Cadherins and catenins as a novel theoretical mechanism in a polyorchid cadaver

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SUMMARY

Polyorchidism is a rare congenital disorder defined as the presence of more than two testicles for which the underlying etiology is unknown. Prior research in this laboratory suggested a new anatomical-functional description for polyorchidism of Type 3 Subgroup B, or supernumerary testis (SNT) attached to the draining epididymis and vas deferens without reproductive potential and SNT located outside the scrotal sac. The purpose of the current investigation was to examine the mechanism underlying the role(s) of cadherins and catenins in the development of polyorchidism using genomic analysis of a cadaveric polyorchid. Formyl Fix Paraffin Embedded tissue samples of a SNT from a 96-year-old polyorchid were prepared using the Accel-NGS 2S Plus DNA Library Kit for whole exome sequencing (*Integrated DNA Technology*, Coralville, IA). Paired end sequencing was carried out using the Illumina NovaSeq 6000 system (*Illumina*, San Diego, CA) using 150 bp reads to an average depth of coverage of 44x. BLAST was used to analyze and compare the SNT sequence to reference genomes in the NCBI database. Sequence analysis of the SNT showed two missense mutations that resulted in single nucleotide variants (SNV) within exons of the N-Cadherin gene (CHD2), NT 23, T to C (Leu 8 to Pro) and NT 2441, A to G (Asn 845 to Ser), respectively. A mutation in the Desmocollin 2 (DSC2) gene was also demonstrated; NT 2393, G to A. The present research suggests a novel biomolecular mechanism based on N-Cad and p120 catenin underlying the development of polyorchidism with application to supernumerary organs in other systems and to metastasis of neoplasms.

Key words: Anatomy – Cadherin – Catenin – Congenital disorder – Genome – Polyorchid – Polyorchidism – Supernumerary – Testes – Testicle

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ABBREVIATIONS

Adenine (A)

Adhesion Junction/Adhesion Junctions (AJ/AJs)

Amino Acid (AA)

Cytosine (C)

Epithelial-Cadherin (E-Cad)

Epithelial-to-mesenchymal transition (EMT)

Guanine (G)

Juxtamembrane domain (JMD)

Leucine (Leu)

National Center for Biotechnology Information (NCBI)

N-Cadherin/Cadherin 2 gene (CDH2)

Neural-Cadherin (N-Cad)

Nucleotide (NT)

Phenylalanine (Phe)

Premoridal Germ Cells (PGCs)

Proline (Pro)

Sex-Determining Region Y (SRY)

Single Nucleotide Variants (SNV)

SRY-Box Transcription Factor 9 (SOX9)

Supernumerary Testis/Testes (SNT/SNTs)

Supernumerary Testis/Testes (ST/STs)

Testis-Determining Factor (TDF)

Testis-Specific Enhancer (TESCO)

Thymine (T)

INTRODUCTION

Polyorchidism is a rare congenital disorder where more than two testicles develop. In 1880, the first histological finding of polyorchidism was found, and in 1895, the first clinical case was confirmed (Lane, 1895; Artul and Habib, 2014; Hassan et al., 2008; Hassan et al., 2014). There are fewer than 200 human cases reported in medical literature. In addition, seven documented cases exist in the animal kingdom: two horses, two dogs, two cats, and a hummingbird (Roca-Ferrer et al., 2015; Tamminen et al., 2012; Witt and Bautista, 2011; Aziz et al., 2016; Talarico et al., 2022). In human polyorchids, diagnosis in patients ranges from 4 weeks to 75 years in age with a median age of 17 years (Mathur et al., 2002; Bergholz, 2009; Mittal et al., 2018; Talarico et al., 2022).

Patients with polyorchidism typically do not present with pain or other discomfort on clinical evaluation. However, if pain is present, it is localized to the scrotum/lower abdomen or the region to where the mass is found (Dollard, 2011; Sakamaoto et al., 2007; Mittal et al., 2018; Otero, 2016). Ultrasound and magnetic resonance imaging can differentiate between polyorchidism and paratesticular lesions (i.e., inguinal lipoma, etc.) (Arslanoglu, 2013). Occasionally, there are some additional abnormalities seen in polyorchids that include testicular maldescent (40%), inguinal hernia (30%), testicular torsion (13%), hydrocele (9%), and malignancy (6%) (Mathur et al., 2002; Tonape et al., 2012; Talarico et al., 2022). Furthermore, polyorchidism is associated with an increased risk of testicular cancer (Talarico et al., 2018; Bergholz et al., 2009).

In the majority of polyorchid cases, the supernumerary testis (SNT) or testes (SNTs) are located on the left side with a 3:1 ratio (left > right) (Sheah et al., 2004). SNTs are typically smaller than the two normal testes (Artul and Habib, 2014; Cohen et al., 2017; Talarico et al., 2022). Prior research has shown that inguinal SNTs without reproductive potential tend to be infiltrated with adipose tissue with increased patient age (Talarico et al., 2022). This has led to the suggestion of a novel anatomical-functional classification in polyorchidism, Type 3, Subgroups A and B (Talarico et al., 2022).

The mechanism(s) of polyorchidism is unknown, but it is hypothesized to result from a malfunction or duplication of the gonadal ridge during embryological development, possibly involving molecular or genetic factors that predispose a patient to developing SNTs.

In embryology, the gonadal ridge is the precursor to the gonads. The gonadal ridge forms during the beginning of testicular development, and is derived from intermediate mesoderm. It is known that its formation is initiated by coelomic epithelial cells on the mesonephros (Yang et al., 2018; Avellar et al., 2019). The Wolffian duct is found in the mesonephros, which contributes to the formation of the epididymis, vas deferens, and seminal vesicles (Yang et al., 2018; Avellar et al., 2019).

