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Evaluation of *BNC2* as a new candidate gene for hypospadias

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In order to evaluate the use of the *BNC2* gene in clinical practice regarding hypospadias, the presence of the impact of mutations in *BNC2* gene in males who had been treated surgically for hypospadias in Sweden (N=413) and controls (N=455) were evaluated. Mutational screening was performed using Sanger sequencing and genotyping by Taqman allelic discrimination, and the findings were evaluated by disease-causing potential using Mutation Taster and PolyPhen. Nine missense mutations were identified, six of which were more common among cases than among controls, one being previously unknown. Six of these genetic variants were predicted to be possibly or probably damaging in mutational predictions and are thus potentially disease-causing. No difference was confirmed in the contribution of these findings in different severity of phenotype. Due to the complexity of the gene structure, the impact of variants was difficult to evaluate, and thus the clinical use of *BNC2* in the management of 46,XY disorder of sex development (DSD) remains limited.

Key words: *BNC2* gene, hypospadias, mutations, polymorphism.

INTRODUCTION

Hypospadias is one of the most frequent congenital malformations in boys. The malformation results from an arrest of the normal development of the urethra, foreskin and ventral aspect of the penis during gestational week 8 to 16. It is characterized by misplacement of the urethral opening, ventrally and proximally from the tip of the glans penis. The site of failure of urethral fold will dictate the position of the abnormal urethral meatus, and thus the severity of hypospadias. Majority of the cases of hypospadias are mild, with the meatus positioned at corona or on the glans penis (Bergman et al., 2015). More severe cases were associated with penile curvature as well as penoscrotal transposition and intersex conditions (Baskin and Ebbers, 2006).

Hypospadias is a multifactorial disorder caused by both genetic and environmental factors. Accordingly, hypospadias sometimes displays a monogenic inheritance pattern. Majority of the cases are sporadic, but in approximately 7%

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of them, familial clustering, has been described, which supports the theory of an underlying genetic effect (Fredell et al., 2002a). Consequently, most hypospadias are sporadic and without a known molecular cause in individual cases. The malformation exhibits an under-masculinization in male fetuses, but mutations in genes involved in the androgen pathway, such as steroid-5-alpha-reductase (*SRD5A2*) and androgen receptor (*AR*), are rare and reported in only a small portion of affected individuals (Hiort et al., 1994; Kon et al., 2015). Other genes that have been associated with hypospadias are *MAMLD1*, which when knocked down in mice reduces testosterone production and exhibits mutations in a few percent of hypospadias patients (Kalfa et al., 2008), *HOXA13*, which may cause hand-foot-genital syndrome (Frisen et al., 2003), *MID1*, in which mutations have been associated with the Opitz syndrome, which is characterized by midline defects and *WT1* gene, in which mutations may lead to 46,XY disorder of sex development (DSD), nephropathy causing renal insufficiency, and Wilms' tumor (Kalfa et al., 2010; Kaltenis et al., 2004; van der Zanden et al., 2012).

A highly conserved gene that has attracted attention lately is the basonuclein 2 (*BNC2*; 9p22.1). It has been established that the protein characteristics of *BNC2* are similar to those of its namesake, *BNC1* (Vanhoutteghem and Djian, 2004), a zinc finger protein highly expressed in skin keratinocytes, which is associated with cell proliferation (Tseng, 1998). The function of *BNC2* is, however, unknown, although it is believed to participate in mRNA processing (Vanhoutteghem and Djian, 2006).

Bhoj et al. (2011) have described the potential role of *BNC2* in urethral development. They found high expression of *BNC2* in fetal periurethral tissues and *BNC2* knockout mice with a high prevalence of distal urethral defects. Furthermore, they performed mutational screening of the major coding exons in 48 males with hypospadias and in controls, and four potentially deleterious variants were identified in three subjects with hypospadias; whereas only one was found in the control group (Bhoj et al., 2011). This study prompted the DSD team to incorporate the *BNC2* gene in mutational screening of 46,XY DSD in our hospital. Kon et al. (2015) recently found one heterozygous missense mutation in *BNC2* when they analyzed the molecular basis of hypospadias among 57 patients.

In order to further evaluate the use of the *BNC2* gene in clinical practice, presence of mutations was screened in it, in a large collection of DNA samples from males with hypospadias and predicted the potential disease-causing effects of candidate polymorphisms.

