotype *and* pesticide exposure. An OR of 1.5 (95% CI 0.7, 3.1) was observed among subjects with the haplotype and no pesticide exposure, indicating that risk was 2.5

times higher (3.7/1.5) among the pesticide-exposed subjects. In contrast, among subjects without the risk-associated haplotype, the odds ratio was 0.9 (95% CI 0.5, 1.6) among subjects who did versus did not have the pesticide exposure.

Similarly, among subjects with risk-associated variants in two or more of the sex steroid-related genes, the ORs were 4.4 (95% CI 1.8, 10.6) and 2.2 (95% CI 1.2, 3.8) among women with or without any pesticide exposure, i.e., 2.0 times higher among babies born to women with pesticide exposure. In contrast, among subjects with risk-associated variants in only 1 of these genes, the ORs for women with and without pesticide exposure were 1.3 (95% CI 0.5, 3.5) and 1.5 (95% CI 0.9, 2.6), i.e., not higher among babies born to women with pesticide exposure; among subjects with no risk-associated variants, the OR for pesticide exposure was not calculated due to small cell sizes (Table 3).

For genes related to genital tubercle development, we did not observe the same pattern; that is, odds ratios tended to be similar among subjects who had a risk-associated genetic variant regardless of whether or not they had pesticide exposure (Table 3).

The RERIs were positive. However, none of their confidence intervals excluded zero; i.e., statistical evidence was lacking that the association of hypospadias risk with the selected pesticides and genetic variants in combination was greater than expected (95% CIs for all RERI's included zero).

Discussion

We examined risks associated with joint exposure to gene variants and pesticides that we previously observed to be independently associated with risk of hypospadias. As expected, the highest risks were among babies with both exposures, indicating 2- to 4-fold increased risks among babies with both exposures. However, our results did not provide statistical evidence that these increased risks were significantly greater than expected on an additive scale, based on risks associated with one exposure at a time. Our inability to detect such a departure may in part reflect the fact that the number of subjects with joint gene and pesticide exposures was relatively small.

Our study is unique in the genetic variants and pesticide exposures it investigated, as well as its examination of these factors in combination; as such, it is challenging to compare it to prior studies. Multiple review papers have discussed the potential contribution of gene-environment interaction to hypospadias risk (Marrocco and others, 2015; Thorup and others, 2014). We are aware of one study that examined gene-environment interaction(van der Zanden and others, 2012). The study reported evidence for interaction between one SNP in *SRD5A2* (rs523349, or V89L) and ‘exogenous estrogen’ exposure (which included oral contraceptive use or intake of soy or linseed-based foods; the OR for both exposures was 8.5, 95% CI 1.1-67.8); the *SRD5A2* SNP and maternal hypertensive disorders (OR 0.6, 95% CI 0.3-1.1); and one SNP in ATF3 rs11119982) and ‘cytokine’ exposure (which included maternal cold, infection or immune-related disease; the OR was 2.8, 95% CI 1.3-6.4).

Our study's strengths include its population-based design and thorough case ascertainment. The registry has the advantage of being based on detailed clinical information at the

population-level. We were able to distinguish cases based on severity but did not do so based on reported minor accompanying genital malformations (e.g., cryptorchidism, micropenis), given that their ascertainment by the registry is less accurate and complete than it is for more major malformations; the potential impact on results is uncertain. Pesticide exposure assessment was more detailed than prior studies but still has limitations, such as being focused on residential proximity to pesticide applications rather than more direct measurements of exposure levels such as serum levels, and inability to consider non-residential exposures. In addition, pesticide exposure was based on residence at birth rather than during the periconceptional period; the extent of error incurred by residential mobility is uncertain but likely to be non-differential (i.e., similar for cases and controls). Data on *DGKK* haplotypes was missing on >15% of subjects, because haplotype characterization required non-missing data on *all* measured variants. We are unaware of any reason to believe that estimates based on the sub-sample with complete data would be biased. Genetic variants in a variety of genes were investigated, but ultimately they may represent a tiny proportion of variants that may contribute to hypospadias risk or the potential negative effects of pesticide exposures. It was not feasible to further extend the amount of genetic information available on each case. A strength of our genetic work is the inclusion of ancestry informative markers; however, adjustment for them did not substantially alter results (data not shown).

This study indicates that babies with genetic variants and maternal pesticide exposures who were previously identified as associated with increased risk of hypospadias were at 2- to 4-fold increased risk of hypospadias. The magnitudes of risk were greatest among babies with joint exposures, but the analysis did not provide statistical evidence for interaction. Our results should be interpreted with caution, given that they represent the first report of joint effects of the studied exposures, and they are based on findings related to independent effects that are also unique.