From a molecular perspective, the cells of male embryos have an X and a Y chromosome. The sex-determining region of the Y chromosome (Yp11) is composed in part of the sex-determining region Y (SRY) gene that encodes for testis-determining factor (TDF) (Cox, 2013; Hoare et al., 2021). In the absence of the SRY gene, the resulting embryo will be female. SRY encodes for the transcription factor that activates the testis-specific enhancer (TESCO) of SRY-Box Transcription factor 9 (SOX9), which is responsible for the differentiation of the Sertoli cells (Fig. 1) (Albrecht et al., 2001). In addition, Sertoli cells are required for testis formation and spermatogenesis. These cells have both endocrine and paracrine roles in spermatogenesis (Iliadou et. al., 2015). Steroidogenic Factor 1 also contributes to the differentiation of Sertoli cells and is encoded by the Nuclear Receptor Subfamily 5 Group A Member 1 (NR5A1) gene (Fig. 1) (Sekido et al., 2008). SOX9 levels are unregulated by SRY. When cellular and tissue levels of SRY and SOX9 reach a sufficient threshold in the genital ridge, SOX9 upregulation causes the development of Sertoli cells (Warr et al., 2012). This initiates morphological changes leading to the development of testicular tissue and incorporation of gametes from the yolk sac in the mature testis (Fig. 1) (Warr et al., 2012).

With reference to Fig. 1 and testicular genesis, it is important to note that the absence of the SRY gene (or presence of an X chromosome instead of a Y chromosome) discussed above differs from Swyer Syndrome (Ostrer, 2019). Swyer syndrome occurs due to mutations of the SRY gene or deletion of the segment of the Y chromosome containing the SRY gene (i.e., SRY gene plus additional gene/ partial gene sequence). In either case, there is no development of Sertoli cells (Fig. 1) and no production of testosterone. The resulting embryo will be phenotypically female with bilateral streak gonads (infertile), but is genetically male. Thus, most of these patients present clinically as "young females" with delayed puberty/amenorrhea (Ostrer, 2019).

Presently, there are several theories regarding the development of polyorchidism. The most widely accepted theory suggests that during development, aspects of mesonephric sections accountable for development of the gonads undergo degeneration or duplication (Yamamoto et al., 2015; Lawrentschuk et al., 2013). When incomplete division occurs, incomplete polyorchidism (i.e., bilobed testicle) is the consequence (Bergholz and Wenke, 2009). *Thus, the question arises, "What molecular or genetic mechanism(s) play roles in the genesis of polyorchids?"*

Is it reasonable to suggest that cadherins (i.e., adhesion proteins) may be involved in polyorchidism? Cadherins are a superfamily of approximately 123 cell adhesion proteins that create linkages to adjacent cells through

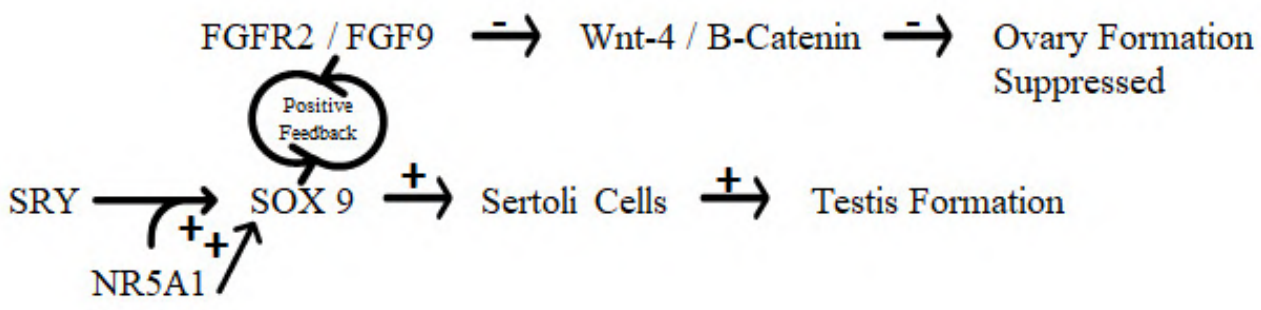

Fig. 1.- Genetic Factors Involved in Testes Development. The Sex-Determining Region Y (SRY) gene encodes for the transcription factor that activates SRY-BOX Transcription Factor 9 (SOX 9). SOX 9 is responsible for the differentiation of the Sertoli cells. Sertoli cells are required for testis formation and spermatogenesis. Nuclear Receptor Subfamily 5 Group A Member 1 (NR5A1) is necessary for functional maturation of Sertoli cells (Kato et al., 2012). The SOX 9 and the positive feedback loop for Fibroblast Growth Factor 9 (FGF9)/ Fibroblast Growth Factor 9 (FGFR2) not only stimulates Sertoli cells but also inhibits β-catenin and Wingless-Type MMTV Integration Site Family Member 4 (WNT-4) which, if uninhibited, would lead to the formation of ovaries. [Abbreviations: Increased expression (+); Decreased expression (-); Sex-Determining Region Y (SRY); SRY-BOX Transcription Factor 9 (SOX 9); Nuclear Receptor Subfamily 5 Group A Member 1 (NR5A1); Fibroblast Growth Factor 9 (FGF9); Fibroblast Growth Factor Receptor 2 (FGFR2); Wingless-Type MMTV Integration Site Family Member 4 (WNT-4)].

extracellular domains (Maître and Heisenberg, 2013). Originally discovered by Masatoshi Takeichi in 1977 (Takeichi, 1977) while studying calcium-dependent cell adhesion in hamster lung tissue samples, cadherins are classified into distinct groups (Table 1). The most studied cadherins are Type I, or classical cadherins, including epithelial-cadherin (E-cadherin, E-Cad) and neural-cadherin (N-cadherin, N-Cad) (Piprek et al., 2019a, 2019b; Piprek et at., 2020).

