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An Examination of the Mechanisms and Treatments of Ciliopathies Triggered by Genes dlg5 and rnf20

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ABSTRACT

In the current society, ciliopathies continue, without fail, to impact a small, but existing portion of the public; classified as a genetic birth defect from mutations in ciliary genes, ciliopathies highly affect the younger generations, indicating an urgency for focus on progressive research in the field. Due to the vast number of possible genetic mutations, this paper centers on a select set of genetic mutations, dlg5 and rnf20, due to the varied amount of mutations that may be cause damage and the variety organs in the body that are impacted when the genes are mutated. The varied characteristics of each gene contribute to mutations developing a specific phenotype, depending on the ciliary aspect and tissue affected; this paper is sought to highlight the increased variability of ciliopathies. Current ciliopathy treatment methods are also investigated, identifying gaps existent and successes, focusing upon genetic therapy advances. Treatment methods are applied to dlg5 and rnf20, as the author proposes effective modes through which increased focus should be applied to devise treatments, in consideration with drawbacks that had been evident which finding treatments for general ciliopathies.

Introduction

Left Right (LR) patterning exhibits an essential significance on the embryonic development of the majority of bilateral invertebrates and vertebrates (Zhang et al., 2020). Normal function of asymmetric organs depends on the accurate positioning of the organs in correspondence with the LR axis. Cilia -cellular vibrating, hair-like structures that sense and generate signals in order to result in tissue homeostasis as well as proper development- generate a leftward flow through the beating of motile cilia to suppress dand5, a nodal antagonist, and signaling of primary cilia (Zhang et al., 2020). The two types of cilia working in combination develop proper LR asymmetry.

Genetic defects result in malformations of LR organization in the form of congenital ciliary birth defects. Out of the 1 in 33 babies born with birth defects in the United States every year, 1 in 10,000 to 20,000 present primarily ciliary dyskinesia (PCD) or ciliopathies (2-3). Unfortunately, according to a recent retrospective study of 151 PCD adults with 35 years as the median age followed for 7 years, all cause mortality was around 5% (Zhang et al., 2020). Therefore, in the severity of symptoms in patients, the topic has been increasingly studied; a variety of aspects of phenotypes have been presented depending on the genetic mutation expressed in the patient. Reflected phenotypes of PCD (in this review) include situs inversus totalis (SIT) as well as heterotaxy (Htx). A congenital condition where the organs in the chest and abdomen are mirrored from normal positions, SIT holds a prevalence in a range of from 1/25,000 to 1/8000 (Zhang et al., 2020). Contrasting to SIT, Htx presents with abnormally arranged internal organs -not in a mirror image- with congenital heart disease observed in 80% of these patients. At a broader scale and as a subgroup of PCD, Kartagener syndrome (KS) is a trilogy of symptoms (nasal polyps, bronchiectasis, and SIT) present in 20% of SIT patients; hydrocephalus is a condition in which the buildup of cerebrospinal fluid enlarges the brain, affecting 2 out of 1,000

births in the United States every year. In a similar fashion, APKD -progressive expansion of the renal cysts- is a renal or kidney related PCD condition, demonstrating PCD's variability in the organs affected.

The phenotypes presented are determined by the target of the genetic mutations affecting the individual. When genetic mutations induce depletion/abnormalities of the scaffold protein dlg5, laterality defects have been identified. Genetic mutations altering dlg5 have modified ciliary structure, resulting in ciliopathies circulating brain and kidney ventricle morphology. Likewise, the histone marker H2Bub1 has presented embryological heart looping abnormalities, during occurrence of the rnf20 complex mutation. Though there are many candidate genes for ciliopathies, the following were analyzed to demonstrate the wide range of genes holding a ciliary relationship.

The following paper highlights the role of genetic mutations in impacting the ciliary scaffold protein dlg5 and protein complex rnf20 to the development of ciliopathies. Multiple ciliary genes are reflected on to demonstrate the variability in candidate genes, inducing a difficulty in identifying a treatment for all ciliopathies. However, the observed knowledge, as focused upon in this paper, provides the scientific community with additional ciliary study in an effort to narrowing their study in being able to target specific genes, allowing for the opportunity for gene therapy such as CRISPR-Cas9 genome editing technology.

