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Identification of Mutation of PKD2 Gene for Autosomal Dominant Polycystic Kidney Disease in Selected Gujarat Population

Chaudhary D[1]*, Patel K[1], Patel R[1], Patel V[1]

[1]S.K.Patel college of Pharmaceutical Education and Research, Ganpat University, Kherva- 384012, Gujarat, India*

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ABSTRACT

Autosomal dominant polycystic kidney (ADPKD) is one of the most common hereditary disease with 1 in 1000 in general population. ADPKD is characterised by formation of fluid filled cysts in both kidneys that leads to progressive renal failure. It is a heterogeneous disorder with mutations in two genes pkd1 and pkd2 gene. In Gujarat state ADPKD families the phenotype and genetic background has not previously been characterised. Therefore, in this study 4 subjects with ADPKD from Institute of Kidney Disease Research Centre, Ahmedabad, and Civil Hospital were identified and investigated for genomic study. The aim was to identify pkd2 gene mutation analysis for selected population. The mutation screening of pkd1 gene is difficult because of its size (around 14 kb) and it contains 46 exons. For the same study purpose we have used ABI 3730 SEQUENCER. The sequence data were compared and contrast within a group as well as with the available source of gene bank NCBI. The mutation co-segregating with ADPKD was identified in all 4 subjects for PKD2 gene. Of the four mutations 2 mutations were frameshift mutation, 1 was nonsense mutation and 1 was missense mutation. The maximum total score was matched with data and found to be few exceptions. Finally the mutation detection was done with help of codon code software with output of point mutation and heterozygous mutation. In selected patient out of 4 three sequencing samples were denoted a point mutation. So our findings reveal that the maximum patients showing were in heredited to polycystic kidney disease.

KEYWORDS

PKD2, Autosomal dominant polycystic kidney disease, ADPKD, Mutation, sequencing

INTRODUCTION

POLYCYSTIC KIDNEY DISEASE

Polycystic kidney disease is a disorder interfere that affects the kidneys and other organs. Clusters of fluid-filled sacs, called cysts, develop in the kidneys and with their ability to filter waste products from the blood. The growth of cysts causes the kidneys to become enlarged and can lead to kidney failure. Cysts may also develop in other organs, particularly the liver.[1]

***Address for Correspondence:**
Darshana Chaudhary
Department of Biotechnology,
S.K.Patel college of Pharmaceutical Education and Research
Ganpat University, Kherva ,
Gujarat, India.
E-Mail Id: darshana.biotech@gmail.com

Figure 1: Cyst formation in Polycystic Kidney Disease Patient

Frequent complications of polycystic kidney disease include dangerously high blood pressure (hypertension), pain in the back or sides, blood in the urine (hematuria), recurrent urinary tract

infections, kidney stones, and heart valve abnormalities. Additionally, people with polycystic kidney disease have an increased risk of an abnormal bulging (an aneurysm) in a large blood vessel called the aorta or in blood vessels at the base of the brain. Aneurysms can be life-threatening if they tear or rupture.[2]

The two major forms of polycystic kidney disease are distinguished by the usual age of onset and their pattern of inheritance. The autosomal dominant form (sometimes called ADPKD) has signs and symptoms that typically begin in adulthood, although cysts in the kidney are often present from childhood. Autosomal dominant polycystic kidney disease can be further divided into type 1 and type 2, depending on which gene is mutated. The autosomal recessive form of polycystic kidney disease (sometimes called ARPKD) is much rarer and is often lethal early in life. The signs and symptoms of this condition are usually apparent at birth or in early infancy.[3]

Basic Research Points the Way to Treatments for ADPKD

Polycystin 1 and polycystin 2 are the normal gene products of the genes which, when mutated, are responsible for PKD1 and PKD2, respectively.[4] Research into the structure and function of the polycystin 1 and polycystin 2 proteins—and what goes wrong when they are not produced in sufficient quantity or accurately—is pointing the way to possible treatments for ADPKD.[5]

Polycystin 1 and polycystin 2 are linked transmembrane glycoproteins found on tubular epithelial cells in the kidney. When they work properly, they inhibit cell proliferation via several pathways. Polycystin 1 has a large extracellular domain that functions as a mechanoreceptor located on the primary cilium of renal tubular cells.[6] Polycystin 1 is linked to polycystin 2, which contains a cation channel highly permeable to calcium. When the mechanoreceptor of polycystin 1 is stimulated by calcium-containing urine flowing through the tubule, the calcium channel of polycystin 2 opens and calcium enters the cell. The trio of calcium flux, growth factors, and cyclic adenosine monophosphate (cAMP) determines the proliferative state of renal tubular cells via the extracellular signal-regulated kinase (ERK) pathway. In addition, the tail of polycystin 1 interacts with tuberin, which regulates the kinase activity of the mammalian target of rapamycin (mTOR) pathway, another pathway for cell proliferation.[7]

When the polycystins are not functioning, as in ADPKD, these proliferative pathways are unopposed. However, proliferation can be countered in other ways. One of the prime movers of cell proliferation, acting through adenylyl cyclase and cAMP, is vasopressin. In genetically produced polycystic animals, two antagonists of the vasopressin V2 receptor (VPV2R), OPC31260 and OPC41061 (tolvaptan), decreased cAMP and ERK, prevented or reduced renal cysts, and preserved renal function. Not surprisingly, simply increasing water intake decreases vasopressin production and the development of polycystic kidney disease in rats.[8] Definitive proof of the role of vasopressin in causing cyst formation was achieved by crossing PCK rats (genetically destined to develop polycystic kidneys) with Brattleboro rats (totally lacking vasopressin) in order to generate rats with polycystic kidneys and varying amounts of vasopressin. PCK animals with no vasopressin had virtually no cAMP or renal cysts, whereas PCK animals with increasing amounts of vasopressin had progressively larger kidneys with more numerous cysts. Administration of synthetic vasopressin to PCK rats that totally lacked vasopressin re-created the full cystic disease.[9]

Aim

This study was undertaken to identify the responsible mutation of PKD2 gene in autosomal dominant polycystic kidney disease (ADPKD) in four gujarat subjects. The following questions were addressed:

1. What are the clinical manifestations with ADPKD patient?

2. What are the mutations associated with ADPKD patient?
3. Type of mutation present in the selected patients group.
4. Evaluation of Identified PKD Nucleotide Alteration.
5. To analyse the pathogenic sequence of renal cyst.