To achieve cell-to-cell binding, the extracellular regions of cadherin molecules composed of five or six domains (Fig. 2A) connect to opposing domains of cadherin on adjacent cells (Fig. 2B). These extracellular domains are linked to an intracellular domain by a transmembrane region. The intracellular (or cytoplasmic) domain interacts with intracellular p120, β-catenin, α-catenin (Shapiro and Weis, 2009). Then, α-catenin binds to F-actin filaments (Shapiro and Weis, 2009).

Two first extracellular domains (EC1) from either cell come together and attach through homophilic binding, with the recognition and selectivity of the opposing cadherin mediated by the Histidine-Alanine-Valine motif (Piprek et al., 2020). The binding takes place primarily through two interactions (Fig. 2). EC1s of both cadherins interact with conserved tryptophan residues and into conserved hydrophobic pockets of the opposing cadherin in a *trans* configuration, which are then further stabilized when docked. It is proposed that the *trans* configuration is characterized by creating a "zipper confirmation" bringing two cells together through the *trans* attachment of the cadherins. This zipper causes an antagonistic tension to the surface tension of the cell, causing easier cell-cell contact (Maître and Heisenberg, 2013). Additionally, the second extracellular domain (EC2) asymmetrically attaches to EC1 of a lateral partner cadherin through a *cis* configuration, allowing further stabilizing a "cluster" or bundle of cadherins for

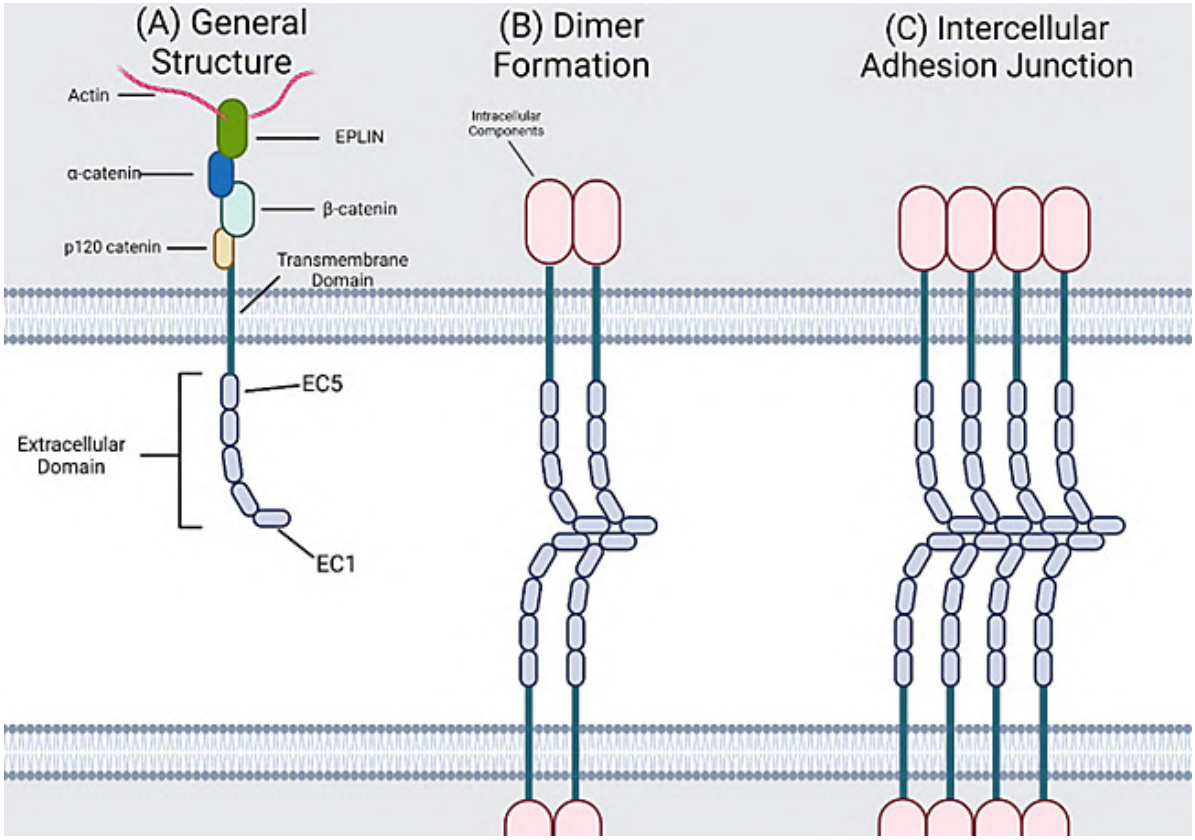

Fig. 2.- General Structure and Interactions of Cadherins. **(A)** General structure of the cadherin protein showing extracellular repeats 1-5 (EC1-EC5) with p120 catenin and beta catenin bound to the intracellular domain, α-catenin bound to β-catenin, and EPLIN facilitating connection between alpha catenin and actin. **(B)** the formation of a cadherin dimer showing to laterally adjacent cadherins interacting with each other and two similar cadherins on opposing molecules in cis conformation. **(C)** Eight cadherins representing a cluster of cadherin complexes forming strong AJs. [Abbreviations: Extracellular repeat (EC); Epithelial Protein Lost In Neoplasm (EPLIN)].

Table 1. Classification of Cadherins, Origins and Associated Systems/Tissues*.

