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RESEARCH ARTICLE

GENETIC ANALYSIS AND PHARMACOGENOMIC STUDY OF HUMAN POLYCYSTIC KIDNEY DISEASE POPULATION

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Abstract

We are in an era of the mass-analysis of genetic information, which will signal the beginning of the study of living organisms on the basis of their most detailed plan: the DNA base-sequence. Throughout the 20th century, the chief epidemiologic impression of polycystic kidney disease (PKD) has been what an early observer described as its outstandingly hereditary character. An Autosomal dominant form of the disease is indeed among the most common genetic disorders affecting about one third of people with diabetes and is caused by a combination of factors including blood sugar control, high blood pressure and inherited factors. The first sign of kidney disease often is protein spilled into the urine called microalbuminuria. Kidney disease may progress to end stage renal disease, a serious condition in which the kidneys fail to remove the wastes from the body and hence must be treated by dialysis or kidney transplant. According to the National Institute of Health, each year more than 50,000 Americans are diagnosed with end stage renal failure [ESRD] disease, and diabetes, which affects an estimated 16 million Americans, is the most common cause. In 1994, the federal government spends $9.3 billion on care for patients with ESRD. According to NIH, African Americans and Native Americans develop diabetes, kidney disease and end stage renal disease at rates higher than average. This is a first attempt study in Indian scenario initiated to clinically analyse the Genetic and Pharmacogenomic study of Human Polycystic Kidney.

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Introduction

The formation of an organism requires coordination of cell behaviors and thus is highly dependent on communication among cells mediated by cell surface receptor proteins and their ligands on the adjacent cells or presented in the extracellular matrix (Clark and Brugge, 1995; Cunningham, 1995; Gumbiner, 1996 and Miller and Moon, 1996). Renal development involves reciprocal inductive interactions between an epithelial structure, the ureter bud (a caudal outgrowth of the mesonephric duct also called the Wolffian duct), and a surrounding mesenchyme, the metanephric blastema. Signals from the tips of ureteric bud epithelium induce the metanephric mesenchyme to undergo a sequence of events leading to its transformation into an epithelial structure that gives rise to the glomerular and tubular epithelia of the mature kidney. On the other hand, the transdifferentiated mesenchyme induces branching morphogenesis of the ureter bud, leading to the development of the collecting duct (CD) system (Davies, 1996 and Vainio and Muller, 1997). As development proceeds, the CD epithelium itself turns from an embryonic inductor into an excretory epithelium composed of two intermingled functionally and morphologically different cell types, the principal (P) cells and the intercalated (IC) cells (Tisher and Madsen, 1996). In the last few years, studies on early stages of renal embryogenesis have revealed a complex cascade of inducting and signaling

events implicating transcription factors, growth factors and their receptors, extracellular matrix constituents, and extracellular matrix degrading enzymes (Lechner and Dressler, 1997; Lelongt et al., 1997 and Wallner et al., 1997). By contrast, still very little is known about the molecular and cellular events that control later stages of renal development. These post - inductive stages include segmental organization and functional maturation of individual nephron segments, branching and growing of the CD, and generation of its cellular heterogeneity (Hanna Debiec, et.al., 1998). The kidney is an organ that functions to reabsorb essential fluid and ions, and this is facilitated by the strictly polarized distribution of numerous transporters, enzymes, and antigens distributed along the 10 distinct segments of the nephron in an epithelial cell type-specific fashion. The polarization of membrane proteins is a critical component in the differentiation of renal tubule epithelial cells and is largely established in the human metanephric kidney before birth (Patricia D. Wilson, et. al., 2000).

Microscopic funnel-shaped tubes called nephrons are responsible for purifying the blood and preventing the build-up of toxins and other wastes. Nephrons are susceptible to damage from many causes including infection, trauma, cancer, autoimmune disease, poisons, aging and genetic predisposition. Since the kidneys have a large reserve capacity and healthy nephrons that have the ability to increase the filtering function, the kidney can compensate for such damage. If the change occurs gradually, such as damage due to aging, the kidneys can continue to function with a little as 25% of the original nephrons. When the number of the functioning nephrons drops below this level, kidney failure occurs; and there are no available treatments to promote the re-growth of kidney tissue. After the kidneys have completed the filtering process, urine passes from them through the ureters and into the bladder where it is stored till it's full and is excreted out through the urethra. For proper function of renal epithelial cells, ion channels, exchangers, and transporters must be localized to functionally and structurally distinct plasma membrane dominas, termed apical and basolateral, that face the nephron lumen and interstitial space, respectively (Almers and Stirling, 1984; Fish and Molitoris, 1994). Studies have shown that both cell-cell and cell-substrate contacts are required to initiate formation of these plasma membrane domains. Subsequently, targeting to and retention of proteins in these domains establishes and maintains the functional polarity of these cells. Cytoskeletal proteins play essential roles in the development and maintenance of structural and functional organization of polarized epithelial cells. Significantly, recent studies of several kidney diseases show changes in the polarized distribution of membrane and cytoskeletal proteins (Fish and Molitoris, 1994).

Polycystic kidney disease is a bilateral disorder that affects approximately 200,000-400,000 persons in the United States. The most common form of the disease is inherited as an Autosomal dominant trait (ADPKD). It typically causes renal insufficiency by the fifth or sixth decade of life. The disease is characterized by the progressive enlargement of a portion of renal tubule segments (proximal, distal, loop of Henle, collecting duct). The tubules enlarge from a normal diameter of 40 um to several centimeters in diameter, causing marked gross and microscopic anatomic distortion. The cause of the cystic change in the tubules is unknown, but current possibilities include obstruction of tubule fluid flow by hyperplastic tubule cells, increased compliance of the tubule basement membranes, and/or increased radial growth of cells in specific portions of the renal tubule. Several studies show that the epithelia of the cysts continue to transport Na+, K+, Cl-, H+, and organic cations and anions in a qualitative fashion similar to that of the tubule segment from which they were derived. ADPKD, then, is a disease in which some gigantic renal tubules, over a period of several decades, impair the function of non-affected nephrons and thereby lead to renal failure. There are three different types of PKD, which vary according to the way people can get the different form. One of the inherited forms is dominant, meaning that those who have the gene from either their mother or father will have the disease. The other inherited form, which is recessive, only develops in those individuals who have copies of the gene from both parents; otherwise, they carry the gene and may pass it to their children, but they themselves may not have kidney disease.

Autosomal dominant PKD is the most common, inherited form. Symptoms usually develop between the ages of 30 and 40, but they can begin earlier, even in childhood. About 90 percent of all PKD cases are Autosomal dominant PKD. **Autosomal recessive PKD** is a rare, inherited form. Symptoms of Autosomal recessive PKD begin in the earliest months of life, even in the womb. Table 1 enumerates the characteristics of Autosomal dominant polycystic kidney disease (ADPKD) and Autosomal recessive polycystic kidney disease (ARPKD). **Acquired cystic kidney disease (ACKD)** develops in association with long-term kidney problems, especially in patients who have kidney failure and who have been on dialysis for a long time. Therefore it tends to occur in later years of life. A landmark advance in PKD research occurred when scientists discovered the genes, known as PKD1 and PKD2, which, when mutated, are responsible for the most prevalent form of PKD. Since the discovery of these causative genes, investigators using three mouse models of the disease have made several advances in understanding PKD. Adult polycystic kidney disease is transmitted as an autosomal dominant trait and affects approximately 1 in 1000

people. Cysts arise from the nephrons and the collecting tubules. Islands of normal parenchymal renal tissue are interspaced between the cysts. Microdissection reveals that the cysts communicate directly with the nephrons and collecting tubules in patients present with hypertension and progressive renal failure after their third decade of life. Uncommonly, autosomal dominant polycystic kidney disease (ADPKD) appears in children, and it is rarely seen in neonates. Of patients with ADPKD, 25-50% have associated hepatic cysts, 9% have associated pancreatic cysts, and 5% have associated splenic cysts; pulmonary cysts occur uncommonly. These extrarenal manifestation are not found in neonates and children.