Objective of the Study Undertaken

This invention is based upon the discovery by the inventors of the PKD_2 gene associated with Autosomal Dominant Polycystic Kidney Disease ("ADPKD"), the "PKD2 gene" or "PKD2", and a novel protein encoded by this gene.[10] The discovery of the PKD2 gene and the protein encoded by the gene will have important implications in the diagnosis and treatment of ADPKD caused by defects in the PKD2 gene.[11]

The present invention also provides a method for diagnosing ADPKD in a subject comprising detecting the presence or absence of a mutated PKD2 gene in nucleic acid of the subject. The method may be used to determine whether persons in the selected population at large have ADPKD, for identifying persons at risk in developing the disease, i.e. relatives of persons with ADPKD, as well as for confirming diagnosis of ADPKD.[12] The method also is useful for diagnosing ADPKD before clinical manifestations of the disease, i.e. the formation of cysts. Accordingly, as used herein, "subject" may be an embryo, fetus, newborn, infant or adult.

The present invention also provides a method for treating ADPKD caused by a mutated PKD2 gene in a subject in need of such treatment comprising the delivery and expression of a functional PKD2 gene into a sufficient number of cells of the subject, preferably bone marrow stem cells, to treat ADPKD in the subject. As used herein, "functional PKD2 gene" is a gene which when incorporated into a cell's nucleic acid expresses a functional gene product, and includes the wild type PKD2 gene as well as variations thereof.[13] The delivery and expression of the functional PKD2 gene may be accomplished by introducing the functional PKD2 gene into the cells or by correcting the mutation(s) in the subject's PKD2 gene. The functional PKD2 gene may be delivered into the subject's cells by a number of procedures known to one skilled in the art, e.g. electroporation, DEAE dextran, cationic liposome fusion (using both monocationic and polycationic lipids), protoplast fusion, DNA coated microprojectile bombardment, injection with recombinant replication-defective retroviruses, homologous recombination, and the like. Accordingly, a stem cell which expresses the PKD2 gene introduced therein through viral transduction, homologous recombination, or transfection is also provided by the present invention.[14]

MATERIALS AND METHOD

Materials

A) Reagents (DNA extraction by john's method)[15]

solution 1(pH 7.6), solution 2, Nonidet $P_{40,}$ Phenol, chloroform, Isoamyl alcohol, Sodium Acetate (3M), Isopropanol, Ethanol (70 %), TE Buffer (0.3X)

B) Composition

Solution I (pH 7.6)

10 mM Tris – 0.303 gm

10 mM KCl - 0.186 gm

10 mM $MgCl_2$ -0.230 gm

Autoclaved and stored at room temperature.

More volume 250 ml with distilled water.

Solution II

10 mM tris – 0.061 gm

10 mM KCl - 0.031 gm

10 mM $MgCl_2$ – 0.048 gm

2 mM EDTA - 0.037 gm

Autoclaved make 50 ml with distilled water

SDS 0.250 gm

(actually added @ 20 mg/ml)

C) Sequencing reagents(ABI 3730 sequencer)

Volume/Amount in 1 Reaction

Use: 1ul BigDye terminator V3.1

100ng -500ng plasmid or 20-50ng PCR product (depends on size)

3.2pmol primer

3.5ul 5x buffer

D) Primers used in Polymerase Chain Reaction[15,16]

Table 1: Primers used in multiplex PCR reaction

Seq name	Run ID	seq text (5'-3')	Length	conc :(if reconstituted in 200µl of T.E/Water) (pm/µl)	% GC
exon 3 FWD	18375	GAGCAGGTTGCTCTGTTTCC	20	217.8	55
REW	18375	TTCTTCTCAGCACCACATCG	20	189.44	50
exon 4 FWD	18375	AATGGTGCTTGGAAAACTGG	20	189.44	50
REW	18375	CCCATGCCTATGTCCTGAAT	20	227.9	50
exon 5 FWD	18375	TGTCCAGGATGGTATTGCCTA	21	235.6	47.61
REW	18375	GGTGCTGGGATAACTGGCTA	20	231.79	55
exon 6 FWD	18375	TGTTCCTGTATTGGGTGCAT	20	182.22	45
REW	18375	TGTCAAGCAAATGGAAAGCA	20	166.12	40
exon 8 FWD	18376	GGACCTGAGAACCACCACTT	20	169.79	55
REW	18375	TTGAATCCTATCCTTTGTGCTC	22	203.91	40.90

E) PCR kit requirements

Table 2: Multiplex PCR reagents

Components	Tube I	Tube II	Tube III	Tube IV
Sterile water	29.0 µl	29.0 µl	29.0 µl	29.0 µl
10X Taq Buffer A	8.0 µl	8.0 µl	8.0 µl	8.0 µl
dNTP's	2.0 µl	2.0 µl	2.0 µl	2.0 µl
DNA template	5.9 µl	5.9 µl	5.9 µl	5.9 µl
Taq DNA polymerase 1u/µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
Total Volume	50.0 mul	50.0 mul	50.0 mul	5 0.0 mul

Methods

Patients with ADPKD (clinical study)

Population study

Patients were recruited through nephrology centers (5 Informed consent was obtained and blood samples collected for DNA isolation from all available family members). At-risk undiagnosed individuals wishing to take part in the study were examined by abdominal ultrasound.