Classification/ Grouping	Cadherin	Origin of Discovery	Associated Tissue
Classical Cadherins			
Type I	E-Cadherin (CDH1)	V79 hamster lung cell line	Various epithelia: lung, immune, salivary, liver, gallbladder, gastrointestinal, endocrine (excluding adrenal), bladder, and reproductive (male & female, excluding ovary)
	N-Cadherin (CDH2)	Neuronal Cells	Neurons (central & peripheral nervous system), cardiac (intercalated discs), thymic epithelium, salivary gland (myoepithelial cells), liver (hepatocytes), gastrointestinal (stomach parietal cells), pituitary (pars intermedia, anterior/posterior lobe), pancreas (islet cells), kidney (proximal tubule), testes (seminiferous epithelium), mammary gland, ovary, oviduct
Type II	P-Cadherin (CDH3)	Mouse embryo, extra-embryonic ectoderm	Placenta, uterine decidua
	R-Cadherin (CDH4)	Chicken retina	Neurons and glial cells
	M-Cadherin (CDH5)	Myogenic mouse cells	Muscle (myoblasts and myotubules)
	T-Cadherin (CDH13)	White adipose tissue	Adipose tissue
	Cadherin 12/BR-Cadherin	Neuronal Cells	Neurons
Desmosomal Cadherins	Desmogliens 1-3	Epidermis	Integument; desmosome containing tissues
	Desmocollins 1-3	Variable tissue origin	Complex stratified tissues
Protocadherins	Pcdh1, Pcdh-2, Pcdh3	Mouse nerve tissue	Neural tissues; neuroblastoma cell lines
ST-Cadherin	HPT-1	Gastrointestinal tract	Liver and intestine; pancreas

*(Amagai and Stanley, 2012; Bornemann and Schmalbruch, 1994; Delva et al., 2009; Gao et al., 2021; Inuzuka et al., 1991; Nose et al., 1986; Pancho et al., 2020; Raya-Sandino et al., 2021; Takeichi, 1977; Yamauchi et al., 2014)

the adhesion process (Fig. 2C) (Harrison et al., 2011). These cadherins contribute to several processes within development, ranging from cell and tissue formation, cancer cell development, as well as helping various signaling pathways (Piprek et al., 2019a, 2019b; Piprek et at., 2020).

E-Cads direct Primordial Germ Cells (PGCs) as they move to the gonadal ridge under controlled expression. Once these cells reach their destination at the gonadal ridge, E-Cad and N-Cad interact heterophillically from the PGCs and the surrounding somatic cells, respectively. In the gonadal ridge, coelomic epithelial cells will undergo the process of epithelial-to-mesenchymal transition (EMT), during which they reduce cadherin expression in favor of less adhesion and promotion of migration (Piprek et al., 2019a, 2019b; Piprek et at., 2020).

The purpose of the present research is to investigate the mechanism underlying the role(s) of cadherins and catenins in the development of polyorchidism using genomic analysis of a cadaveric polyorchid.

MATERIALS AND METHODS

Cadaveric Material

With the consent given from the Anatomical Gift Association of Illinois, the study was conducted on a 96-year-old male cadaver at the Advanced Human Cadaver Laboratory at North Park University (Chicago, Illinois, USA). The anatomical donor was embalmed on October 18, 2019, and prosection began on August 27, 2020. Medical history and hospital records were unavailable. Throughout the study, procedure for care and use were performed in accordance with both state and federal guidelines.

Tissue Preparation

STs and SNTs were excised from the cadaver and placed into trays which were then moved to a labeled tissue cassette. As previously described in Talarico et al. (Talarico et al., 2022), increments of 5 x 5 mm, sagittal sections of the left and right ST and SNTs were obtained using a No. 22 scalpel blade. The samples collected were placed into containers with Formal Fixx (*Thermo Scientific Shandon,* Hampton, NH, USA). After a 24-hour period, each sample was then immersed in a container with 70% EtOH for 72 hours. After, each sample was paraffin embedded, sectioned, and processed at the University of Chicago Molecular Diagnostic Laboratory Center (*University of Chicago*, Chicago, IL, USA), where DNA extraction took place.

DNA Extraction

Formyl Fix Paraffin Embedded (FFPE) samples were prepared using the Accel-NGS 2S Plus DNA Library Kit for whole exome sequencing (*Integrated DNA Technology*, Coralville, IA) according to the manufacturer's protocol. The DNA concentration used to establish the library was a minimum of 500 ng.

Whole Genome Sequencing

Paired end sequencing was carried out using the Illumina NovaSeq 6000 system (*Illumina*, San Diego, CA, USA) using 150 bp reads to an average depth of coverage of 44x (i.e., there were 44 "reads" for each base). Sequencing data was converted into FASTQ format and demultiplexed using the bcl2fastq software from Illumina. Paired end reads were aligned to the GRCh37/hg19 reference genome using the Burrows Wheeler Alignment algorithm (Li, 2013). BAM files were processed and variants called using GATk's Haplotype Caller best practices pipeline, including duplicate reading marking and base quality score recalibration (DePristo et al., 2011).

RESULTS

General Summary

Sequence analysis of the SNT shows two missense mutations that result in single nucleotide variants (SNV) within exons of the N-Cadherin gene (CHD2). A mutation in the Desmocollin 2 (DSC2) gene was also demonstrated. All identified SNVs were translated into amino acid substitutions in the polypeptide sequence.

Genomic Analysis

Thymine to Cytosine

One mutation in the SNT genome occurs at Chr18(GRCh37), exon: 1, NT: 25756964, or NM_001792.4, NT 23, thymine (T) to cytosine