Within the paper, all sections of proteins, kinases, etc, are divided into subsections. The subsections begin with background knowledge on the candidate gene as well any unique characteristics pertaining to it. Its phenotypic impact, impact on cilia, and other gene specific impacts developed including on aspects such as cellular pathways are next discussed. Depending on the experimentation able to be run, results of tests conducted to see how the different components of the focused protein complex interact are also included in some sections. The discussion portion of the paper centralizes on comparisons between the varied candidate genes reflected and content related opinions on the articles reviewed. The paper will end with current event relations and future steps as presented in the conclusion.

Basic Nature of Cilia

Introduction to Cilia

The first accounts of cilia had been discovered, as early as 1676, by Leeuwenhoek, due to observations of ciliary motility (Satir, 2017). Through the years, further observations identified the 2 divisions of cilia including primary and motile cilia. Both ciliary groups consist of a foundation known as the basal body, built by triplet microtubules. Further up from the basal body, the axoneme contains 9 pairs of outer microtubules (9+0 axoneme), as well 2 central microtubules (9+2 axoneme), only in motile cilia. The motility of motile cilia allows for its ability in select functions including the repression of negative regulators, which further aid in working alongside primary cilia, to allow the activation and flow of ciliary relevant processes. With a variety of ciliary characteristics in consideration, it is significant to analyze the major impact that occurs in an organism when cilia are affected, as provided in this paper.

Ciliopathies and dlg5

Characteristics of Presented Study and dlg5

Experiments have observed the ciliopathy gene, dlg5. Dlg5 or discs large 5 is part of the membrane-associated guanylate kinase family. It regulates Hippo, sonic Hedgehog (Shh), and transforming growth factor through (TGF-B) signaling (Marquez et al., 2020). Depletion of dlg5 leads to many patient phenotypes including congenital anomalies of the kidney and urinary tract (CAKUT). The common phenotype across all patients was

found to be ciliary loss in a tissue specific manner. This supported that dlg5 was in fact a ciliopathy gene with numerous phenotypic responses, as it can be inherited through de novo, heterozygous, homozygous, and compound heterozygous variants. The gene, dlg5, holds significance in orchestrating ciliary function that heads many phenotypes and actions including kidney/brain structure, Shh signaling, and cilia polarization. Without dlg5, many functions that happen in providing homeostasis wouldn't occur.

Characteristics of Presented Study and rnf20

Genetic variants affect ciliary function resulting in a chain of reactions that present phenotypes. The observed, identified variants impact the genes: rnf20, rnf40, ube2b, developing primarily CHD (Robson et al., 2019). All listed genes are involved in chromatin modification or remodeling termed histone 2B monoubiquitination. Histone 2B monoubiquitination conjugates histone and lysine through the utilization of the protein, ubiquitin - consisting of 3 enzymes (Ubiquitin activating enzyme, Ubiquitin conjugating enzyme, Ubiquitin ligase). This creates the upstream transcriptional regulator, H2Bub1 which functions in controlling the tissue specific expression of cilia genes (Robson et al., 2019). The rnf 20/40 complex mediates H2Bub1, meaning mutations affecting rnf20/40 result in decreased/abnormal expression of H2Bub1 (Figure 1). Rfx3 presents as a ciliary protein coding gene that regulates transcriptionally regulating cilia genes. Since H2Bub1 triggers rfx3 transcription, depletion of it negatively impacts Rfx3. This leads to abnormal cilia motility and the phenotype of incorrect cardiac left right patterning is presented. A 'domino effect' of impacts, beginning from the rnf20 genetic mutation is observed; this effect is highlighted in Figure 1, showcasing the impact of rnf20 on H2Bub1, to result in impact on the body (Figure 1).