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Table 1

Descriptive characteristics of hypospadias cases (n=189) and unaffected male controls (n=390), California, 1991-2003.

	Percent of Cases (n)	Percent of Controls (n)
Maternal race-ethnicity		
Non-Hispanic white	48 (90)	37 (143)
Hispanic	38 (72)	46 (180)
Others	14 (27)	17 (67)
Maternal education		
< High school	29 (54)	41 (159)
High school	31 (58)	34 (131)
> High school	40 (75)	25 (96)
Unknown	1 (2)	1 (4)
Maternal age		
< 25 years	39 (73)	52 (202)
25-34 years	49 (93)	40 (155)
35 or more years	12 (23)	8 (33)
Number of previous live births		
0	50 (94)	33 (129)
1	29 (55)	31 (122)
≥ 2	21 (40)	36 (139)
Unknown	0 (0)	0 (0)
Infant birthweight		
≤ 2500 g	33 (63)	6 (23)
> 2500 g	66 (124)	94 (367)
Unknown	1 (2)	0 (0)
Gestational age at delivery		
< 37 weeks	27 (51)	9 (37)
≥ 37 weeks	71 (135)	84 (327)
Unknown	2 (3)	7 (26)

Table 2

Frequency of genetic variant and pesticide exposure variables among case (n=189) and male control (n=390) infants.*

	Percent of cases (n)	Percent of controls (n)
Risk-associated genetic variants		
DGKK high-risk haplotype		
Yes	10 (19)	6 (22)
No	74 (139)	74 (290)
Missing	16 (31)	20 (78)
Risk-associated SNPs in sex steroid genes		
Any	83 (156)	72 (282)
None	16 (31)	26 (100)
Missing	1 (2)	2 (8)
Risk-associated SNPs in genital tubercle genes		
Any	28 (52)	16 (61)
None	69 (131)	81 (314)
Missing	3 (6)	4 (15)
Risk-associated pesticide exposure		
Any	13 (25)	12 (45)
None	87 (164)	88 (345)

[1] Details regarding definitions of genetic and pesticide variables are available in the Methods. In brief, for *DGKK,* subjects were designated as having a risk-associated haplotype or not; for sex steroid-related genes, 2 or more indicates that subjects had risk-associated variants in 2 or more different genes in this set of genes; and for genital tubercle-related genes, 'Any' indicates that subjects had risk-associated variants in any of the genes in this set. Pesticide exposure was based on residential proximity to commercial agricultural application of pesticides that we previously identified as being associated with hypospadias risk.

Table 3

Joint association of genetic variants and residential proximity to pesticide applications with hypospadias risk.[1]

	Presence of risk-associated genetic variants	Exposure to risk-associated pesticides	No. cases	No. controls	Odds ratio (95% CI)[2]	RERI (95% CI)[3]
DGKK	Any	Yes	5	3	3.7 (0.8, 16.5)	2.4 (−3.2, 8.0)
	Any	No	14	19	1.5 (0.7, 3.1)	
	None	Yes	16	36	0.9 (0.5, 1.6)	
	None	No	123	254	Reference	
Sex hormone-related genes	2+	Yes	16	14	4.4 (1.8, 10.6)	3.1 (−0.7, 6.9)
	2+	No	62	109	2.2 (1.2, 3.8)	
	1	Yes	7	16	1.3 (0.5, 3.5)	0.4 (−0.9, 1.7)
	1	No	71	143	1.5 (0.9, 2.6)	
	0	Yes	2	15	-	
	0	No	29	85	Reference	
Genital tubercle-related genes	Any	Yes	7	7	2.3 (0.8, 6.8)	0.2 (−2.4, 2.8)
	Any	No	45	54	1.9 (1.2, 3.2)	
	None	Yes	18	37	1.1 (0.6, 2.1)	
	None	No	113	277	Reference	

[1] Details regarding definitions of genetic and pesticide variables are available in the Methods. In brief, for *DGKK,* subjects were designated as having a risk-associated haplotype or not; for sex steroid-related genes, 2 or more indicates that subjects had risk-associated variants in 2 or more different genes in this set of genes; and for genital tubercle-related genes, 'Any' indicates that subjects had risk-associated variants in any of the genes in this set. Pesticide exposure was based on residential proximity to commercial agricultural application of pesticides that we previously identified as being associated with hypospadias risk.

[2] Odds ratios are not reported for comparisons that involved cell sizes <3. Odds ratios are adjusted for genetic markers for ancestry (i.e., ancestry informative markers, as described in the Methods) and maternal self-reported race-ethnicity.

[3] RERI = Relative excess risk due to interaction, which reflects departure from additivity of effects of exposure to pesticides and presence of genetic variants; a value of '0' represents the null (no departure from additivity).