MATERIALS AND METHODS

Clinical material

Four hundred and thirteen (413) males who had been surgically treated for hypospadias in Sweden were included in the study. The Ethics Committee of Karolinska Institutet approved the study first in 1995. All samples were obtained after oral or written information were given to the parents that gave oral consent. These samples have been collected continuously since 1996. Initially and until 2008, the consent was only oral. The original approval of the study covered all the years that the study took place, but since 2008, when an extended application on additional congenital malformations was requested, the consent have also, according to new rules from the National Ethics Committee been documented in the patient's medical chart. According to the rules from the National Ethics Committee, there is no demand for an explicitly written consent and the Ethics Committee of Karolinska Institutet have specifically approved oral consent from parents.

Both sporadic cases and familial (one per family) ones were included with varying phenotypic severity ranging from mild glandular hypospadias to perineal hypospadias with an intersex condition. Initially, 236 cases of the ranging phenotype were sequenced for mutational analyses. Phenotypes were grouped according to severity as glandular (N=35), penile (N=55), or penoscrotal/perineal (N=77) and, in 22 cases, the severity of hypospadias was unknown. An additional 47 males with familial hypospadias, with mainly a glandular phenotype, were included. Further analyses using Taqman were performed regarding two variants, which led to the inclusion of 177 additional cases.

Control samples of DNA from healthy volunteer anonymous blood donors and placenta tissue from the Karolinska University Hospital were analyzed using both standard sequencing and Taqman. The number of controls ranged from 76 to 455, depending on whether sequence variants that were identified in cases were controlled for by Sanger sequencing or Taqman. The Ethics Committee of Karolinska Institutet approved the study.

DNA extraction

Genomic DNA from the clinical sample was extracted from peripheral blood or penile tissue obtained during surgery using the standard phenol chloroform extraction protocol or the Gentra Purogenekit (Qiagen, Maryland, USA).

Mutational analysis

Exons 1 to 6 of *BNC2* were PCR-amplified and primers were designed to cover the entire coding sequence, including flanking intronic sequences (primer-3; http://primer3.wi.mit.edu) (Vanhoutteghem and Djian, 2007; Bhoj et al., 2011). PCR products were purified using Shrimp Alkaline Phosphatase and Exonuclease I (Fermentas Life Sciences). Sanger sequencing was performed on both strands, using standard cycle-sequencing reactions with the BigDye terminator v3.1 cycle sequencing kit (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems), and run on an ABI3130 Sequencer (Applied Biosystems). Chromatograms were analyzed using SeqScape v3.7 (Applied Biosystems) and compared with reference sequence ENST00000380672. All positive findings were confirmed by resequencing from the original DNA solution.

Genotyping

Genetic variations that were identified in the materials used and previously of unknown frequency in a healthy population were further analyzed using TaqMan allelic discrimination assays according to recommended protocols. The results were analyzed using the 7900 HT Fast-Real-Time PCR system (Applied Biosystems) and SDS 2.2 (Applied Biosystems). Only successful samples are reported (≥92% success rate in patients).

Four SNPs (rs62540608, TMP_ESP_9_16435990, rs3739715,

rs189895388) and three gene variants without rs number (c.1002, c.1239, and c.1738) were further evaluated in the cases and controls. No parents of the patients were genotyped.

Mutation prediction

The functional significance of all amino acid substitutions found in the *BNC2* gene was assessed *in silico* using Mutation Taster (http://www.mutationtaster.org) and Polyphen (http://genetics.bwh.harvard.edu/pph2/). The compiled results were classified according to the following categories: (1) Benign, if the missense mutation was predicted to be benign both by PolyPhen (score < 0.2) and Mutation taster; (2) Possibly damaging, if the missense mutation was predicted to be disease-causing by either Mutation Taster or PolyPhen (score >0.75); (3) Probably damaging, both PolyPhen (score >0.75) and Mutation Taster predicted the variant to be disease-causing. The conservation and 7X regulatory potential (UCSC genome browser, human NCBI/hg18 and GRCh37/hg19) were also assessed.

RESULTS

By sequencing, 20 genetic variants were identified in the *BNC2* gene in 115 out of 236 cases with hypospadias (Table 1 and Figure 1). Seventeen polymorphisms were previously known, thus three were novel, one of which was a missense mutation. Allelic variants were found most frequently in exon 5. Findings of this study are grouped as the results of the mutational prediction *in silico* subsequently.