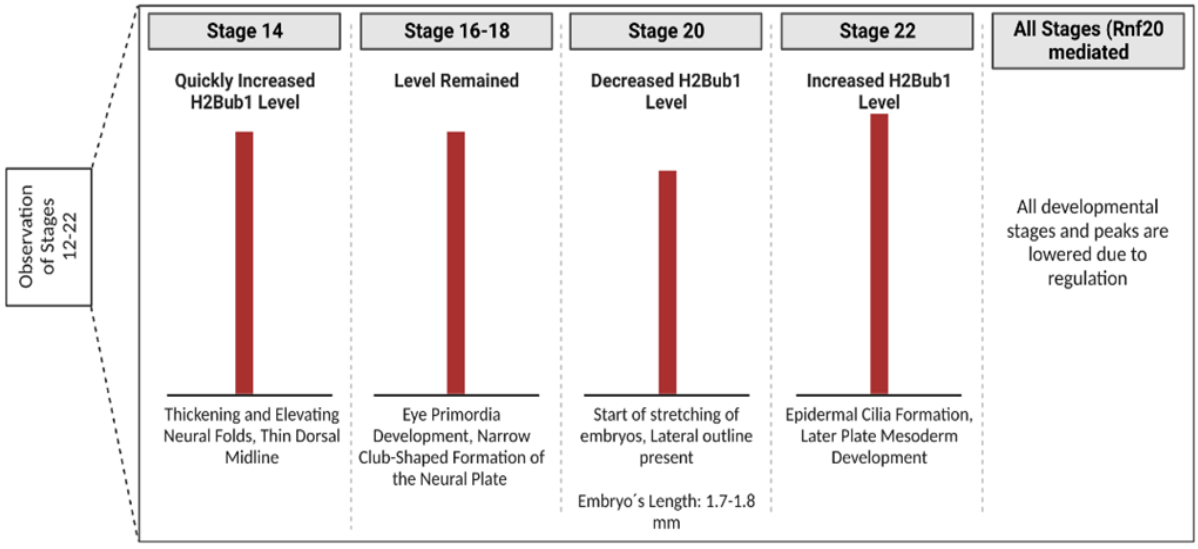

Figure 1. H2Bub1 levels during stages 12-22 with and without rnf20 mediation. Supports ideology of rnf20 mediating H2Bub1, due to decreased levels during rnf20 insertion.

Patient Phenotypes

During a study of dlg5 mutations, specific patients were selected. The proband or person who serves as a starting point for a family genetic study was a 21 week gestational male fetus with skeletal abnormalities as well as other cardiac and kidney phenotypes present (Marquez et al., 2020). The fetus had a de novo variant (variant that is present for the1st time in a family) in the gene dlg5. The 4 examined patients from 3 unrelated families identified as having different variants of dlg5. Patient 1 (father) and 2 (son) were heterozygous for the novel variant and presented similar symptoms such as ureteropelvic junction (Marquez et al., 2020). Patient 3 (female) had heterozygous dominant variant and had progressed to stage 3 chronic disease and showed slightly dilated cerebral ventricles as well. Patient 4 -had their last follow up at 7 months of age- and showed hydrocephalus as well as coarse facial features. The wide range of patients, phenotypes, and variant types highlight the numerous possibilities and effects of dlg5 mutations (Marquez et al., 2020).

In regards to rnf20 mutations, CHD presents as a major consequence (Robson et al., 2019). In a case of 3 CHD patients, monoallelic mutations (rnf20 and UBE2B de novo and rnf40 inherited) had been present affecting the rnf20 core complex (Robson et al., 2019). In another scenario, all patient phenotypes with DNMs affecting H2Bub1 presented the full CHD effect (Robson et al., 2019). One specific rnf20 patient had continuous pulmonary infections, chronic respiratory failure, as well as hydrocephalus; tracheal cilia biopsy revealed absent inner dynein arms (Robson et al., 2019). This demonstrates the various versions of phenotypes present from the same general mutation.

Both rnf20 and dlg5 mutations encompassing a plethora of different phenotypes highlights the nature of ciliopathies in impacting various organs and having numerous types of mutations.