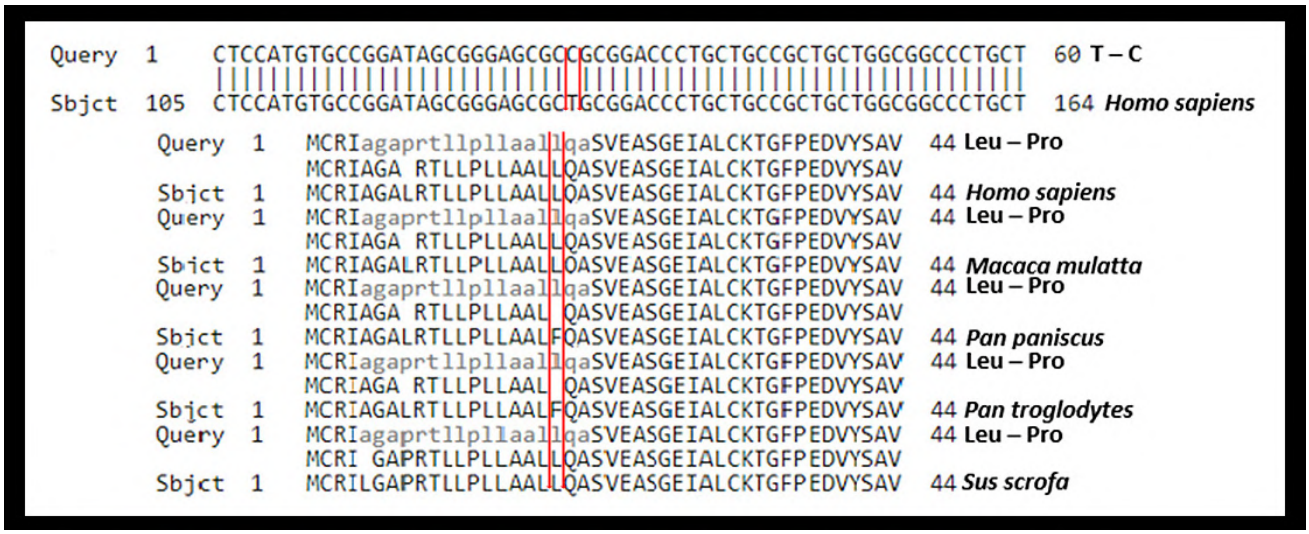

Fig. 3.- Thymine to Cytosine Substitution in the SNT Genome of N-Cadherin. BLAST alignments for SNV NM_001792.4 NT 23 T to C, against Human (homo sapiens: NM_001792.4) as well as AA translation Leu – Pro, against Human (Homo sapiens: AAH36470.1), Rhesus monkey (Macaca mulatta: XM_028838055.1), Bonobo (Pan paniscus: XM_034943719.1), Chimpanzee (Pan troglodytes: XM_523898.6), and Pig (Sus scrofa: XP_020951864.1).

(C) (Walsh et al., 2022a). When translated this nucleotide substitution results in the replacement of leucine (Leu) 8 to proline (Pro) (NP_001783.2) (Walsh et al., 2022a). This data is shown in Fig. 3.

The normal nucleotide sequence for CDH2 in humans against the sequence with the identified SNV, T 23 to C is shown in Fig. 3. Further, the translated AA sequence for Leu-Pro against multiple species N-Cad sequences are compared showing conservation of the normal sequence in all but the sequence for Pig (*Sus scrofa*), which matched the Leu-Pro AA substitution. Blasting of the sequence in the NCBI Database also showed an additional substitution in the sequences for *Pan paniscus* (Bonobo) and *Pan troglodytes* (Chimpanzee), Leu 19 to Phenylalanine (Phe).

Asparagine to Serine

The second mutation found in CDH2 occurs at NM_001308176.1, exon 16, NT 2441, dbSNP: rs2289664 A to G (Walsh et al., 2022b). Translation of this SNV results in the replacement of asparagine (Asn) 845 to serine (Ser) (NP_001295105.1) (Walsh et al., 2022b) (Fig. 4). The alignment of the translation product compared against known species sequences is shown in Fig. 5.

Fig. 4 shows the normal nucleotide sequence against the sequence of the identified SNV from full exome sequencing data compared to multiple species. Conservation of the normal nucleotide sequence is seen differing from the SNV 2441 A to G. Also present is the variation of Bonobo and Chimpanzee with a substation of SNV 852 C to G.

The translated result of the identified SNV resulting in Ser replacing Asn at position 845 (AAH36470.1) is shown in Fig. 5. This substitution is observed to occur in the cytoplasmic region of N-Cad which as shown in Fig. 5 is a conserved sequence across multiple similar species. Shown in the AA translation, the previously indicated variation in Bonobo and Chimpanzee for the NT sequence is synonymous and results in the same AA in the polypeptide.

Desmocollin 2 (DSC2)

Genomic sequencing of the specimen also identified a mutation in DSC2. This mutation occurs at NM_004949.3, Exon: 15, NT: 2393, G to A, dbSNP: rs61731921 (Walsh et al., 2022c). [Sequence alignment is not shown.]

DISCUSSION

Overall Summary of Results

In previous work done in this laboratory, a unique case of tetraorchidism suggested a novel classification of polyorchidism and demonstrated SNTs could be more prevalent due to misdiagnosis as lipomas (Talarico et al., 2022). The purpose of this study was to explore a potential link between cadherins and polyorchidism, because the mechanism underlying the development of polyorchidism is unclear (Bergholz and Wenke, 2009; Artul and Habib, 2014; Cohen et al., 2017). Through full exome sequencing of the SNT, this research discovered two missense mutations within the CDH2 gene (Leu 8 to Pro and Asn 845 to Ser). Since the