Polycystins represent an expanding family of membrane proteins composed of two subfamilies, polycystin I-like and polycystin 2-like molecules. PC1, encoded by PKD1, is predicted to be a 460-kDa integral membrane glycoprotein with a very large extracellular amino terminus, 11 transmembrane domains, and a small intracellular carboxyl terminus (Anonymous, 1995 and Hughes, J, et.al., 1995). PC1 is found in the plasma membrane or in the cell-cell junction of cultured cells and in tissues (Geng, L, et.al., 1996, Ibraghimov-Beskrovnaya, O, et.al., 1997 and Peters, D, et.al., 1999). PC1 has recently been shown to function as a G protein-coupled receptor (Delmas, P, et.al., 2002), although its ligand(s) has not been identified. PC2, the product of PKD2, is predicted to encode an integral membrane protein of 110 kDa with an EF-hand domain at its carboxyl terminus (Mochizuki, T, et.al., 1996). Sequence homology to other ion channels suggests a pore-forming capacity of PC2. Since mutations in PC1 and PC2 result in similar phenotypes and PC2 is able to interact with PC1 through its coiled-coil domain in vitro (Qian, F, et.al., and Tsiokas, L, et.al., 1997), it has been speculated that PC1 and PC2 form a functional complex. PDK1 and PKD2 are two recently identified genes that are responsible for the vast majority of autosomal polycystic kidney disease, a common inherited disease that causes progressive renal failure. Mutations in the PKD-1 gene are responsible for 85% of cases of ADPKD, which affects 500,000 patients in the U.S. and leads to ESRD. Polycystin-1 is highly expressed in focal adhesions at the basal surface of migrating ureteric bud epithelial cells of normal developing kidneys. In normal adult kidneys polycystin-1 is down regulated and seen in the cell-cell adherens junctions of medullary collecting tubules. The developmental state can be modeled in vitro in sub confluent cultures. Analysis of the function of the normal and mutant polycystin-1 proteins is under study.

Various approaches exist in humans to determine whether a gene or genomic region influences a particular disease or phenotype. Recently it has been emphasized that in comparison to the traditional linkage approach, association studies might be more powerful to detect genes for polygenic diseases. To determine possible candidates genes, animal models provide valuable insights both in the pathogenic mechanism and genetic basis of diseases. The constantly increasing knowledge of nucleotide sequence and map position of genes, both in animals and humans, make it now possible to determine homologous regions in humans. In particular, the chromosomal localization of genes and their order tends to be conserved between mammalian species. By identifying a genetic link, the narrowing down of the regions of DNA and Nucleic acids in the body is made easy thus reducing the complication to a greater extent (Jeffrey P. Gardner, et.al., 2000). Karyotype is a photomicrograph of chromosomes arranged according to a standard classification (http/karyotyping.tripod.com/). It is a test used to identify and evaluate the size, shape and number of chromosomes in a sample of body cells. This test gives clues to problems associated with a person's growth, development, and body function PCR is used in molecular biology and genetic disease research to identify new genes. Due to the high sensitivity and particularity of PCR, scientists have employed it as an essential tool for improving human health and human life. Medical research and clinical medicine are profiting from PCR mainly in two areas: detection of infectious disease organisms, and detection of variations and mutations in genes, especially human genes. Since PCR can amplify unimaginably tiny amounts of DNA, even that from just one cell, physicians and researchers can examine a single sperm, or track down the elusive source of a puzzling infection. These PCR-based analyses are proving to be just as reliable as previous methods-sometimes more so-and often much faster and cheaper

The entire set of human genes is now available. This represents an irresistible amount of data that breached the bioinformatics gap that lay between biologists and their understanding of genetics. DNA is the "genetic blueprint" that determines the genotypic make-up of each). In its barest form, DNA consists of two strings of nucleotides, or bases (abbreviated A, C, G, and T), wound around each other. The bases composing DNA have specific binding capabilities: A always binds to T, and C always binds to G. These binding capabilities are useful for scientists to understand since, if the nucleotide sequence of one DNA strand is determined, complementary binding allows the sequence of other strand to be deduced. Thus, DNA sequencing reveals crucial variations in the nucleotides that constitute genes and these mutational changes can produce disease and even death by forcing the genes to produce

abnormal proteins, or sometimes no proteins at all. The need for ongoing development of new drugs needs no emphasis in light of the current global situation of health and disease. Traditionally, the process of drug development has revolved around a screening approach, as nobody knows which compound or approach could serve as a drug or therapy. Such almost blind screening approach is very time-consuming and laborious. The shortcoming of traditional drug discovery; as well as the allure of a more deterministic approach to combating disease has led to the concept of "Rational drug design." Rational drug design is a more focused approach, which uses information about the structure of a drug receptor or one of its natural ligands to identify or create candidate drugs. The three-dimensional structure of a protein can be determined using methods such as X-ray crystallography or nuclear magnetic resonance spectroscopy. Armed with this information, researchers in the pharmaceutical industry can use powerful computer programmes to search through databases containing the structures of many different chemical compounds. The computer can select those compounds that are most likely to interact with the receptor, and these can be tested in the laboratory.

In structure-based drug design, the three dimensional structure of a drug target interacting with small molecules is used to guide drug discovery. Structure-based drug design represents the idea of seeing exactly how the molecule interacts with its target protein. This structural information can be obtained with X-ray crystallography or nuclear magnetic resonance spectroscopy (NMR). Ideally, these two techniques complement one another. However, most companies that are specializing in structure-based drug design focus on only one method of structure determination, at least initially. Originally, structure-based drug design was equated with de novo design or building a molecule from the ground up. The active site of the protein was a space to be filled with a molecule that complemented it in terms of shape, charge, and other binding components. Combinatorial chemistry is the best thing that ever happened to structure-based drug design. Purely random combinatorial chemistry has evolved into focused combinatorial libraries, which can be considered an abstract form of structure-based drug design Researchers realized that specific knowledge of the target could guide the power of combinatorial chemistry to rapidly make many compounds. Docking is the computational procedure in which a potential drug molecule or ligand is placed in the active site of the target molecule. Using models of the binding energy of various parts of the molecules, the interaction between the two can be evaluated to find if there is likely to be a good 'fit'.) The procedure can also be used to find the best location for a ligand in the active site, and this can guide the addition of side chains that can improve the fit, a process known as structure-based drug design. Almost all research teams working in drug discovery will make use of this type of software, according to Quantum

METHODOLOGY

The blood samples were collected using a heparanised vacutainer from 78 subjects of 8 families. After preliminary screening using Biochemical and Ultrasound scan studies they were categorized as PKD (experimental) or control subjects. The Subject Number, Life Status, Nature of Death, Family Number, Generation Number, Offspring Type, Clinical Status, Subject Name and Age were attributed to the 78 subjects. Hydragel Protein (E) K20 Serum Protein Kit was used for the separation and quantification of serum protein by Agarose gel electrophoresis. Cell Dyne 6000 Analyzer was used to analyze the hematological parameters. Routine Cytogenetic analysis were made on the blood cells following 72 hours of incubation in RPM1 1640 culture medium containing a B-cell mitogen, the phytohaemagglutination (PHA). The standard Giemsa and Trypsin G banding technique of Sea bright (1971) was used for Cytogenetic analysis. Metaphase plates were photographed using an Applied Imaging Cyto Vision Automated Karyotyping System Version 3. PCR by SSCP using standardized method was demonstrated for all 78 subjects for five Exons, namely Exon 15 (Nucleotide position 29039 – 29523), Exon 15 (Nucleotide position 29476 – 29847), Exon 18 – 20 (Nucleotide position 32835 – 33314), Exon 25 – IVS 26 (Nucleotide position 38978 – 39675) and Exon 29 – 30 (Nucleotide position 41579 – 41915) of PKD 1 gene for critically excluding experimental subjects which showed a shift in the banding pattern for DNA Sequencing. DNA Sequencing for 12 samples, 10 experimental subjects [samples which showed a shift in the banding pattern] and two control subjects was done commercially from Bangalore Cenei Pvt. Ltd.