Effect on Cilia When Mutated

Effect of rnf20 on Ciliary Motility

During laterality development in the LRO, motile cilia are significant in repressing dand5 expression; dand5 is a nodal antagonist that inhibits the nodal branches of the TGFB signaling pathway (Robson et al., 2019). When analyzing the state of motile cilia, during rnf20 depletion, it is seen that there is no observed change in cilia length, number, or LRO orientation. Cilia number as well as ratio of motile/immotile cilia remained normal. However, when examining LRO cilia motility, there is a significant decrease in rotation frequency (Robson et al., 2019). Analysis of the Xenopus embryonic epidermis' MCCs that normally move extraembryonic fluid by beating coordinately, reveals normal ciliary distribution on the rnf20 morphant MCCs surface; however, impaired and "stiff " cilia motility with a limited range of motion is present in rnf20 morphants. In patients with the rnf20 variant, Xenopus epidermal cilia ultra structure often presented shortened/absent IDAs in 8/8 examined (Robson et al., 2019). This highlights that rnf20 typically promotes effective ciliary motility through the mediation of ciliary IDAs.

Lack of Cilia Polarization

Vital in happenings such as directional motile ciliary beating, cilia polarization holds much significance. The process of cilia polarization begins when a brisk bulk fluid flow is created over the embryo's surface from multiciliated cells in the Xenopus embryonic epidermis. In order to give cilia access to extraembryonic fluid, multiciliated cells originate at the epidermis' basal layer and migrate apically to insert between epithelial cells at the epidermal surface (Figure 2) (Marquez et al., 2020). However, depletion of dlg5 has been found to affect polarization of cilia within the cell. The effect of dlg5 depletion on the cell was examined through dnah9 (a marker of ciliated cell fate). When dnah9 was stained in a dlg5 MO injected side and control, the staining

of dnah9 was continuously weaker on the dlg5 MO injected side (Marquez et al., 2020). In dlg5 morphants, apical migration was unable to occur. This resulted in cells labeled for ciliated cell fate to be clustered beneath the epidermal layer in the form of secretory cells (Figure 2) (Marquez et al., 2020). This notion was supported with ciliary loss originating from the lack of multiciliated epidermal cells on the surface (Figure 2) (Marquez et al., 2020).