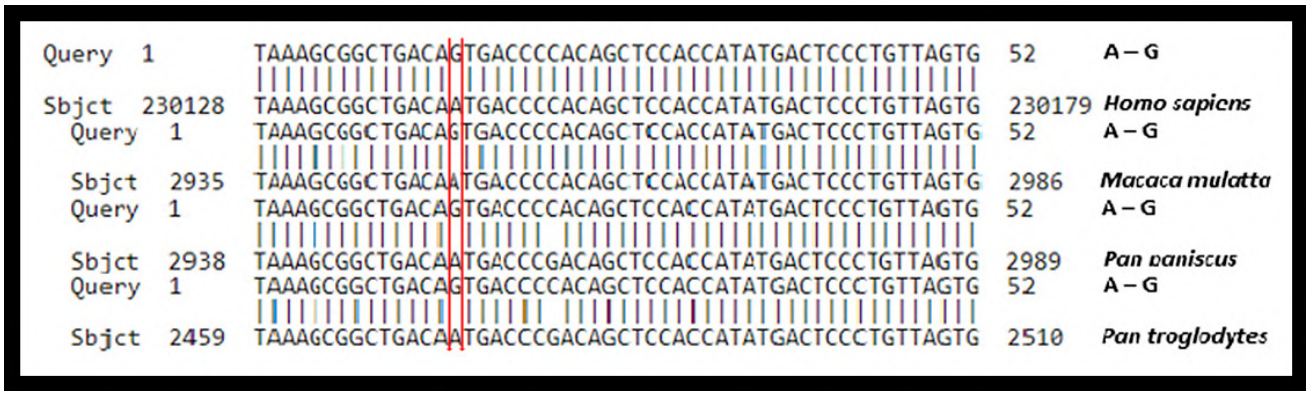

Fig. 4.- Adenine to Guanine Mutation in N-Cadherin of SNT. BLAST alignments for NM_001308176.1 2441 A to G, against Human (Homo sapiens: NG_011959.1, Rhesus monkey (Macaca mulatta: XM_028838057.1, Bonobo (Pan paniscus: XM_034943719.1, and Chimpanzee (Pan troglodytes: XM_016933484.2).

Leu 8 to Pro mutation occurs in the prodomain region of N-Cad, the prodomain region is cleaved off of the cadherin molecule; resulting in the activation of the final product. Due to the prodomains cleavage, it is not present in the final product of the functional protein that is inserted into the cell membrane (Latefi et al., 2009). This mutation does not likely play a role in polyorchidism and will not be addressed. The Asn 845 to Ser mutation occurs in the cytosolic portion of the N-Cad, which could cause improper binding to the p120 catenin. These findings present a *novel* model for the development of polyorchidism building on the gonadal ridge theory. Finally, the current study provides support for the association between cryptorchidism, polyorchidism and cancer.

N-Cadherin and p120 Catenin

As previously stated, full exome sequencing of cadaveric SNT tissue identified two mutations in the CDH2 gene. The first of the N-Cad mutations (Leu-8 > Pro) occurs in the prodomain of the cadherin protein, which is cleaved off during protein maturation in the late Golgi Apparatus (Latefi et al., 2009). Based on this, it is reasonable to suggest that there is little functional change given its absence in the final functional protein structure, as well as its distance from the cleavage site, given the location of the mutation at Leu 8 of the ~130 AA prodomain (Koch et al., 1993).

The second N-Cad mutation (Asn-845 > Ser) is located in the cytosolic domain of the cadherin protein that is a highly conserved sequence of the cadherin protein family (Kister et al., 2001). Specifically, the region referred to as the juxtamembrane domain (JMD). This region of N-Cad and other classical cadherins is responsible for the binding of p120 catenin (Fig. 2) (Kourtidis et al., 2013).

p120 catenin is a member of the catenin family of armadillo repeat (conserved alpha helix repeats) containing proteins that have multiple regulatory functions in Rho GTPase function, nuclear signaling, and regulation/maturation of adhesion junctions (AJs) (Kourtidis et al., 2013). The p120 gene contains 4 alternatively spliced exons and 4 different transcriptional start sites that can produce 64 different isoforms of the p120 protein. Loss of functional p120 can have mild-to-significant effects based on affected tissue types (Hernández-Martínez et al., 2019). p120 is also highly regulated by multiple serine, threonine, and tyrosine sites that can be phosphorylated (Kourtidis et al., 2013; Fukumoto, 2007). Specific functions of each isoform have not been well fully investigated. However, there is a known change in the ratio of p120 isoform expression during EMT (Kourtidis et al., 2013; Zhang et al., 2014).

Celomic epithelial cells of the gonadal ridge proliferate and undergo EMT resulting in the

Fig. 5.- Translation of A to G Mutation in N-Cadherin Changes Asparagine to Serine. BLAST alignment for amino acid sequence of SNV NM_001308176.1 NT 2441 A to G, against Human (Homo sapiens: NP_001295105.1), Rhesus Monkey (Macaca mulatta: XP_014977198.1), Chimpanzee (Pan troglodytes: XP_0716788973.1), Bonobo (Pan paniscus: XP_034799610.1), and pig (Sus scrofa: XP_020951864.1).

formation of the gonadal ridge (Piprek et al., 2020). During this time, alternate expression of p120 catenin results in reduced adhesion in favor of cell motility (Acloque et al., 2009). Based on the mutation (Asn 845 > Ser) found in N-Cad in the present study, it is reasonable to suggest that the potential for p120 to decouple from N-Cad (Fig. 6) results in reduced clustering activity and potential endocytosis yielding a reduction of available N-Cad in the cell membrane. Impairment of cellular adhesion at this stage of gonad development could initiate the detachment between the proliferating somatic cells of the gonadal ridge as they encapsulate migrating PGCs. Therefore, it is reasonable to suggest that reduced adhesion of somatic cells at this stage of gonadal development could result in aberrant division of the gonadal ridge yielding SNTs. Another potential outcome would be the incomplete separation of the gonadal ridge resulting in partial separate development and formation of a bilobed testicle or incomplete polyorchidism (Hekmatnia et al., 2016).

Support of the Proposed Model from Studies on Neoplasms

It has been shown that during EMT, there is a decrease in E-Cad expression. The effects of this are weakened cell AJs which allow for detachment and migration of individual cells. Also seen during EMT is an increase in N-Cad expression (Saénz-de-Santa-María et al., 2020). “N-cadherin expression, which is thought to contribute to a stroma-oriented cellular adhesion profile leading to more motile, invasive and metastatic cell phenotypes.” In cancer, EMT is pivotal to supporting metastasis, chemoresistance, and tumor stemness (i.e., tumor cell ability to proliferate and differentiate) (Loh et al., 2019). Furthermore, elevated levels of soluble N-Cad have been found in the serum of cancer patients, specifically those with cancers of the prostate, breast, or urinary bladder (Cao et al., 2019).