RESULTS

15 families were initially selected for the present study. After personal counseling to seek their consent, 8 families comprising of 78 subjects of F1, F2 and F3 generation consented to co-operate for the study amidst social and family constraints.

TABLE 1 : CHARACTERISTIC FEATURES OF AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE (ADPKD) AND AUTOSOMAL RECESSIVE POLYCYSTIC KIDNEY DISEASE (ARPKD)

Characteristic	ADPKD	ARPKD
Inheritance	Autosomal dominant	Autosomal recessive
Incidence	1/500 to 1/1000	1/6000 to 1/40000
Gene (chromosome)	*PKDI* (Chr 16); *PKD2* (Chr 4)	PKHD1 (Chr 6)
Age of onset of ESRD	53 yr (*PKD1*); 69 yr (*PKD2*)	Infancy/Childhood usually
Location of renal cysts	All nephron segments	Collecting ducts[a]
Extrarenal manifestations	Hepatic cysts/pancreatic cysts	Biliary dysgenesis
	Cerebral & aortic aneurysms	Hepatic fibrosis
	Cardiac valvular abnormalities	Portal hypertension
	Systemic hypertension	Systemic hypertension
Protein name	Polycystin-1; Polycystin-2	Fibrocystin/Polyductin
Protein size	Polycystin-1; 4302 amino acids	4074 amino acids and alternative shorter forms
	Polycystin-2; 968 amino acids	
Protein structure	Polycystin-1; Integral membrane protein, multiple Ig-like domains, similar to egg jelly receptor	Transmembrane protein (and possible secreted forms), multiple TIG/IPT domains, as occur in hepatocyte growth factor receptor and plexins
	Polycystin-2; Integral membrane protein, similar to TRP channel	
Tissue distribution	Polycystin-1 and -2: Widespread	Kidney, pancreas, and liver
Subcellular localization	Polycystin-1: Plasma membrane, cilia[b]	Unknown
	Polycystin-2: Endoplasmic reticulum, cilia	
Function	Polycystin-1: ? Receptor, forms ion channel when co expressed with polycystin-2	? Receptor
	Polycystin-2: Calcium-activated cation channel	

[a] Cysts appear transiently in proximal tubules during fetal development (90).
[b] Based on localization in male-specific sensory neurons in *C. elegans* (66).

BIOCHEMICAL ANALYSIS

Biochemical Screening was done for all the 78 subjects in the hospital to detect the values for all the 18 biochemical components in the blood which served as a tool to differentiate the control subjects from the experimental subjects. The experimental subjects showed a significant increase or decrease in value than the normal range with special reference to Blood Urea and Serum Creatinine level, the index factors thereby confirming the positivity of the clinical status of the subject. Based on the results received from hospital by Biochemical screening, the subjects were segregated as control subjects (48) and experimental subjects (30) from a total live sample size of 78. Table 2 comprises of the normal values of all the 18 Biochemical parameters analyzed in the blood for a confirmatory test. Subject No 33 revealed a peculiar manifestation of Hematuria condition (Blood in the urine). [Plate 1 depicts the Hematuria condition in Subject No 33].

PEDIGREE ANALYSIS

A pedigree chart / Biogenetic tree was constructed in accordance with the linkage analysis pattern of Roser Torra, et.al., 1996 with slight modifications to update and suite the present study. Pedigree chart was mainly constructed to trace the linkage pattern and the rate of PKD gene expression from F1 to F2 and subsequently to F3 generation. Assuming the positivity of PKD condition to one of the parent in the F1 generation, the PKD gene transfer rate was calculated for the F2 and F3 generation (Table 3 & Figure 1). Plate 2A and B gives an overall view of the pedigree chart of the 8 families comprising of 89 subjects taken for the study.

KARYOLOGY / CHROMOSOMOLOGY

Karyotyping analysis was carried out for few chronic experimental subjects. Since PKD is characterized by the alteration at the gene level, contrasting variation was not encountered in the metaphase plate of the karyotype. Plate 3 depicts the Karyotype of a chronic experimental subject.

PCR ANALYSIS BY SSCP

Table 4 depicts the percentage rate of control and experimental subjects selected for PCR analysis by SSCP method for Exon 15 (Nucleotide position 29039 – 29523), Exon 15 (Nucleotide position 29476 – 29847), Exon 18 – 20 (Nucleotide position 32835 – 33314), Exon 25 – IVS 26 (Nucleotide position 38978 – 39675) and Exon 29 – 30 (Nucleotide position 41579 – 41915). Among the 30 experimental subjects, 10 showed a shift in the banding pattern which formed the criteria for selection for DNA Sequencing. Plate 4 reveals the Agarose gel showing high molecular weight genomic DNA isolates from blood samples. Plates 5 – 9 are Representative Agarose gels for PCR product of Exon 15 (Nucleotide position 29039 – 29523), Exon 15 (Nucleotide position 29476 – 29847), Exon 18 – 20 (Nucleotide position 32835 – 33314), Exon 25 – IVS 26 (Nucleotide position 38978 – 39675) and Exon 29 – 30 (Nucleotide position 41579 – 41915). Plates 10 – 14 are Representative SSCP gel analysis of Exon 15 (Nucleotide position 29039 – 29523), Exon 15 (Nucleotide position 29476 – 29847), Exon 18 – 20 (Nucleotide position 32835 – 33314), Exon 25 – IVS 26 (Nucleotide position 38978 – 39675) and Exon 29 – 30 (Nucleotide position 41579 – 41915).