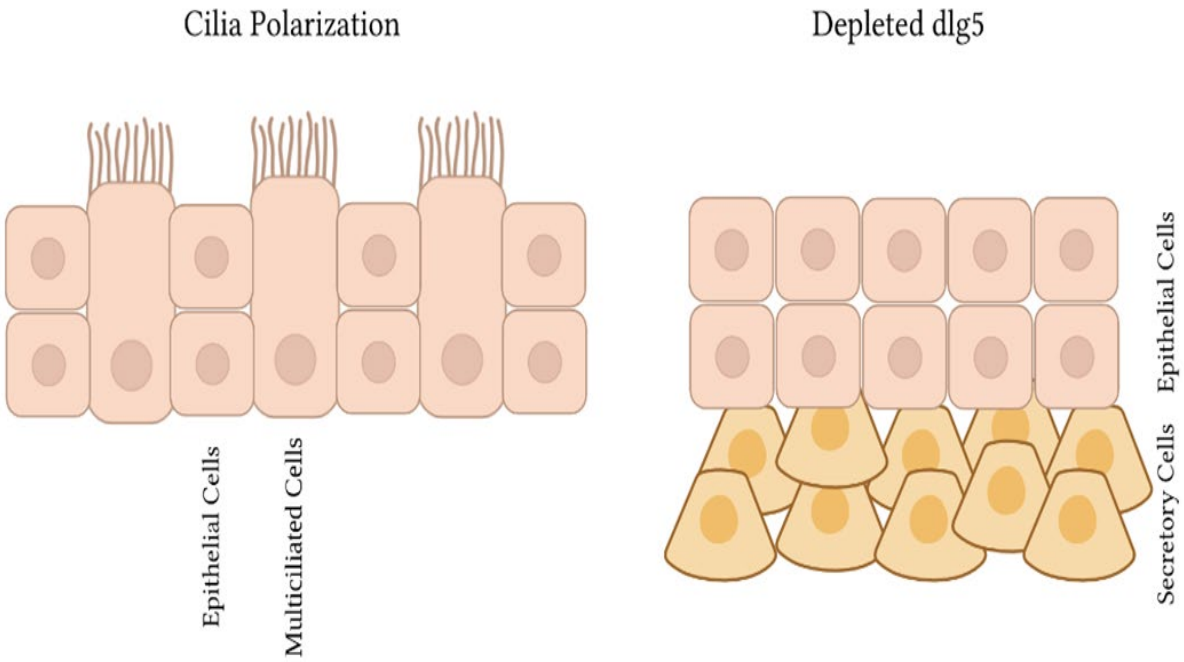

Figure 2. Demonstrates the contrast between normal ciliary polarization and the effect of dlg5 depletion. With the inability to migrate from the basal layer to the epithelial cell layer, there is a lack of cilia in the multiciliated epithelial cells. Abnormalities in the polarization results in a cluster of secretory cells below the epithelial cells.

Decreased Shh Signaling

Along with ciliary polarization, dlg5 depletion had continued to impact other specific cellular happenings. Shh signaling presented as one affected as well. Shh signaling or the sonic hedgehog signaling pathway is a network that functions in the regulation of developmental processes such as multicellular embryonic growth and patterning. Cilia perform in vertebrates as the central organelle in sonic hedgehog pathway transduction. In regards to dlg5, it has been reported that dlg5 is located at cilium bases and regulates Shh signaling, meaning dlg5 depletion negatively impacts Shh signaling which therefore affects ciliary function. The correspondence of dlg5 depletion to Shh signaling was examined through the state of foxf1 (a well established Shh target gene), condition of ptch1 expression (mirrors activity of Shh in the neural tube and brain), the expansion of pax6 (that normally expands its expression under the repression of dlg5 in the lateral margin of the neural tube) (Marquez et al., 2020).

Relevance of foxf1 to Shh signaling remained high. As a Shh target gene, foxf1 dysfunction would occur with failure of Shh signaling. Therefore, foxf1 presented itself as a readout for Shh signaling in the pronephros in correspondence to dlg5 depletion and a decrease of foxf1 was observed (Marquez et al., 2020). In control and dlg5 morphant embryos, Ptch1 expression mirrored Shh signaling in the brain ventricles and found

a ptch1 reduction when dlg5 was depleted (Marquez et al., 2020). Furthermore, as it is understood that pax6 expression expands under Shh signaling repression, the effect on pax6 under dlg5 depletion was tested (Marquez et al., 2020). With a pax6 promoter driven GFP expression, an increase of Pax6 expression was observed on the ventricular floor in a transgenic Xenopus line (Marquez et al., 2020). This then concludes that dlg5 depletion leads to decreased Shh signaling.

Effect on Body

Effect of rnf20 on LR Development

Across all vertebrates, LR patterning has been conserved; therefore Xenopus was utilized to analyze LR development during knockdown of rnf20 by injecting morpholino oligo (MO). At the one cell stage, rnf20 knockdown resulted in edema or swelling by stage 40, preventing heart looping analysis; therefore rnf20 MO was injected in one cell of the two cell embryo. By injecting only one blastomere at the two cell stage in Xenopus, it allows for scoring of cardiac looping along with rnf20 knockdown to be targeted to either the embryo's right or left side (Robson et al., 2019).

During observation period, being that the left side is crucial in ciliary left right patterning, abnormal heart loops from left sided injection suggested rnf20 functioning in LR development through LRO cilia (9% in right and 16% in left). In order to determine the specific step of LR development where rnf20 is needed, LR asymmetry markers pitx2c and dand5 were utilized. Normally expressed in the left lateral plate mesoderm, pitx2c expression presented at stage 28 during rnf20 depletion. Left sided injection has been observed with a greater amount of pitx2c expression abnormalities shown as 53% left side depletion compared with 26% right side depletion; the overall results mirror the heart looping observations, showcasing rnf20's ciliary defects impacting cardiac looping (Robson et al., 2019).

dlg5 Impact on Kidney and Brain

On a broad spectrum, locations in the body that express dlg5 include the brain, pronephros, heart, and neural tube. The gene, dlg5, heads the implementation of Sonic Hedgehog- a protein that establishes the midline in the brain (Marquez et al., 2020). Depletion of dlg5 was tested in Xenopus embryos. Clinical phenotypes present after depletion of dlg5 include hydrocephalus and cystic fibrosis with ciliary loss in both tissues. In order to examine this effect, MO knockdown and FO CRISPR knockout of dlg5 at the one-cell stage of tadpole stage embryos was conducted. This resulted in anasarca or a serious condition of fluid accumulation in an interstitial space. Enlarged kidney tubules as well as fewer cilia were present (Marquez et al., 2020).