The genetic study was carried out over 10 patients with ADPKD that required medical assistance at the institute of kidney disease and research centre (IKDRC) during the period between 2011 and followup until march 2012; one member of each family knew for certain of the hereditary character of this disease. Samples from live family members were taken from those patients in whom mutation of the *PKD2* gene was observed. The patients had been diagnosed with ADPKD according to clinical and radiological criteria. The samples were taken after informed consent. Main clinical aspects of patients and relatives with ADPKD where the *PKD2* gene mutation was observed were analysed. Clinical histories provided information on all these data. The main parameters analysed were age of disease onset, aspects related to initial clinical manifestations of the disease, to clinical manifestations occurring during evolution of the disease, to extra-renal manifestations, and to survival and morbidity and mortality.

DNA isolation from 2 ml blood (johns method)[16]

Total genomic DNA was extracted from blood samples using john's method

1. Mix 5 ml of blood with 5 ml of sol I and 120 µl nonidet P_{40} to lyse the cell.

2. Mix well by inverting several times and centrifuging at 1500 rpm for 10 minutes.

3. Discard the supernatant and resuspend the pellet gently in 400 µl of solution II to lyse the nucleus. Mix well.

4. Add equal volume of saturated phenol (400 µl) to the suspension. Now transfer to micro centrifuge tubes and centrifuge at 11,000 rpm for 20 minute at 4˚ C.

5. Transfer the upper phase to a clean micro centrifuge tube and add equal volume (400 µl) of saturated phenol chloroform isoamyl alcohol (25:24:1).

6. Centrifuge the tubes at 11, 000 rpm for 1 min at 4 ˚ C and transfer the upper phase to another micro centrifuge tube.

7. Add equal volume (400 µl) of chloroform isoamyl alcohol (24:1).centrifuge the tube at 11,000 rpm for 2 min at 4 ˚ C and transfer the upper phase to another micro centrifuge.

8. Precipitate the DNA by adding 1/ 10^{th} volume of solution sodium acetate (3M) and equal volume of isopropanol. Keep in 4 ˚ C for 1 hour or precipitate using absolute alcohol.

9. Mix the tube gently and centrifuge at 11, 000 rpm for 10-15 min at 4-8˚C to form DNA – pellet.

10. Discard the isopropanol carefully and wash the pellet with 70% alcohol for at least 3 times (500 µl).

11. Air dry the pellet for overnight.

12. Resuspend the pellet after complete drying in 200 µl of 0.3X TE buffer and incubate at 65˚C for 1 hr in a water bath.

13. Store DNA sample at -20˚ C till further use.

Primer Designing

Primer3

Primer3 is a free online tool to design and analyze primers for PCR and real time PCR experiments. Primer3 can also select single primers for sequencing reactions and can design oligonucelotide hybridization probes. The online tool constitutes some important features like primer detection, cloning, sequencing and Primer listing.

Multiplex PCR to simultaneously amplify more than one locus using multiple primers in single tube.

Setting of the PCR:

1) Add following reagent to the PCR tube in the following order:
2) Mix the contents gently and layer the reaction mix with 50 µl of mineral oil, to prevent evaporation.
3) Carry out the amplification in a thermocycler for 30 cycles using the following reaction conditions:

94 ºC	94 ºC	54 ºC	72 ºC	72 ºC
5.0 minute	30 seconds	30 seconds	30 seconds	7.0 minutes
Denaturation	X 35 cycles			Final extraction

4) Following PCR amplification, add 5 µl of gel loading buffer to each of the
5) PCR tubes.
6) 5) Tap the mixture thoroughly and wait for a few seconds for the 2 layers to Separate.
7) Carefully pipette out 15 µl of reaction mixture (avoiding mineral oil layer) and load onto 1.5 % agarose gel.
8) Load 10 µl of the ready to use marker provided. Note down the order in which the samples have been loaded.
9) Run the samples at 100 volts for 1-2 hours till the tracking dye (bromophemol blue) reaches ¾th of the length of the gel.
10) Visualize the gel under UV transilluminator.

Agarose Gel Electrophoresis

Preparation of Agarose Gel (1.5 %):

1) Prepare 1X TAE by diluting appropriate amount of 50X TAE buffer (for one experiment, approximately 200 ml of 1X TAE is required. Make up 4 ml of 50X TAE to 200 ml with distilled water)
2) Weigh .75 gm of Agarose and add 50 ml of 1X TAE. This gives 1.5 % of agarose gel.
3) Boil till agarose dissolves completely and clear solution results.
4) Mean while place the combs of electrophoresis set such that it is approximately 2 cm away from the cathode.

5) Pour the agarose solution in the central part of the tank when temperature reaches approximately 60°C. Do not generate air bubbles. The thickness of the gel should be around 0.5 to 0.9 cm. Keep the gel undisturbed at room temperature for agarose to solidify.
6) Pour 1X TAE buffer into the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface.
7) Gently lift the combs, ensuring that wells remain intact.
8) Connect the power cord to the electrophoretic power supply according to the convention.
9) Load the samples in the wells in the desired order.
10) Set the voltage to 50V and switch on the power supply.
11) Switch off the power when the tracking dye from the well reaches the ¾th of the gel. This takes approximately 1 hour.

Quantifying DNA using Nanodrop® ND-1000[17]