TABLE 2: NORMAL VALUES OF BIOCHEMICAL COMPONENTS IN BLOOD

S.NO.	NORMAL VALUES OF BIOCHEMICAL COMPONENTS IN BLOOD	KEY TO ABBREVIATIONS
1	BLOOD UREA NITROGEN [BUN] : - 20 mg/dl	IU – International Unit
2	SERUM CREATININE : 0.8 – 1.4 mg/dl	L – Liter
3	BLOOD GLUCOSE TEST : 64 – 128 mg/dl	dl – Decilitre = 0.1 litre
4	TOTAL CHOLESTEROL : 100 – 240 mg/dl	g/dl – gram per deciliter
5	SERUM TOTAL PROTEIN : 6.3 – 7.9 g/dl	mg – milligram
6	SERUM SODIUM : 136 – 144 mEq/L	mmol – millimole
7	POTASSIUM TEST : 3.7 – 5.2 mEq/L	mEq – milliequivalents
8	SERUM CHLORIDE : 101 – 111 mmol/L	
9	SERUM BICARBONATE : 15 – 20 mmol/L	
10	SERUM URIC ACID : 4.1 – 8.8 mg/dl	
11	SERUM ALBUMIN : 3.9 – 5.0 g/dl	
12	SERUM GLOBULIN : 1.4 – 2.0 g/dl	
13	TOTAL BILIRUBIN : 0.2 – 1.9 mg/dl	
14	SGOT [SERUM GLUTAMIC OXALOACETIC TRANSAMINASE] – NORMAL RANGE VARIES, IU/L	
15	SGPT [SERUM GLUTAMATE PYRUVATE TRANSAMINASE] – NORMAL RANGE VARIES, IU/L	
16	SAP [SERUM ACID PHOSPHATASE] – NORMAL RANGE VARIES, IU/L	
17	SERUM CALCIUM : 8.5 – 10.9 mg/dl	
18	SERUM PHOSPHOROUS : 2.4 – 4.1 mg/dl	

TABLE 3: PEDIGREE ANALYSIS AND GENE TRANSFER PERCENTAGE OF CONTROL AND EXPERIMENTAL SUBJECTS FROM F1 TO F2 AND F3 GENERATION

GENERATIONS	NORMAL EXPIRY	PKD EXPIRY	CONTROL SUBJECTS	EXPERIMENTAL SUBJECTS
F1	5	6	3	2
F2	-	1	28	14
F3	-	-	16	14
TOTAL	5	7	47	30
PERCENTAGE	5.61%	7.86%	52.83%	33.70%

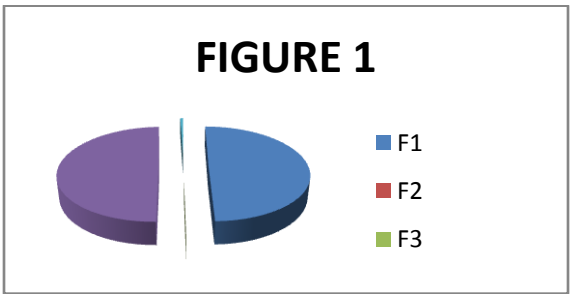


TABLE 4: SAMPLE SELECTION FOR PCR ANALYSIS BY SSCP METHOD [78 SUBJECTS]

	CONTROL SUBJECTS	EXPERIMENTAL SUBJECTS	EXPERIMENTAL SUBJECTS SELECTED FOR DNA SEQUENCING *	CONTROL SUBJECTS SELECTED FOR DNA SEQUENCING *
TOTAL	48	30	10	2
PERCENTAGE	61.53%	38.47%	83.33%	16.67%

DNA SEQUENCING

DNA Sequencing for 12 samples, 10 experimental subjects [samples which showed a shift in the banding pattern by SSCP] and two control subjects (Table 5) was done commercially from Bangalore Genei Pvt. Ltd, for Exon 25 – IVS 26 (Nucleotide position 38978 – 39675), Exon 18 – 20 (Nucleotide position 32835 – 33314) and Exon 29 – 30 (Nucleotide position 41579 – 41915). Comparison of the analyzed PKD 1 regions with standard sequence of PKD 1 gene and its present protein was performed by using the basic BLAST search and blastn in nr database of Genebank and blastz in Swissport. Substitution mutations of Transition and Transversion type and Heterozygous condition were observed in both the experimental and control subjects at the same nucleotide positions common to all and also at different positions in the nucleotide within the selected exons. Plates 15 – 20 shows the Transitions, Transversiion and Heterozygous condition identified by DNA sequencing of control subjects for Exon 25 – IVS 26 (Nucleotide position 38978 – 39675), Exon 18 – 20 (Nucleotide position 32835 – 33314) and Exon 29 – 30 (Nucleotide position 41579 – 41915).

TABLE 5: SAMPLE SELECTION FOR DNA SEQUENCING

Family No.	Generation No.	Offspring Yes = 1 No = 0 2 = Parent	Status N = Normal P = PKD	Subject No.	Shift in SSCP Gel Y = Yes N = No
1	2	0	N	8	N
1	2	1	P	10	Y
1	2	1	P	11	Y
1	3	1	P	23	Y
2	2	1	P	30	Y
3	3	1	P	37	Y
3	2	1	N	34	N
4	2	1	P	41	Y
5	2	1	P	55	Y
6	2	1	P	70	Y
7	1	2	P	76	Y
8	1	2	P	87	Y

PLATE 1 : HEMATURIA

PLATE 3 : KARYOTYPE

PLATE 2A : PEDIGREE CHART

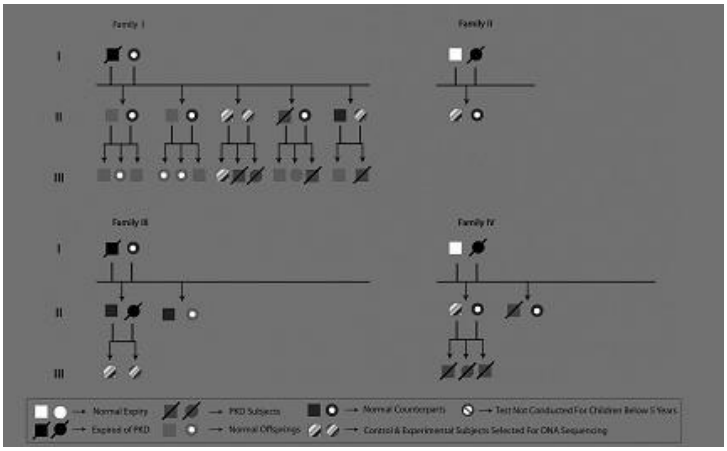


PLATE 2B : PEDIGREE CHART

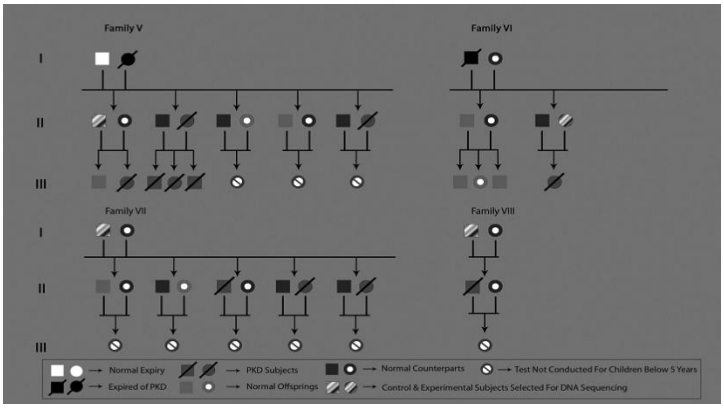
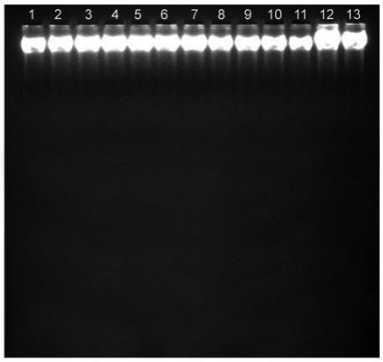


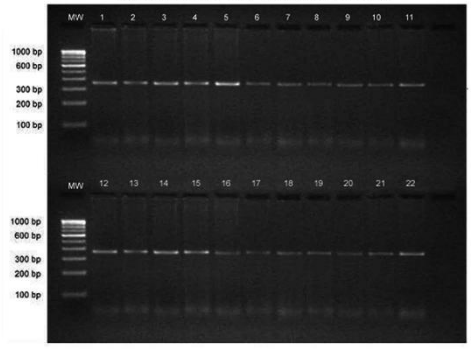
PLATE 4 - AGAROSE GEL SHOWING HIGH MOLECULAR WEIGHT GENOMIC DNA ISOLATES FROM BLOOD SAMPLES