Probably damaging

We identified three missense mutations that were predicted to be probably damaging in mutational simulations: (c.2051C>T, p.P684L), (c.2768C>T, p.A923V), and (c.2789A>G, p.D930G) in 15 boys with hypospadias (Table 2). All three variants have been described previously and allelic frequencies in the general population were available. The frequency of p.P684L (0,4)was higher incases than in the general population and was not detected in any of the healthy controls. The frequencies of p.A923V and p.D930G were lower or comparable in cases compared with the controls and the general population, suggesting that these variants do not cause hypospadias. However, p.D930G was only found in one case that was homozygous, whereas only heterozygotes were found in the controls or in the previously described populations (Table 1). Bhoj et al. (2011) have previously described p.A923V as “tolerated” using SIFT analysis. In our analyses it was predicted to be tolerated using SIFT, but to be damaging using both PolyPhen-2 and Mutation Taster. Based on these results and the high conservation of this region, we cannot rule out the fact that the variant might be damaging; however, since it is more common among controls than among cases, we do not consider it to have a causative effect in hypospadias.

Possibly damaging

Three missense mutations were predicted to be possibly damaging: (c.916C>G, p.P306A), (c.1738G>A, p.G580R), and (c.1868C>A, p.P623H). In one boy with Robinow syndrome, a change was identified in p.P306A; this boy was also a carrier of p.A923V, which was predicted to be damaging. The missense mutation in p.G580R was identified in one boy with several other malformations, including anal atresia, cardiac malformations, and polydactyly. The change in p.G580R has not been described previously and did not affect any of the controls in our analyses; p.P623H was identified in two boys, one of whom also had an associated cri-du-chat syndrome.

Both p.P306A and p.P623H have been described previously and occurred less frequently or just as frequently in cases as in controls or the general population. Both of these variants were predicted to be damaging according to the SIFT analysis, as reported by Bhoj et al. (2011). However, our results indicate that they are only possibly damaging when further simulation analyses are taken into account.

Benign

Majority of our findings were predicted benign, 11 being synonymous mutations (c.84A>T, p.A28A), (c.501C>T, p.V167V), (c.519C>T, p.S173S), (c.936C>T, p.F312F), (c.1002A>G, p.P334P), (c.1239T>C, p.D413D), (c.1545A >G, p.S515S), (c.1947C>T, p.T649T), (c.2202C>T, p.G734G), (c.2478A>G, p.L826L), and (c.2739C>T, p.R913R), and three were non-synonymous (c.171G>T, p.Q57H), (c.1240C>G, p.L414V), and (c.2929A>G, p.I974V).

The changes in p.P334P and p.D413D were previously unknown, each affecting one individual, and none of these changes were present in controls.

In total, there was an even distribution of findings among penoscrotal/perineal (64%), penile (64%) and glandular (69%) hypospadias. Mutational findings where less frequent among familial cases (43%). The frequency of findings defined as probably damaging was slightly higher in penile (9%) and penoscrotal/perineal (9%) cases than in individuals with glandular (6%) hypospadias.

We tested for statistical difference between the phenotypes using the Fischer exact test; there was no significant difference. The findings in the familial cases were all predicted to be benign.

DISCUSSION

In this study, mutational screening of the *BNC2* gene in 413 males with hypospadias was performed to evaluate the potential clinical use of *BNC2* in molecular diagnostics of hypospadias. By sequencing *BNC2* in 236 affected males, nine missense mutations were found with six of

Table 1. Data on all mutational findings, including calculated allelic frequencies among cases and controls. Disease-causing potential prediction based on PolyPhen and Mutation Taster.