For Nucleic Acids

1. Turn on the software by clicking on the icon. (Note: there is no ON/OFF switch on the instrument) Choose your "User" folder for storage of your data.
2. The first panel allows for the selection of the required analysis and the "Nucleic Acid" button is on the top left of the screen.
3. The Module startup panel comes up on the screen. For this step, the pedestals are cleaned and a water sample is loaded in order to initialize the instrument. Refer to the manual for illustration of how to load the samples (page 3-1).
4. In order to prepare a report of all readings, the "Recording" button must be pressed. The report can log either 12 or 32 measurements.
5. Choose "DNA, "RNA" or other analysis before doing the "blank" measurement.
6. Before making measurements, a blank must be measured and stored. Place a fresh sample of RNase Free water on the pedestal and press "Blank". Then place a fresh sample on the pedestal and enter "Blank" as the sample name. A straight line should appear on the screen. If this baseline is not flat repeat the "Blank" measurement until it is. Clean the pedestals between readings with a Kimwipe. [***Note: the measurement cycle takes about 10 seconds and is accompanied by some clicking sounds***]
7. Samples can now be measured and stored. The sample names can be recorded in the window and will be indicated on the report page.
8. Remove the sample from the pedestals using a Kimwipe. The pedestals can also be wiped with a wet Kimwipe and then dried between samples.
9. The "PrintScreen" button will give a printout of the spectrum of the sample from the attached printer but makes a large image and uses lots of toner!!!
10. The "Show Report" button will display all the readings associated with the current report. There are 3 options within this window..Save – saves the report as a .jpg; Print – prints the report to the default printer; Exit – returns to the specific application module.
11. The "Re-blank" option establishes a new reference which is used for the calculations of all subsequent samples.
12. The "Exit" command closes all the application modules and supporting options. After clicking the 'Exit' button the user has 10 seconds to cancel the command. If no action is taken, the command is carried out. Note: All measurement data is automatically saved

to an archive file and requires no user action.

13. Always clean the pedestals after all samples are read and do a measurement on a fresh replicate of the blanking solution to confirm that the pedestal is clean.

Sequencing using ABI 3730 Capillary Sequencer[18]

All the coding regions for pkd2 gene were screened. The amplified bands were sequenced in one direction on automatic fluorescent sequencer ABI 3730.

Mutation Detection by Codon Code Aligner[19]

Mutation screening (Sequencing) using codon code aligner:Sequencing was used to confirm and investigate variants. All sequencing analysis was subcontracted to Cogenics® (Cogenics, Meylan, France) and performed by Sanger sequencing method. Capillary sequencing is performed using Big Dye*. Terminator chemistry and sequences are delineated with Applied Biosystems 3730 × l platforms (http: //www.beckmangenomics.com/documents/services/GS_Sanger_Sequencing.pdf). Samples were prepared by diluting 20µl PCR product in 30 µl of ultrapure water. Primers were diluted to obtain a concentration of 2 mM as recommended by the subcontractor.

Finding mutations

When working with sequence traces from genomic PCR sequencing, Codon Code Aligner can help you to find and analyze homozygous and heterozygous mutations ("SNPs"), both point mutations and insertions and deletions *(of course, you can also look for homozygous mutations by simply defining discrepancies and gapsas features).*

RESULT AND DISCUSSION

Assessment of Clinical Characteristics

GFR Measurement and Estimation

GFR was centrally determined at the laboratory of the Clinical Research Center at patient inclusion and one year apart by using the iohexol plasma clearance technique. GFR was determined by the plasma clearance of iohexol. Briefly, on the morning of renal function evaluation, 5 ml of iohexol solution (Omnipaque 300, GE Healthcare, Milan, Italy) was injected intravenously over 2 minutes. Blood samples were then taken at 120, 180, 240, 300, 360, 420, and 480 min for patients with expected mGFR≤40 mL/min, and at 120, 150, 180, 210, and 240 min for those with expected mGFR>40 mL/min. Blood iohexol plasma levels were measured by high-performance-liquid chromatography. The clearance of iohexol was calculated according to a one-compartment model (CL_1) by the formula: CL_1 = Dose/AUC, where AUC is the area under the plasma concentration-time curve. According to Bröchner-Mortensen, plasma clearances were then corrected by using the formula $CL = (0.990778 \times CL_1) - (0.001218 \times CL_1^2)$. GFR values were then normalized to 1.73 m^2 of body surface area (BSA).

Kidney Size

The normal kidney size is 11 – 13 cm in legnth 6 cm wide and 4 cm thick.

The adpkd patients ultrasonographic imaging shows increased in the size of kidney

With 14×6.3 in the left kidney and 14×6.5 in the right kidney.

The largest kidney size is shown by patient number 2 with size 16 x7.7 cm in left kidney and 16×7 cm.

Creatinine

Most of the patints showed increased creatinine levels with 1.88, 1.33, 1.14, 1.13 mg / dl in comparison to normal creatinine level 0.6 – 1.2 mg / dl.

Hypertension in ADPKD Patients

Hypertension is the most common complication in pkd. It may be the primary signal for seeing a doctor. Most of the patients are hypertensive with 150 systolic and 90 diastolic bp.

Table 3: Clinical characteristics of polycystic kidney disease patients

Family	Age of affected member (years)	Sex	Serum creatinine (mg/dl)	B.P	Ultrasound imaging(cm) Size of cyst	GFR ml/min	Vol. (cc)	Size of kidney (cm) (ultrasonography)	
								Left	Right
1	50	F	1.810	150/90	1.5	40	1026	14 x6.3	14.5 x6.5
2	48	F	1.33	145/90	4.3	35	1076	16 x7.7	16 x7.6
3	31	M	1.13	160/95	1.8	55	1056	14.4 x5.7	13.1 x5.6
4	45	F	1.14	130/85	3.4	25	1290	13 x4	12 x3.6

Cause of Hypertension in PKD

Kidneys are important organs in body .they are responsible for filtering blood and keep fluid and electrolyte balanced as well as secret some hormones. Healthy patients can secret renin that can help maintain blood pressure at stable levels. However in pkd, the cyst will enlarge continuously supporting the surrounding nephrons. As a result renal ischemia and anoxia. When the kidneys can not get enough supply of oxygen, they will secret more renin than normal, which stimulate secretion of angiotensin. The blood vessels contract and the bp rises.

Treatment

The drugs given in the treatment of ADPKD patients are ACE inhibitor (enalapril, cilazapril)

Angiotensin receptor (losartan), mTOR (sirolumus) to decrease the size of kidney.