Agarose gel showing High Molecular weight Genomic DNA Isolates from blood samples



PLATES 5 : REPRESENTATIVE AGAROSE GELS FOR PCR PRODUCT OF EXON 15 (NUCLEOTIDE POSITION 29039 – 29523)

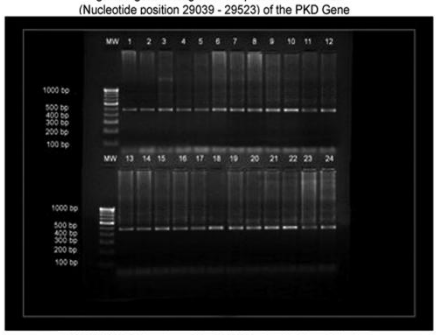
Agarose gel showing the PCR product of Exon 15 (Nucleotide position 29476 - 29847) of the PKD Gene



LANE MW : Molecular weight DNA Marker (100 bp ladder)
LANE 1-22: PCR Amplicon of Exon 15

PLATES 6 : REPRESENTATIVE AGAROSE GELS FOR PCR PRODUCT OF EXON 15 (NUCLEOTIDE POSITION 29476 – 29847)

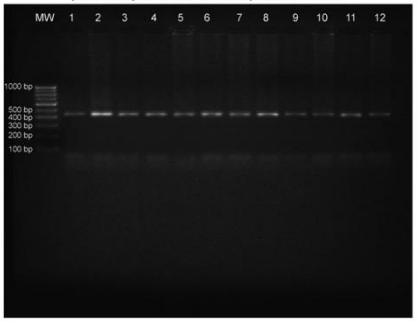
Agarose gel showing the PCR product of Exon 15 (Nucleotide position 29039 - 29523) of the PKD Gene



LANE MW : Molecular weight DNA Marker (100 bp ladder)
LANE 1-24: PCR Amplicon of Exon 15

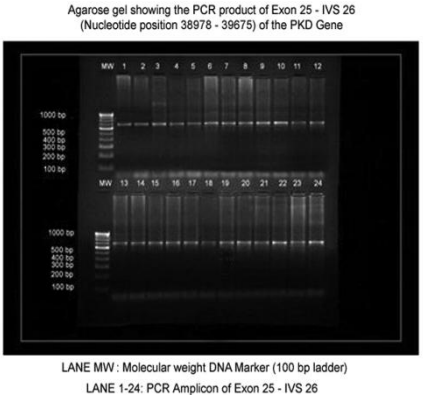
PLATES 7 : REPRESENTATIVE AGAROSE GELS FOR PCR PRODUCT OF EXON 18 – 20 (NUCLEOTIDE POSITION 32835 – 33314)

Agarose gel showing the PCR product of Exon 18 - Exon 20 (Nucleotide position 32835 - 33314)of the PKD Gene

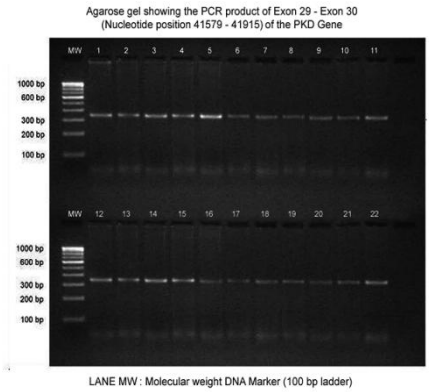


LANE MW : Molecular weight DNA Marker (100 bp ladder)
LANE 1 - 12 PCR Amplicon of Exon 18 - Exon 20

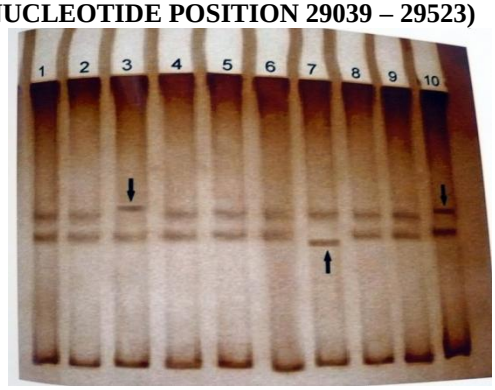
PLATES 9 : REPRESENTATIVE AGAROSE GELS FOR PCR PRODUCT OF EXON 25 – IVS 26 (NUCLEOTIDE POSITION 38978 – 39675)



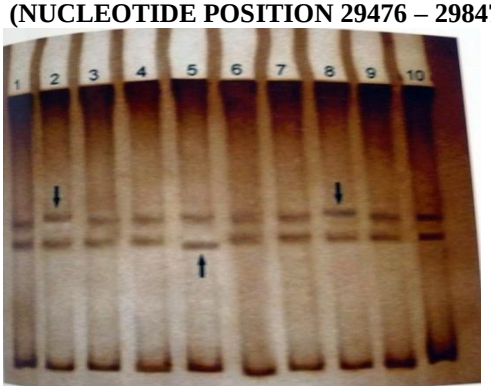
PLATES 8 : REPRESENTATIVE AGAROSE GELS FOR PCR PRODUCT OF EXON 29 – 30 (NUCLEOTIDE POSITION 41579 – 41915)



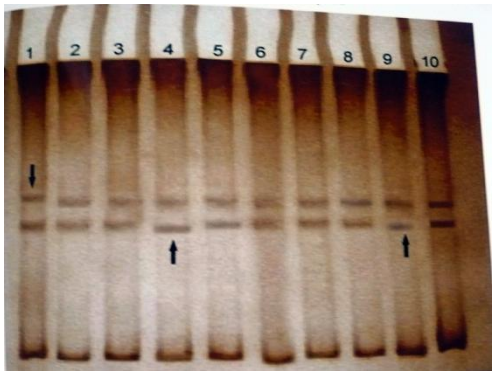
PLATES 10 : REPRESENTATIVE SSCP GEL ANALYSIS FOR PCR PRODUCT OF EXON 15 (NUCLEOTIDE POSITION 29039 – 29523)



PLATES 11 : REPRESENTATIVE SSCP GEL ANALYSIS FOR PCR PRODUCT OF EXON 15 (NUCLEOTIDE POSITION 29476 – 29847)



PLATES 12 : REPRESENTATIVE SSCP GEL ANALYSIS FOR PCR PRODUCT OF EXON 18 - 20 (NUCLEOTIDE POSITION 32835 - 33314)



PLATES 13 : REPRESENTATIVE SSCP GEL ANALYSIS FOR PCR PRODUCT OF EXON 25 IVS 26 (NUCLEOTIDE POSITION 38978 – 39675)

PLATE 14 : REPRESENTATIVE SSCP GEL ANALYSIS OF EXON 29 – 30 (NUCLEOTIDE POSITION 41579 – 41915)

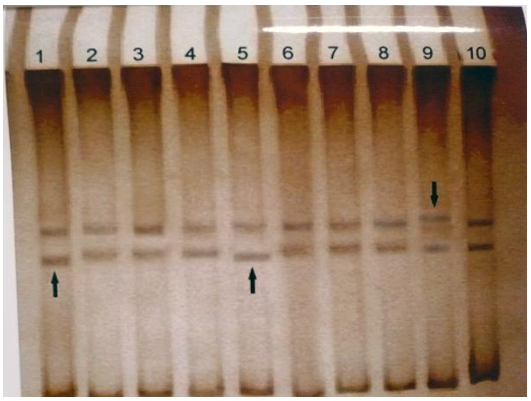


PLATE 15 : TRANSITIONS AND TRANSVERSIONS OBSERVED IN DNA SEQUENCING FOR EXON 18 – 20 (NUCLEOTIDE POSITION 32835 – 33314)