Protein	RSnumber	Case	Control	Population rate[†]	Disease-causing potential
		N (%)	N (%)	All/Eur	Predicted effect
p.A28A	rs76485966	4/236 (1.7)[a]	0/80 (0)	4.5/0.3	Benign
p.Q57H	rs145011045	1/236 (0.2)	0/83 (0)	0.0/0.1	Benign
p.V167V	rs143821778	1/236 (0.2)	0/95 (0)	0.0/0.1	Benign
p.S173S	s149019822	1/236 (0.2)	0/95 (0)	0.0/0.1	Benign
p.P306A	rs114964332*	1/236 (0.2)	0/117 (0)	0.2/0.0	Possibly damaging
p.F312F	rs77464990	1/236 (0.2)	0/117 (0)	1.1/0.0	Benign
p.P334P	n.a.	1/236 (0.2)	0/361 (0)	n.a	Benign
p.D413D	n.a.	1/236 (0.2)	0/455 (0)	n.a	Benign
p.L414V	rs148873573*	7/236 (1.5)	3/94 (1.6)	0.2/0.4	Benign
p.S515S	rs117470554	17/236 (3.8)[b]	9/94 (4.8)	2.6/5.1	Benign
p.G580R	n.a.	1/236 (0.2)	0/443 (0)	n.a	Possibly damaging
p.P623H	rs114596065*	2/236 (0.4)	1/82 (0.6)	0.4/0.0	Possibly damaging
p.T649T	rs62540608	7/384 (0.9)	8/444 (0.09)	0.2/0.7	Benign
p.P684L	rs138187836	2/236 (0.4)	0/76 (0)	0.1/0.0	Probably damaging
p.G734G	TMP_ESP_9_16435990	1/236 (0.2)	0/361 (0)	0.0/0.0	Benign
p.L826L	rs3739715	65/406 (8.4)[c]	48/356 (6.9)[d]	6.8/6.7	Benign
p.R913R	rs189895388	1/236 (0.2)	0/361 (0)	0.0/0.0	Benign
p.A923V	rs117452684*	12/236 (2.5)	14/159 (4.4)	1.2/3.0	Probably damaging
p.D930G	rs41268965	1/236 (0.4)[a]	0/159 (0)	0.2/0.7	Probably damaging
p.I974V	rs35005898	6/236 (1.3)	2/159 (0.6)	0.3/0.7	Benign

*Previously described by Bhoj et al. (). [a]All cases homozygous. [b]One case homozygous. [c]Three cases homozygous. [d]One control homozygous.

Table 2. Detailed information on phenotype of the cases with variants predicted damaging in mutational simulation.

Subject			Detected non-synonymous variant		Disease-causing potential
ID	Phenotype	Other birth defects	cDNA	Protein	Predicted effect
H1	Distal penile	-	c.2051 C>T	p.P684L	Probably damaging
H2	Distal penile	-	c.2051 C>T	p.P684L	Probably damaging
H3	Glandular	-	c.2768 C>T	p.A923V	Probably damaging
H4	Unknown	-	c.2768 C>T	p.A923V	Probably damaging
H5	Perineal	Robinow syndrome	c.2768 C>T	p.A923V	Probably damaging
H6	Distal penile	-	c.2768 C>T	p.A923V	Probably damaging
H7	Penoscrotal	-	c.2768 C>T	p.A923V	Probably damaging
H8	Penoscrotal	-	c.2768 C>T	p.A923V	Probably damaging
H9	Glandular	-	c.2768 C>T	p.A923V	Probably damaging
H10	Penoscrotal	-	c.2768 C>T	p.A923V	Probably damaging
H11	Distal penile	-	c.2768 C>T	p.A923V	Probably damaging
H12	Penoscrotal	-	c.2768 C>T	p.A923V	Probably damaging
H13	Penoscrotal	-	c.2768 C>T	p.A923V	Probably damaging
H14	Perineal	Short stature	c.2768 C>T	p.A923V	Probably damaging
H15	Distal penile	-	c.2789 A>G	p.A923V	Probably damaging

which occurred more frequently in cases than in the controls, and one was previously unknown. Six of the genetic variants were predicted to be possibly or probably damaging and potentially disease-causing; however, some of these findings occurred just as frequently or even less frequently in cases than in controls, implying that the variants are not causative regarding hypospadias. Four of these missense mutations have been described previously