As discussed by Cao et al. (2019), breast cancer, pancreatic cancer, melanoma, multiple myeloma, lung cancer, prostate cancer and squamous cell carcinoma have all been found in connection

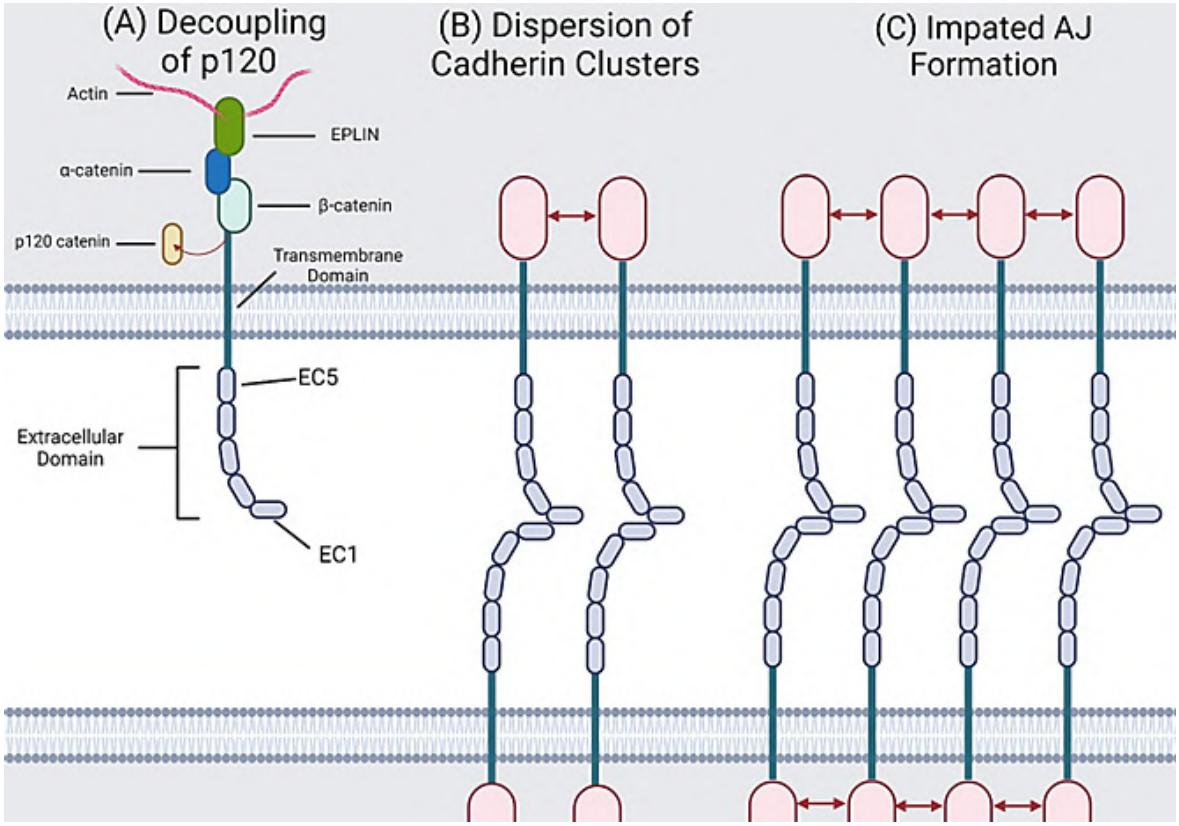

Fig. 6.- Suggested Mechanism for Cadherin in the Genesis of Polyorchidism. **(A)** Decoupling of p120 catenin from adhesion complex due to N-Cad mutation in JMD. **(B)** shows separation of adjacent cadherin dimers due to lack of clustering activity normally facilitated by p120 catenin. **(C)** shows how lack of clustering activity fails to allow the formation of clustered AJs. [Abbreviations: Extracellular Repeat (EC); Epithelial Protein Lost In Neoplasm (EPLIN); Adhesion Junction (AJ); Juxtamembrane domain (JMD)].

to increased expression of N-Cad. When N-Cad expression is increased, collective migration occurs in tumor cells. In contrast, deletion of the intracellular domain, or just the β-catenin binding domain of N-Cad will result in higher incidences of individual migration, or cancer cells detaching and migrating from their cell clusters (Mrozik et al., 2018). β-catenin is one component of the N-Cad protein complex, connecting the transmembrane portion to the actin cytoskeleton. It is plausible that the missense mutation discovered in the present study occurs within the same region of the protein complex, resulting in a similar disruption as deletion of β-catenin.

The *novel* model presented here suggests a relationship between the development of polyorchidism and cancer metastasis based on two criteria. The first supportive evidence comes from the genomic sequencing of a cadaveric SNT that showed a mutation in the cytoplasmic domain of N-Cad. This mutation was located at a point within the sequence where the N-Cad binds to p120 catenin. Because "membrane expression and lateral clustering of N-Cad is dependent upon p120 catenin" (Mrozik et al., 2018), it is reasonable to suggest that the mutation identified in the present work might impact how efficiently N-Cad can provide intercellular adhesion (Fig. 6 and Fig. 7). This investigation suggests that mutant N-Cad affects the binding of p120 (Fig. 6) resulting in abnormal separation of the gonadal ridge and expression of the polyorchid phenotype (Fig. 7). Therefore, using the same reasoning this *novel* mechanism may promote a similar detachment of cells in tumors, resulting in neoplastic migration.

The second supporting evidence for this model is that polyorchids have a higher risk for testicular cancer (Nurfajri et al., 2021; Talarico et al., 2018; Talarico et al., 2022). Prior research done by Mrozik (Mrozik et al., 2018) also reports that aberrant expression of N-Cad is a "well-documented feature of epithelial malignancies". Epithelial cells in the testes, also known as germinal epithelium, exist lining the walls of the seminiferous tubules. One of the many structures that N-Cad is responsible for is the arrangement of the seminiferous epithelium (Piprek et al., 2020). Thus, a loss of adhesive capabilities due to a cytoplasmic mutation of N-Cad in the germinal epithelium could be linked to both testicular cancer and polyorchidism.