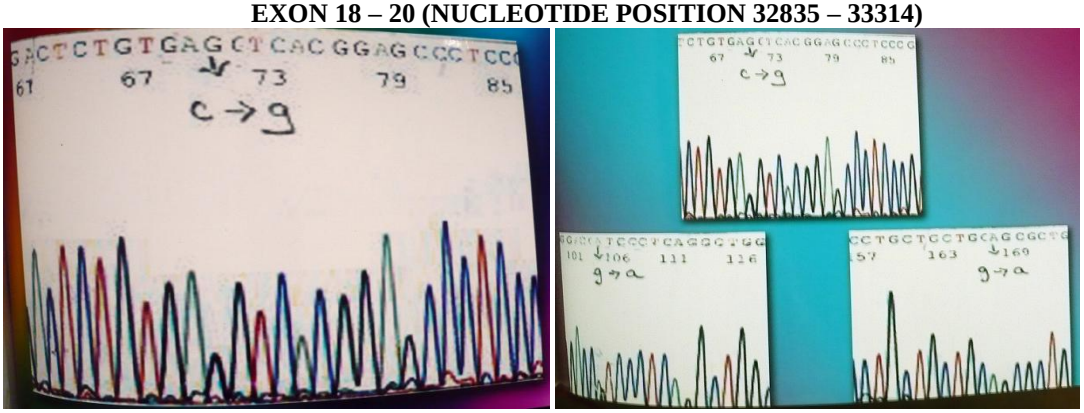


PLATE 16 : TRANSVERSIONS OBSERVED IN DNA SEQUENCING FOR EXON 25 – IVS 26 (NUCLEOTIDE POSITION 38978 – 39675)

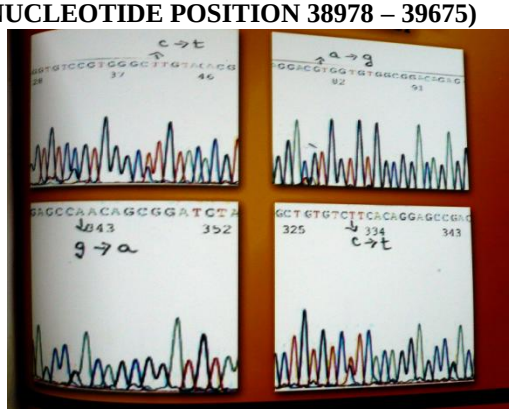
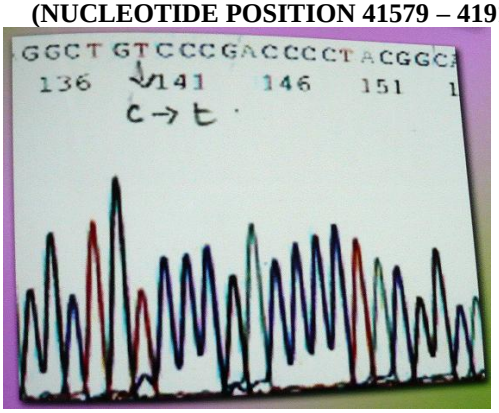
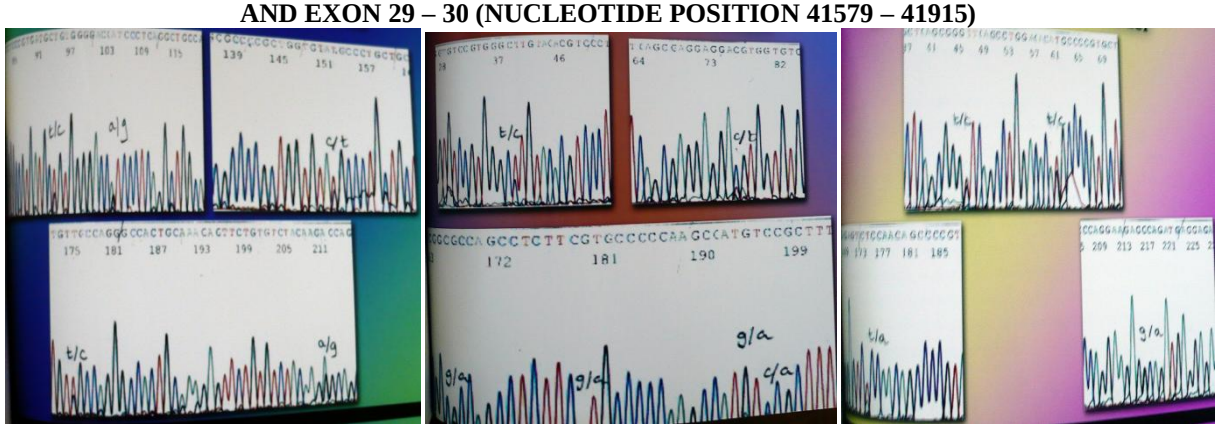


PLATE 17 : TRANSVERSIONS OBSERVED IN DNA SEQUENCING FOR EXON 29 – 30 (NUCLEOTIDE POSITION 41579 – 41915)



PLATES 18 - 20 : HETEROZYGOUS CONDITION OBSERVED IN DNA SEQUENCING FOR EXON 18 – 20 (NUCLEOTIDE POSITION 32835 – 33314), EXON 25 – IVS 26 (NUCLEOTIDE POSITION 38978 – 39675) AND EXON 29 – 30 (NUCLEOTIDE POSITION 41579 – 41915)



DRUG DESIGN

Research work undertaken in Han: SPRD rat support a significant therapeutic potential of EGFR tyrosine kinase inhibition in ADPKD. Based on the above findings, molecules for epidermal growth factor receptor (EGFr) were taken from the Enzyme database Brenda. This database maintains a list of numerous enzymes with their characteristic properties which are experimentally derived ones. For our current study, we researched for (EGFr) and found the following 8 EGR inhibitors from the Enzyme Database Brenda which might prove to be a promising tool for the future in combating ADPKD. These inhibitors are usually shown in two dimensional structures, but since enzymes are three dimensional structure and their active sites are also being three dimensional entity we converted these two dimensional molecules into three dimensional structures using DS viewer.

STATISTICAL METHOD

Statistical Analysis was implemented as an effective tool to analyze the significant difference between various study variables of the observed data between the normal and PKD subjects who were taken for the study.