After dlg5 depletion, hydrocephalus was noted from a loss of midline structures analogous to the foramen of Monro -between paired lateral ventricles and third ventricle of the brain- and human cerebral aqueduct. Within the ventricular system, beating cilia create fluid flow. Surprisingly, this flow persisted in the cerebral ventricles even after dlg5 depletion (Marquez et al., 2020). This indicates residual cilia function or choroid plexus driven flow that prevails. Motile cilia's prevailing in beating presented in cerebral flow, had failed to persist in other aspects including the major embryological process of ciliary polarization.

Analyzing dlg5 and rnf20

Depletion of dlg5 impacts essential pathways and processes in the human body. For example, this gene is significant in relation to affecting Shh signaling -a pathway significant in growth regulation. In regards to structure of impact, dlg5 directly affects ciliary function, compared to rnf20's depletion triggering a domino effect, impacting its counterpart ciliary genes that ultimately present effect on ciliary action.

Regardless of the mode of impact severe phenotypic impacts are ensued when the reviewed candidate genes are mutated. Consequences of mutated dlg5 genes have involved hydrocephalus and cystic fibrosis. It is also found that cancer can even result from abnormal dlg5variations of Shh signaling. Likewise, protein complex rnf20's depletion corresponds to cardiac looping abnormalities. Defects in laterality have been found to be harmful, with higher mortality identified in CHD patients struggling from laterality anomalies, in comparison to CHD patients without laterality anomalies. Therefore, considering the detrimental characteristics both mutations present, it is significant that existing treatment methods are provided to corresponding patients and experimentation is pursued to devise more effective treatment strategies.

Treatments

There remains no cure to treat ciliopathies, due to their increased level of impact from a wide variety of mutations. Autosomal dominant polycystic kidney disease remains the only ciliopathy with a treatment that is internationally approved; limitations exist even with this treatment, Tolvaptan; when used for more than 30 days, the liver has experienced extensive harm and injury (McIntyre, 2013). Therefore, this highlights the limited success in current treatments, for the great variety of ciliopathies.

Neverthless, the development of new genetic therapy advancements and treatments aimed to alleviate symptoms, has allowed for the pathway towards a lessened impact of ciliopathies on an individual.

Upon current experimentation, the delivery of cilia-related genes has involved adeno-associated virus, lentivirus, and adenovirus. The least invasive locations for gene delivery have been found to be the visual and olfactory systems. In regards to the visual systems, a successful advancement of cilia-related gene therapy had occurred at the National Eye Institute (NEI), where a gene therapy had been created to rescue retinal degradation from Leber Congenital Amaurosis (LCA), a disease resulting in early childhood blindness. Through the utilization of retinal organoids that consist of normal retinal structure and function, it has been studied that a type of LCA mutation is one that impacts the NPHP5 gene. It codes for a protein that functions at the primary cilium to regulate proteins that enter the cilium, with the help of the CEP290 protein. Under normal circumstances, a healthy retina would consist of light sensing molecules, known as opsins, being housed in photoreceptor outer segments (NIH Researchers, 2022). The photoreceptor outer segment is a select variety of primary cilium, meaning deficiencies in the NPHP5 and CEP290 protein from LCA, impacts ciliary function (NIH Researchers, 2022). Therefore, with knowledge of the cause of the ciliopathy, gene augmentation was able to be applied, aiding patients with this mutation.

Similarly, nephronophthisis, a disorder that develops inflammation and scarring of the kidneys, has shown successful results of the use of CRISPR/Cas9 to treat NPH, in correspondence to *in vitro* systems. Through the gene editing of nephrocystins, genes that develop nephronophthisis, CRISPR/Cas9 had restored cilia phenotypes in iPSC-derived kidney organoids (Stokman et al., 2023). However, a complication faced that has stalled delivery of this treatment to the patient has been difficulties in delivering to the kidney and the occurrence of an immune response.