NANODROP® ND-1000 SPECTROPHOTOMETER (QUANTIFICATION)

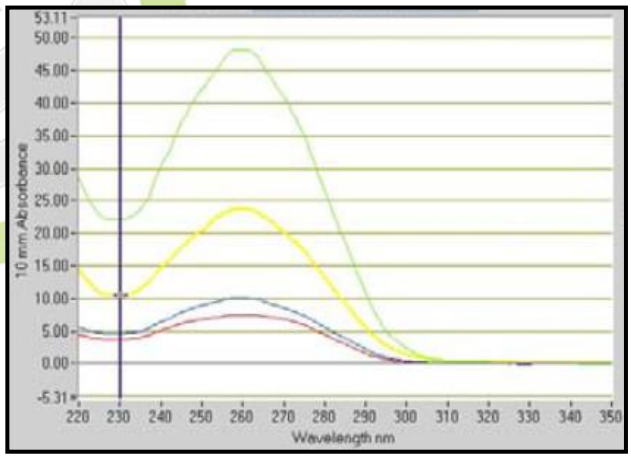


Figure 2: Plot of absorbance vs wavelength.

Table 4: NANODROP 1000 (DNA CONC ng/ µl)

Sample No	DNA CONC(ng/µl)	RATIO (260/280)
1	30	1.95
2	26	1.96
3	35	1.86
4	30	1.88

Note: **260/280**

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. Some researchers encounter a consistent 260/280 ratio change when switching from a standard cuvette spectrophotometer to the NanoDrop® ND-1000 spectrophotometer. The three main explanations for this observation are listed below:

MULTIPLEX PCR

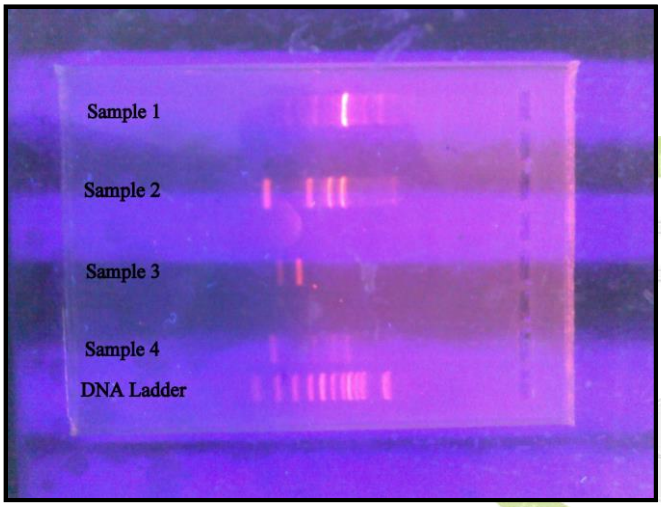


Figure 3 Gel Electrophoresis of amplified PCR Product

Sequence Similarity Analysis Using Blast X

Sequence for sample no 1(SEQUENCE ID NO 1)

GCGCCGGGAAGAAAGGAACATGGCTCC
TGAGGCGCACAGCGCCGAGCGCGGCGC
CGCGCACCCGCGCGCCGGACGCCAGTGA
CCGCGATGGTGAACTCCAGTCGCGTGCA
GCCTCAGCAGCCCGGGGACGCCAAGCG
GCCGCCCGCGCCCCGCGGCTGATGGCTG
GCTGCGCGGCCGTGGGCGCCAGCCTCGC
CGCCCCGGGCGGCCTCTGCGAGCAGCGG
GGCCTGGAGATCGAGATGCAGCGCATCC
GGCAGGCGGCAGCGCGGGACCCCCCGG
CCGGAGCCGCGGCGTTGGGAAGAAAGG
AACATGGCTCCTGAGGCGCACAGCGCCG
AGCGCGGCGCCGCGCACCCGCGCGCCGG
ACGCCAGTGACCGCGATGGTGAACTCCA
GTCGCGTGCAGCCTCAGCAGCCCGGGGA
CGCCAAGCGGCCGCCCGCGCCCCGCGGC
TGATGGCTGGCTGCGCGGCCGTGTTTGG
GAAGCCCT

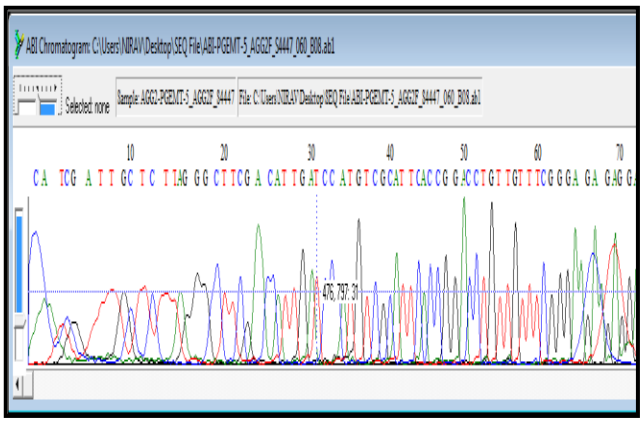


Figure 4 a) ABI File of Seq1

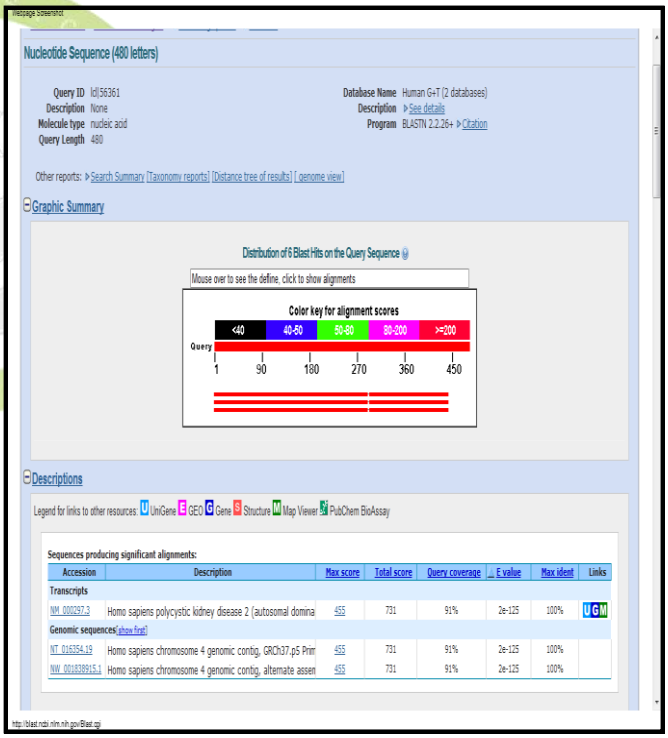


Figure 4 b) BLAST graphical view. A simple graphical overview of the hits found aligned to the query sequence. The alignments are color coded ranging from black to red as indicated in the color label at the top. BLAST table view. A table view with one row per hit, showing the accession number and description field from the sequence file together with BLAST output scores.