Significance of the Current Work

N-Cad has been known to promote cell survival, migration, and invasion relative to progression of neoplasms (Yu et al., 2019). Further, it has been

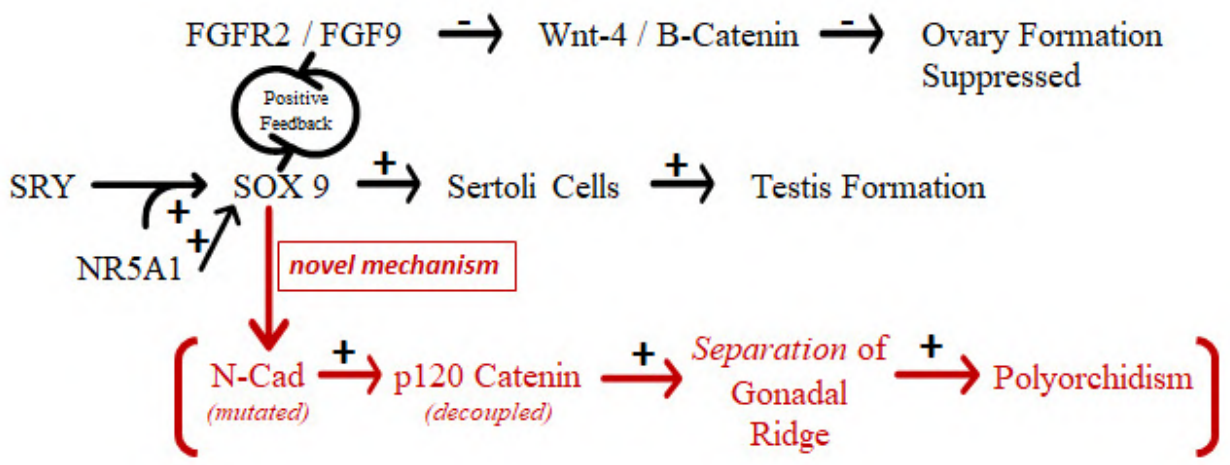

Fig. 7.- Suggested Development of Polyorchidism in Association with Mutated N-Cadherin. The traditional pathways for gonadal development are shown in "black" (also see Fig. 1). In the novel mechanism proposed in this investigation (shown in "dark red"), a mutation in N-Cad can lead to decoupling of p120 catenin causing migration of cells from the gonadal ridge to another location, thus resulting in polyorchidism. [Abbreviations: Increased expression (+); Decreased expression (-); Sex-Determining Region Y (SRY); SRY-BOX Transcription Factor 9 (SOX 9); Nuclear Receptor Subfamily 5 Group A Member 1 (NR5A1); Fibroblast Growth Factor 9 (FGF9); Fibroblast Growth Factor Receptor 2 (FGFR2); Wingless-Type MMTV Integration Site Family Member 4 (WNT-4); N-Cadherin (N-Cad)].

shown that elevated (i.e., expressed) levels of N-Cad from neoplasms are associated with poor prognosis (Mariotti et al., 2007). In prior research done by Mrozik et al. (2018), over-expression of an N-Cad mutant, where the extracellular domain was fused to the anti-binding domain of α-catenin impeded the movement of follower cells. This demonstrates that N-Cad-actin linkage is vital for efficient, collective cell migration. In the novel mechanism presented, a mutation for N-Cad could have the same result. In the gonadal ridge, coelomic epithelial cells undergo EMT where AJ expression is reduced in favor of greater motility. With the mutant N-Cad discovered in this work, impaired binding of p120 catenin likely results in the reduction of AJ binding strength. Thus, it is reasonable to suggest that a decrease in N-Cad clustering fosters an environment for decreased AJ binding strength. This can lead to increased motility of individual cells of the gonadal ridge, resulting in aberrant division and the development of polyorchidism. This same mechanism could apply in some instances of metastatic neoplasms.

Future Work

Genomic analysis of a SNT lead to the discovery of a *novel* N-Cad mutation. Using this N-Cad mutation and known factors involved in testicular genesis, a new molecular model was suggested for the development of polyorchidism. This work lays the foundation for future studies at the molecular and genetic level to further refine all factors and their mechanistic roles in the genesis of this pathology. As suggested by the current work, future studies may also show the application to oncologic models, as well as pathologies in other organ systems.

Additional Findings

An additional finding from genomic sequencing of the male polyorchid in the present work identified a mutation in DCS2. This mutation is known to be associated with arrhythmogenic right ventricular cardiomyopathy, type II. The cause of death of the polyorchid was charted as cardiomyopathy. A survey of the scientific literature did not document any known association between polyorchidism and this mutation in DCS2.

Limitations

The research was conducted on a postmortem, 96-year-old male anatomical donor. Access to the comprehensive medical, social and occupational histories of the donor were not available. It is unknown if the donor was aware of the condition, if he had a family history of polyorchidism or reproductive challenges. Further, secondary to embalming solutions used in this donor and the time from initial embalming, DNA extraction from the STs did not yield a library for analysis. However, the NCBI database allowed the utilization of the BLAST with known, unaltered genomic sequence for unaffected human and animal testes. Lastly, because of the limited number of polyorchids, this study represents the genomic analysis of only one subject.

CONCLUSION

The etiology of polyorchidism is currently unknown, however, the present work establishes a plausible genetic theory based on N-Cad and p120 catenin. This work may apply to the development of supernumerary organs in other systems and in the development and metastasis of neoplasms. Finally, this work establishes a strong foundation for further exploration into the molecular mechanics of cell adhesion as it relates to findings in gross anatomy.

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