This has mirrored the major obstacles to expanding gene therapy for ciliopathies, particularly seen by the limited locations of the body that are able to receive effective cilia gene delivery. A major characteristic of many different types of ciliopathies has also encompassed affecting many different tissues, and having varied severity of phenotypes in each. Current modes of gene therapy haven't shown effectiveness in treating different

types of tissue, since a therapy finding success in one tissue, may function well in a different tissue (McIntyre, 2013).

Other forms of major treatment have involved targeting ciliary signaling pathways such as pathways using G-protein coupled receptors. Ciliary GPCRs have been significant in producing cyclic AMP, which has promoted renal ciliopathies (McIntyre, 2013). Therefore, by targeting this pathway, it has lowered extensiveness, particularly renal cyst formation, in renal ciliopathies.

Applying Treatments to rnf20 and dlg5

Due to the harmful effects of the mutated forms of rnf20 and dlg5, the development of treatments holds significance to aid the individuals affected. With no treatments currently existing, the author of this paper examines the obstacles to devising treatments and current outlooks for possible pathways toward treatment options.

After consideration of the gaps existing in current treatment methods for ciliopathies, the difficulties in finding treatments for individuals with affected rnf20 and dlg5 genes lie similar. Both genes affect multiple tissues, meaning current gene therapy would be ineffective in treating all affected types of tissues.

However, regardless of the drawback, there highlights promise in utilizing antisense oligonucleotides (ASOs), synthetic nucleotides in 12 and 30 nucleotides in length, used to bind with RNA, to target and correct the genetic defect itself. The binding of the ASO to mRNA would change the expression of the mRNA, allowing for the intended ciliopathy phenotype to be induced (Devlin, 2023). With further experimentation focused upon expanding the described treatment, is also proposed that focus is upon targeting pathways relevant to both genes. For example, the Hedgehog Signaling Pathway or Shh signaling showcases to be abnormal, when dlg5 is mutated. Shh signaling is significant in promoting renal ciliopathies as any variations in this pathway that mutated genes such as with dlg5 produce, has found to develop harmful side effects. When targeting Shh signaling, if thoughts of silencing the pathway were highlighted to lessen the promotion of the ciliopathy, it is significant to note to consider that other bodily functions, regarding homeostasis, that continue to depend upon the normal functioning of the signaling will be affected.

Conclusion

With around 190 ciliopathy disease causing genes have been identified (Focsa et al., 2021), 2 of these ciliary genes have been focused upon in this paper. All of the analyzed genetic mutations impact ciliary structure and function. In many cases, this abnormality affects pathways dependent on ciliary motility, primarily the Nodal signaling pathway -due to cilia's relationship with laterality. As observed in the patient, the pathway or cellular process affected presents phenotypes including hydrocephalus as well as congenital heart disease. (Hierck et al., 2023).

Due to the possibility of patients being affected by any of the currently identified genes and others that have not been identified yet, it is crucial to expand study in the analysis of the identification, behavior, and effect of ciliary genes. Through the compilation and growth of this knowledge, further steps can be taken through treatments or therapy being developed.

The treatments discussed represent the progress that has been made in ciliopathy treatment research, as well as the complications that continue to exist. Due to, as the Multiple System Atrophy Coalition reflects, "diseases that impact a greater portion of the general public [being] more likely to receive funding", costs for treatment remain expensive and research is often limited (Rare Disease, 2018). Even if the number of patients impacted by ciliopathies remains low, those affected continue to be dependent on research being done, highlighting the urgent and significant need for focus on this field.

During future accounts, it is hoped to be investigated on targeting gene therapy for more tissues in the body, and finding a balance in silencing or altering pathways that promote ciliopathies, but preventing disruption of homeostasis simeoultaneously. With the increase of variety of ciliary phenotypes, it urges a *call to action* of the experimentation of further candidate ciliary genes being topics of interest. Evolution of the study of life will only continue as time does; it is in humanity's hands how fast and with what purpose this evolution pursues.

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