Result

The query sequence is represented by the *numbered red bar* at the *top* of the figure. Database hits are shown aligned to the query, *below* the red bar. Of the aligned sequences, the most similar are shown closest to the query. In this case, there are three high-scoring database matches that align to most of the query sequence. The next twelve bars represent lower-scoring matches that align to regions of the query, from about residues 1-100. The *cross-hatched parts* of these bars indicate that the two regions of similarity are on the same protein, but that this intervening region does not match. The high scoring is shown in between 100-300.

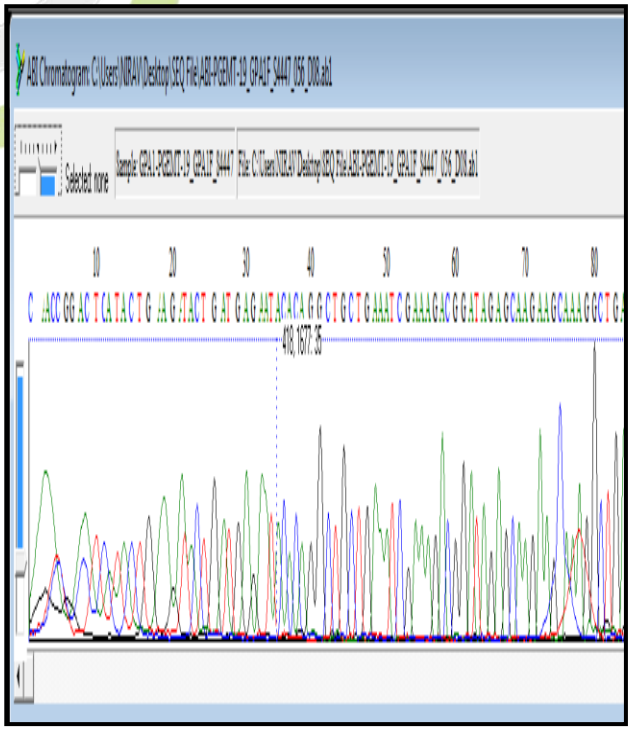
Each line is composed of four fields: (*a*) the gi number, database designation, Accession number, and locus name for the matched sequence, separated by vertical bars (*b*) a brief textual description of the sequence, the definition. This usually includes information on the organism from which the sequence was derived, the type of sequence (e.g., mRNA or DNA), and some information about function or phenotype. The definition line is often truncated in the one-line descriptions to keep the display compact; (*c*) the alignment score in bits.

Higher scoring hits are found at the top of the list; and (*d*) the E-value, which provides an estimate of statistical significance.

The graph shows that the maximum score is of 455 out of total score 731. The ident value is 100 % which means i=our sequence matches to most of the other sequences and the E value is 2e-125 which shows that our sequence is mutated

Sequence for Sample No 2(SEQUENCE ID NO 2)

GCGCCGGGAAGAAAGGAACATGGCTCC
TGAGGCGCACAGCGCCGAGCGCGGCGC
CGCGCACCCGCGCGCCGGACGCCAGTGA
CCGCGATGGTGAACTCCAGTCGCGTGCA
GCCTCAGCAGCCCGGGGACGCCAAGCG
GCCGCCCGCGCCCCGCGGCTGATGGCTG
GCTGCGCGGCCGTGGGCGCCAGCCTCGC
CCCGGGCGGCCTCTGCGAGCAGCGGGGC
CTGGAGATCGAGATGCAGCGCATCCGGC
AGGCGGCAGCGCGGGACCCCCCGGCCG
GAGCCGCGGCGTTGGGAAGAAAGGAAC
ATGGCTCCTGAGGCGCACAGCGCCGAGC
GCGGCGCCGCGCACCCGCGCGCCGGACG
CCAGTGACCGCGATGGTGAACTCCAGTC
GCGTGCAGCCTCAGCAGCCCGGGGACGC
CAAGCGGCCGCCCGCGCCCCGCGGCTGA
TGGCTGGCTGCGCGGCCGTGTTTGGGAA
GCCCTTGCGCCGGGAAGAAAGGAACAT
GGCTCCTGAGGCGCACAGCGCCGAGCGC
GGCGCCGCGCACCCGCGCGCCGGTTGGC
TGACCTAGTCAATGGTGAACTCCAGTCG
CGTGCAGCCTCAGCAGCCCGGGGACGCC
AAGCGGCCGCCCGCGCCCCGCGGCTGAT
GGCTGGCTGCGCGGCCGTGGGCGCCAGC
CTCGCCGCCCCGGGCGGCCTCTGCGAGC
AGCGGGGCCTGGAGATCGAGATGCAGC
GCATCCGGCAGGCGGCAGCGCGGGACC
CCCCGGCCGGAGCCGCGGCGTTGGGAAG
AAAGGAACATGGCTCCTGAGGCGCACA
GCGCCGAGCGCGGCGCCGCGCACCCGCG
CGCCGACGCCAGTGACCGCGATGGTGAA
CTCCAGTCGCGTGCAGCCTCAGCAGCCC
GGGGACGCCAAGCGGCCGCCCGCGCCCC