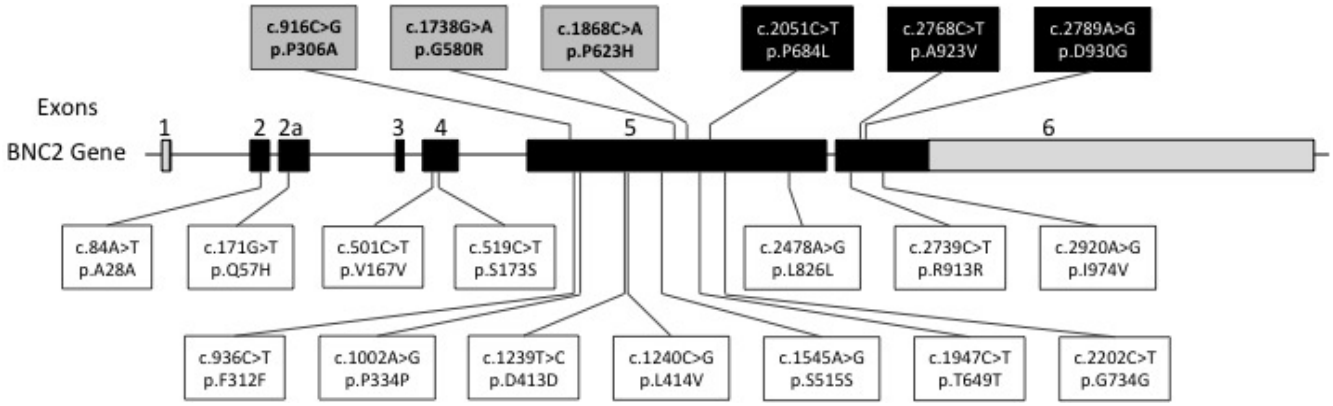

Figure 1. The black filled boxes represent the coding regions, and the light gray filled boxes denote the untranslated regions. The positions of the variants found in the *BNC2* are shown. Numbers indicate the number of exons in *BNC2*. Conclusion indicating disease-causing potential: filled with white background, benign; filled with gray, possibly damaging; filled with black, probably damaging.

previously in patients with hypospadias (Bhoj et al., 2011). No difference was confirmed in the distribution of mutational findings between different phenotypes.

Disturbances in developmental pathways, such as midline fusion and skin development, could, apart from defects in androgen signaling, cause hypospadias. Malformations involving the midline, such as cleft-lip-palate and congenital heart malformations, are known to be more frequently associated with hypospadias (Fredell et al., 2002b; Wu et al., 2002). *BNC2* is mainly expressed in renal, intestinal, uterine, and testicular tissue (Vanhoutteghem and Djian, 2006), but also in keratinocytes (Romano et al., 2004) and periurethral tissue during development (Bhoj et al., 2011). It has been confirmed that *BNC2* is important for the proliferation of craniofacial mesenchymal cells during embryogenesis in mice and it has been suggested that human craniofacial abnormalities may result from a lack of *BNC2* (Vanhoutteghem et al., 2009). Previous studies have demonstrated the importance of *BNC2* in urethral development, since knockout mice develop typical hypospadic features (Bhoj et al., 2011), potentially due to defects in skin development and midline fusion.

The *BNC2* gene spans 461 kb on chromosome 9 (9p22.1) and possesses 6 promoters (1 major and 5 minor), 4 polyadenylation sites, and a total of 23 exons (1, 1d, 1b, 1c, 2, 2f, 2b, 2a, 2g, 2b, 2a, 2g, 2b, 2l, 2c, 2e, 3, 3a, 4, 4a, 5, 5c, 5d, 5a, 5b, 6). Each promoter, splice site, and polyadenylation addition site has been suggested to be used independently, with the potential to generate up to approximately 90,000 variants encoding >2000 different proteins (Vanhoutteghem and Djian, 2007). Thus, none of the 23 exons is present in all *BNC2* mRNA isoforms, all exons can therefore be considered as alternative. The most abundant *BNC2* mRNA variants are stable and translated into proteins as demonstrated by PCR and transient transfection in HeLa cells (Vanhoutteghem and Djian, 2007).

With regard to the unknown function and large number of potential transcripts of *BNC2,* the significance of occasional mutational findings and their role in the pathogenesis of hypospadias are difficult to evaluate. Among the three variants predicted probably damaging in this study only one was more common among cases compared to controls (p.P684L); it was present in two cases with penile hypospadias and none of the controls. Thus, it is believed that the use of *BNC2* in current clinical practice regarding hypospadias is limited.

To further evaluate the potential pathogenic effect of sporadic variants in *BNC2,* it would first of all be desirable to determine which isoforms of *BNC2* are expressed in penile tissue during development. Secondly, functional studies on *BNC2* would be useful to assess the effect of our findings regarding the pathogenesis of hypospadias.

Conclusion

In this study, *BNC2* have been screened for mutations in order to investigate its potential clinical use in the diagnostics of XY, DSD. Several potential disease-causing SNPs that are more abundant in males with hypospadias than in controls have been identified, but we have not identified any certain phenotype-causing mutations. Due to the complexity of the gene structure and our occasional findings, the clinical use of *BNC2* was assessed in the management of 46,XY DSD as being limited.

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Conflict of interests

The authors have no conflicts of interest to declare.

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