DRUG TRIALS ARE UNDERWAY FOR PKD

TOLVAPTAN

Polycystic Kidney Disease or PKD destroys a person's kidneys and there is no cure, but the Food and Drug Administration has just fast-tracked a new drug that is being tested at the Mayo Clinic in Rochester, Minn. Gary DeGrande's family legacy is a frightening one. He inherited PKD from his father, who got it from his mother. "Then the result is either dialysis or transplantation," DeGrande said. "There's really no treatment for PKD. There is no cure for PKD." The Mayo Clinic is hoping to change that. It is one of several sites testing what could be the first drug to control the cysts of PKD. The drug is called Tolvaptan. Maras has taken the drug in a preliminary trial and is now enrolled in a three-ear study at Mayo. Dr. Vincente Torres, a PKD expert, said he has seen the drug work on mice. "So the fact that it works in animals with mutations in the same genes that cause the disease in humans makes us be cautiously optimistic that we will have good results." While the drug is not a cure, it holds the promise of a healthier future for more than 500,000 Americans and 12 million people worldwide. It is too late for DeGrande. Six months ago, his wife gave him one of her kidneys, but the drug may spare his son from the ravages of PKD. Tolvaptan appears to be safe and it is already approved by the FDA for other conditions. The only side-effect reported is thirtiness

NEUGENE

Most human diseases arise from the increased function or dysfunction of genes within the body, either that of pathogens, such as viruses, or of one's own genes. The Human Genome Project has paved the way toward identifying the genes associated with most major human diseases and determining the sequence of their genetic codes. Using modern methods of chemical synthesis, compounds can be prepared that recognize target gene sequences in a pathogen or pathogenic process. When these compounds bind tightly to the disease-causing sequence, the genetic process is inhibited, thus disabling the pathogen or pathogenic process. This is called antisense technology because the strands of genetic material that get translated into a protein are called "sense"

strands, and so "antisense" drugs are meant to stop that translation process. In clinical studies, NeuGene antisense compounds have displayed advantageous pharmaceutical properties in stability, specificity, efficacy, delivery and safety. The Phase 1b clinical trial represented the first time NEUGENES had been given to adult patients with substantial impairment of kidney function, which is important for drug clearance and metabolism. The single-center study, performed at Legacy Good Samaritan Hospital in Portland, Oregon, showed that significant renal impairment in trial participants did not substantially impact how patients distributed and cleared NEUGENES from the body. This supports the use of NEUGENES as a potential safe and effective treatment for the PKD disease. In this clinical trial, three groups of eight patients with PKD and various degrees of kidney function impairment were exposed to increasing doses of AVI's proprietary NEUGENE antisense drug. The primary purpose of the study was to compare the way the drug is distributed in, and cleared from, the blood in PKD patients compared to normal healthy volunteers. A second important objective was to determine the safety of this drug in this clinical setting. Side effects were noted as minimal and not related to the study drug. In addition, kidney function was not adversely affected. Further, blood distribution in patients was not significantly different from that of normal volunteers.

EGF INHIBITION FACTOR – THE GREAT BOON

Epidermal growth factor (EGF) has an important role in the expansion of renal cysts. Epithelial cells from cysts from patients with the autosomal dominant form and from those with the autosomal recessive form are unusually susceptible to the proliferative stimulus of EGF. Moreover, cyst fluids from the former group of patients contain mitogenic concentrations of EGF, and this EGF is secreted into the lumens of cysts in amounts that can induce cellular proliferation (Du, J. and Wilson, PD, 1995). The over expression and abnormal location of EGF receptors on the apical (luminal) surface of cyst-lining epithelia creates a sustained cycle of autocrine-paracrine stimulation of proliferation in the cysts (Du, J. and Wilson, PD, 1995).

The clinical variability in the rate of progression of autosomal dominant polycystic kidney disease (ADPKD) has been attributed to genetic heterogeneity, though environmental factors and modifying genes very likely play an important role as well. The association examined between clinical outcome, defined by age at onset of end-stage renal disease (ESRD) in 46 ADPKD patients, revealed a polymorphism in the epidermal growth factor receptor (EGFR) gene, a candidate modifying gene. EGFR is a key element in renal tubular proliferation. Studies carried out in 46 unrelated patients with ADPKD and ESRD and 58 healthy controls confirmed the prevalence of PKD 1 mutations in patients. Results revealed the allelic frequencies of the EGFR polymorphism to be different in the ADPKD sample and the control population (G2=17.19; P=0.009). In particular, the frequencies of the 122 and 118bp length alleles had a different distribution (P=0.010 and P=0.047 respectively). Patients with the 122bp length polymorphism had ESRD at an earlier age, but this finding was not statistically significant. Thus these findings suggest an association between the EGFR microsatellite polymorphism and ADPKD. However, it is difficult to establish which alleles are protective and which harmful. Thus, these 8 EGFR lead molecules which are derived from this study if taken to the next level of drug testing and clinical trial practices may serve to be a promising tool to reduce the cyst growth in PKD in Indian population thereby delaying the onset of ESRD and may spare the offsprings or future generations from the ravages of PKD. A larger, multicenter study may help to clarify these results and replicate preliminary finding of an association between ADPKD and the EGFR polymorphism thereby proving the efficacy of these lead molecules which are derived from this study (Figures 1 – 6).

FIGURE 1 : PHARMACOPHORE

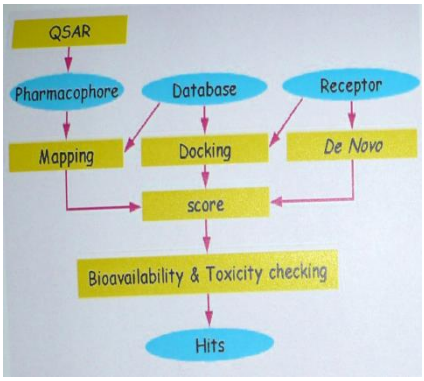


FIGURE 2 : BREAKUP OF PHASES IN CLINICAL TRIALS

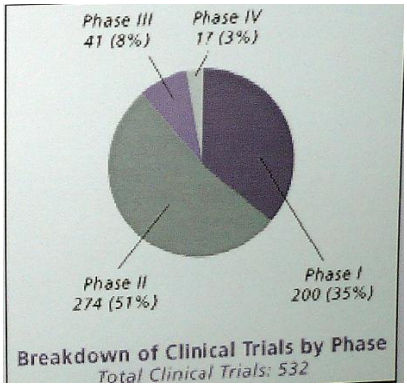


FIGURE 3 : CLINICAL TRIAL CHART

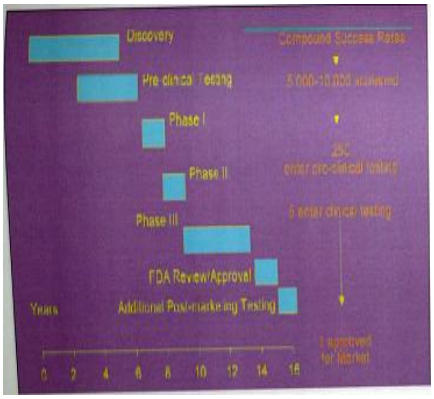


FIGURE 4 : PHASES OF REGULATORY IN CLINICAL TRIALS

FIGURE 5 : RANDOMIZATION DEVELOPMENT

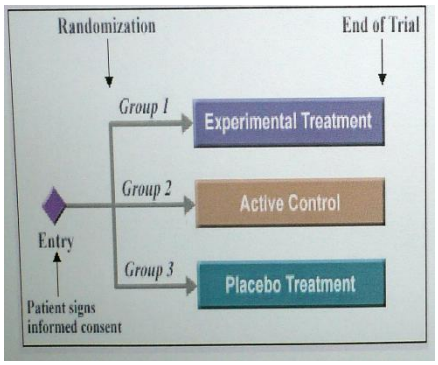
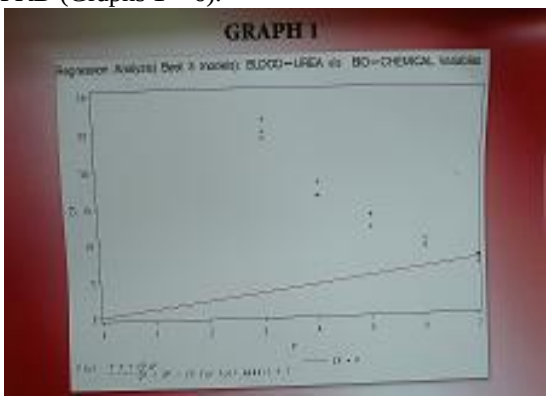
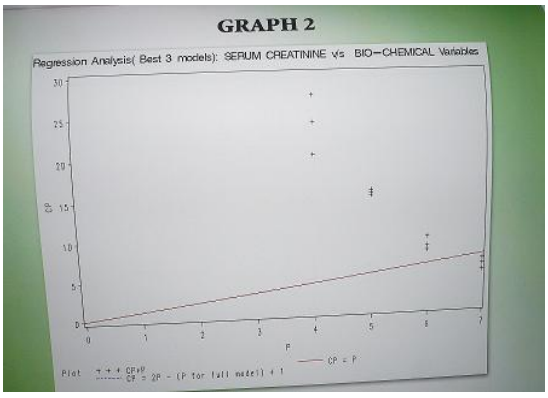