GCGGCTGATGGCTGGCTGCGCGG

Figure 5 a) ABI File SEQ2

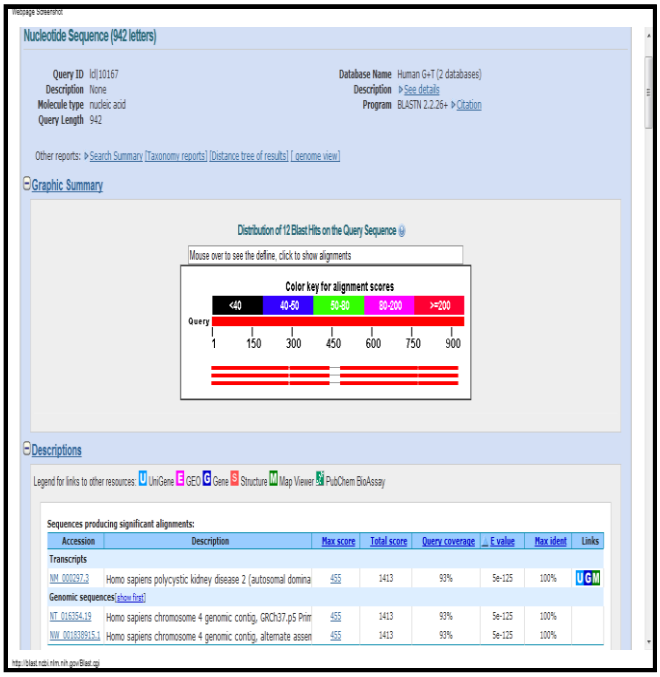


Figure 5 b) BLAST graphical view. A simple graphical overview of the hits found aligned to the query sequence. The alignments are color coded ranging from black to red as indicated in the color label at the top. BLAST table view. A table view with one row per hit, showing the accession number and description field from the sequence file together with BLAST output scores.

Results**:** This sequence has shows maximum score of 168 and maximum indent of 68%.

The E value is 5e-125 which shows that our sequence does not match to the query sequence and it has got mutation.

Sequence for Sample No 3 (SEQUENCE ID NO 3)

TGGGGCAAGATGCTATCCCAAATCTCTC
TGACAACAAAACTCATTCTTATCACTCTA
CTATTTTCATAGAGTTGCCAAATGCTTGG
TTATGCAAACGATGCAGGCAGGGGCAAG
ACAGCGGCTGAGCTTGGAACTTTTTCAG
AGATGTTTCCTTTGCTTTTAGTTCACAGA
AGGCTCCTTATTGGATGGGCTGTACTGG
AAGATGCAGCCCAGCAACCAGACTGAA
GCTGACAACCGAAGTTTCATCTTCTATG
AGAACCTGCTGTTAGGGGTTCCACGATT
ACGGCAACTCCGAGTCAGAAATGGATCC
TGCTCTATCCCCCAGGACTTGAGAGATG
ATTGGGGGAGATCTAGGTACGAATCTGG
TTCAGGAACGGGTGACGCTATTGCTACA
AACTTTTCTTTACAGGAATAAAGCATGG
CATTTTGCAGACTTCGAAGCCCCGACCG
ATAACTA

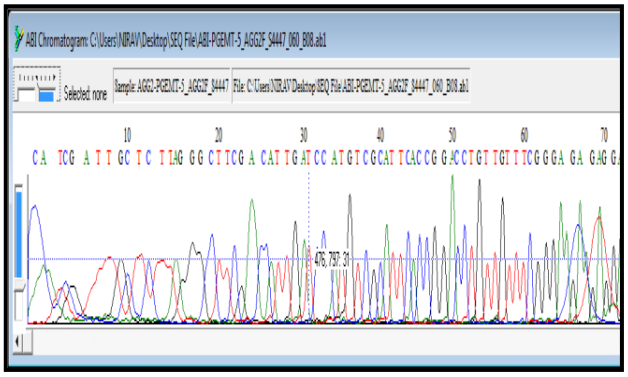


Figure 6 a) ABI File SEQ 3

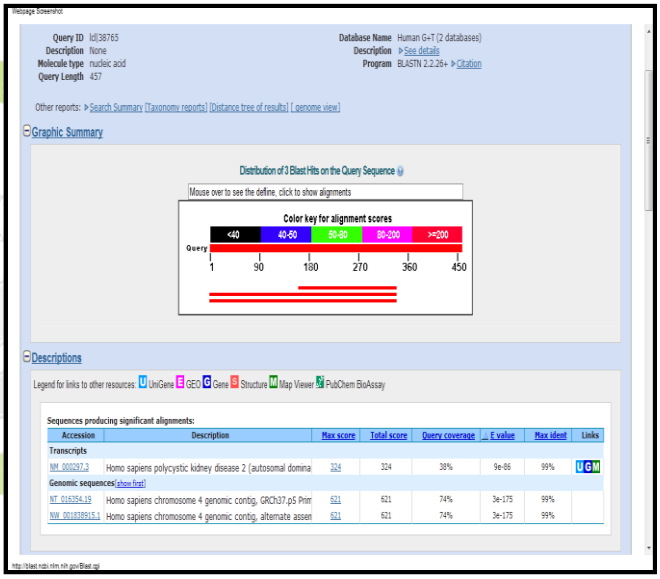


Figure 6 b) BLAST graphical view. A simple graphical overview of the hits found aligned to the query sequence. The alignments are color coded ranging from black to red as indicated in the color label at the top. BLAST table view. A table view with one row per hit, showing the accession number and description field from the sequence file together with BLAST output.

The graphical output (shown in figure) gives a quick overview of the query sequence and the resulting hit sequences. The hits are colored according to the obtained alignment scores.

The sequence of this gene shows resemblance to our gene product polycystic kidney disease gene.

The maximum score is 234, the E value is 9e-80 which shows that the mutation is present in our sequence, the indent value is 98%.