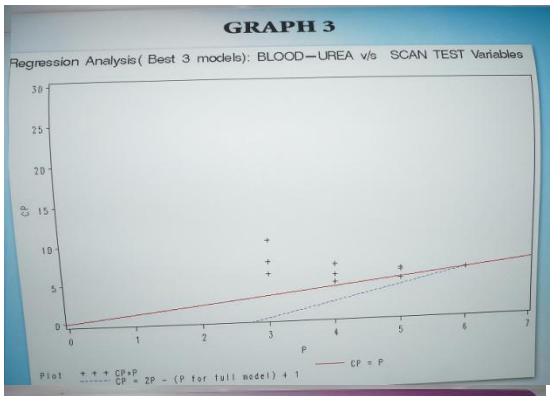
FIGURE 6 : NEUGENE ANTISENSE CLINICAL

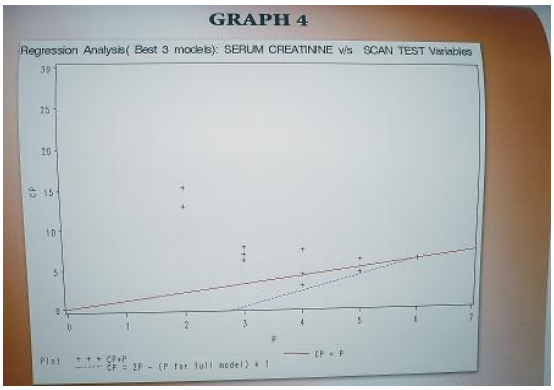
STATISTICAL SIGNIFICANCE

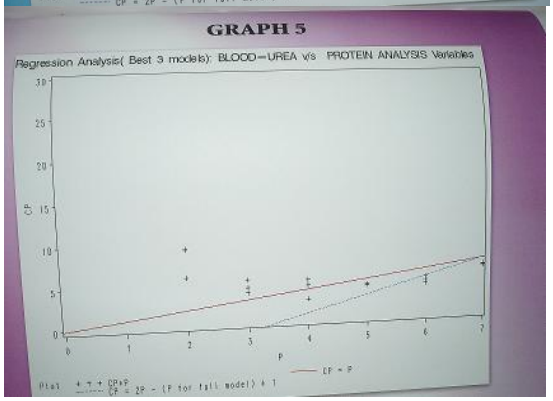
Statistical tools incorporated in the present study revealed the existence of significant amount of difference between normal and PKD subjects within the study variables and helped to identify the variables which significantly influence the dependent variables such as Blood Urea and Serum Crreatinine in the PKD subjects which can pave the way and prevent the stress of undergoing laborious clinical tests to confirm the positivity of clinical status of PKD (Graphs 1 – 6).

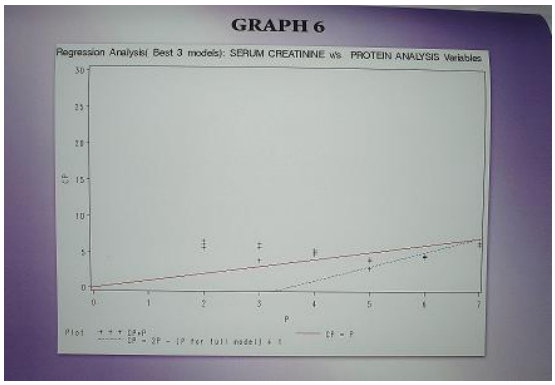












SUMMARY

The significant advances in understanding the molecular basis of adult dominant Polcystic Kidney Disease have generated intense interest and have provided investigators with important research opportunities. The present investigation, largely fundamental and a pioneering study in Indian population is likely to generate, in the foreseeable future, a variety of possible strategies for molecular interventions in clinical research. This being a first attempt study is initiated to confirm the Western data with the Indian data regarding the location, mutation and genetic alteration of PKD1 gene and also to trace for novel mutations through PCR and DNA Sequencing in the selected subjects of the Indian population. Clinical screening by Biochemical, Ultrasonography, Protein Profile and Hematological diagnosis were confirmed with Molecular Analysis. The method of Seabright (1971) adopted for G – banding of chromosomes and Karyotyping failed to serve as a promising tool in the determination of the genetic alteration of PKD1 gene and hence PCR Analysis by standardized SSCP method and DNA Sequencing aided in unraveling the mutations in the control and experimental subjects selected for the present study. The designing of target lead molecules to control cyst growth in PKD was facilitated and was made accessible through the Brenda Enzyme Database System. The development of statistical models proved to be an important tool to predict the index variables, Blood Urea and Serum Creatinine for PKD without undergoing the strenuous procedure of clinical testing. The important area of investigation was the identification of novel mutations of human ADPKD1 which elucidated the interactions between PKD susceptibility loci, genetic determinants and cellular and molecular mechanisms which resulted in disrupted normal kidney function. The need for ongoing development of new drugs needs no emphasis in light of the current global situation of health and disease. Traditionally, the process of drug development has revolved around a screening approach, as nobody knows which compound or approach could serve as a drug or therapy. Such almost blind screening approach is very time-consuming and laborious. The shortcoming of traditional drug discovery; as well as the allure of a more deterministic approach to combating disease has led to the concept of "Rational drug design". Nobody could design a drug before knowing more about the disease or infection process than past. The first necessary step is the identification of a molecular target critical to a disease process or an infectious pathogen. Then the important prerequisite of "drug design" is the determination of the molecular structure of target, which makes sense of the word "rational". In fact, the validity of "rational" or "structure-based" drug discovery rests largely on a high-resolution target structure of sufficient molecule detail to allow selectivity in the screening of compounds. Target molecules designed to combat PKD though may be in its infancy, may prove to be a promising tool in the future to control cyst growth and may spare the offsprings or future generations from the ravages of PKD. Although certain areas of research in PKD are already receiving careful study,

the timely opportunities to discover more about the etiology and pathogenesis in particular, and the related cellular and molecular mechanisms that determine kidney function in general, need to be addressed in a more elaborative manner. Future studies can be undertaken to understand the phenotype/genotype correlation and disease phenotypes in different genetic backgrounds, i.e., gender, race, and ethnicity. Specific role of genes in determining severity of disease and extra-renal manifestations and predicted impact of mutations on proteins could be the immediate next endeavor to be taken up. Substantial difficulties exist in the translation of fundamental insight into therapeutic methods, including the slow rate of progression of the disease in patients and the difficulty of monitoring cyst growth. The present research study aimed at the identification of lead molecules which could pave the way for therapeutic opportunities and gene targeted strategies to prevent the progressive rate of cyst growth. Thus, with the help of the identification of lead or target molecules to combat PKD, innovative therapeutic interventions is not beyond our reach.

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