Sequence for Sample No 4 (SEQUENCE ID NO 4)

CAACCGGACTCATACTGAAGATACTGAT
GAGAATACACAGGCTGCTGAAATCGAAA
GACGGATAGAGCAAGAAGCAAAGGCTG
AAAAGCATATTCGGAAGCTTTTGCTACT
TGGTGCTGGGGAATCTGGAAAATCTACA
ATTTTTAAGCAGATAAAACTTCTATTCCA
AACGGGATTTGATGAAGGAGAACTAAA
GAGCTATGTTCCAGTCATTCATGCCAAT
GTCTATCAGACTATAAAATTATTGCATG
ATGGAACAAAGGAGTTTGCTCAAAATGA
AACAGATTCTGCTAAATATATGTTATCTT
CTGAAAGTATTGCAATTGGGGAGAAACT
ATCTGAGATTGGTGGT

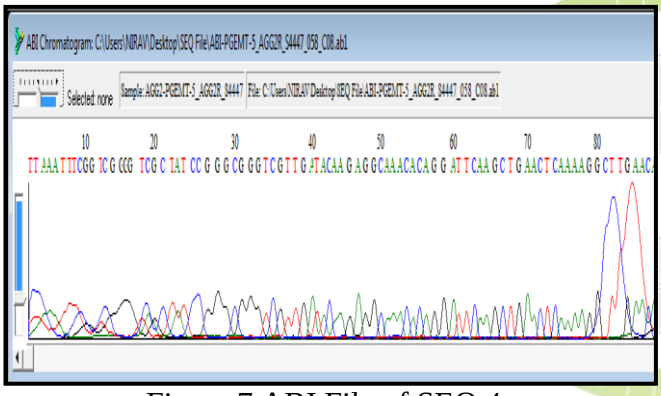


Figure 7 ABI File of SEQ 4

Result: The blastn result of this sequence show significant similarity to our gene because the E value for this sequence is 5e-25 which shows that there is mutation in this gene.

Multiple Sequence Alignment of Four Sequences

Figure 8 a) Multiple seq alignment, BLAST graphical view. A simple graphical overview of the hits found aligned to the query sequence. The alignments are color coded ranging from black to red as indicated in the color label at the top.

Figure 8 b) Multiple seq alignmtent, BLAST table view. A table view with one row per hit, showing the accession number and description field from the sequence file together with BLAST output scores.

Figure 8 c) Multiple sequence Alignment view of BLAST results. Individual alignments are represented together with BLAST scores and more

Result

The multiple sequence alignment of four sequences with reference seq shows the E value 0 which shows that there is almost no chance that alignment can occur by chance.

The total score is 1362 bits out of total score 3749.

The identities are 768 / 781.The gaps show the deletions or insertions. In the alignment view one can manually inspect the individual

alignments generated by the BLAST algorithm. This is particularly useful for detailed inspection of the sequence hit found (subject) and the corresponding alignment. In the alignment view, all scores are described for each alignment and the start and stop positions for the query and hit sequence are listed. The strand and orientation for query sequence and hits are also found here. In most cases, the table view of the results will be easier to interpret than tens of sequence alignments.

Mutation Screening of PKD2 Gene

Result

Mutation screening was performed by direct sequencing using ABI 3730 from patients blood. The whole pkd2 coding region was investigated from four ADPKD families. The mutations found were listed in the above table (6.4)

Table 5: Mutations and Polymorphism in pkd2 gene

Sub	Mutation	Location	Nucleotide change	Effect on Coding sequence
1	Nonsense mutation	EXON 6	C to T	Arg stop at 309
2	Insertion/ deletion	EXON 4	insertion of C at 480	frameshifting after 78
3	Inertion/ deletion	EXON 8	del of C at 930	frameshifting after 325
4	Missense	EXON 5	T to G at 451	Try to Gly 83

Nonsense Mutations

One nonsense mutation was identified in our sample. The nucleotide change is from C to T that substitute arginine at stop codon 309.

Mutations Causing Frameshifting

In subject 2 the mutation is caused by insertion of C at position 480 causing frameshifting after codon 78.

Similarly in subject 3 there is deletion of C at 930 causing frameshifting after codon 325.

Missense Mutation

The only change observed in subject 4 was T to G at substitution in exon 5 converting try to gly changing codon 83.

Discussion

Retrieving information from the following website www.pkdb.mayoclinic.edu, we have compared our mutations with that of the reported mutations. The comparison shows that the mutations found in Gujarat families are novel.

In addition to functional studies the spectrum of mutations leading to ADPKD may provide insight into the role of proteins.

Evaluation of Identified PKD Nucleotide Alterations

Several novel nucleotide sequence alterations in the PKD2 genes have been identified that are associated with ADPKD. The mutations in PKD2 were found by direct sequencing of the genes and the pathogenicity of the mutations determined using a combination of various analyses and algorithms. The mutations in the PKD2 genes identified as pathogenic can be used to detect and/or predict the occurrence of ADPKD in an individual. This is important clinically in diagnostic and prognostic analysis of the genes for ADPKD.[23]

Accordingly, the invention relates to methods of detecting or predicting the occurrence of ADPKD in an individual. In one aspect, the present invention relates to a method of detecting or predicting the occurrence of autosomal dominant polycystic kidney disease (ADPKD) in an individual comprising detecting the presence of one or more nucleotide sequence alterations in a PKD2 gene having the nucleotide sequence of SEQ ID NO 3 wherein said one or more alterations are selected from the group consisting of: a deletion of C at nucleotide position 930 and SEQ ID NO 2 consisting of insertion of C at nucleotide position 480.

Numerous novel nucleotide alterations in PKD have been identified (see Table 6.4). These sequence alterations were then evaluated to determine whether they were pathogenic, this is, resulted in an altered PKD gene product (e.g., protein, polypeptide). A "nucleotide sequence alteration" or "nucleotide alteration" or "mutation" refers to a nucleotide sequence modification including one or more substitutions (transitions or transversions), deletions (including loss of locus), insertions (including duplications), translocations, inversions and/or other modifications relative to a normal PKD gene (e.g., SEQ ID 2 or SEQ ID NO:3). Thus, a nucleotide alteration/change in a PKD2 nucleotide sequence (e.g., DNA or mRNA) can be a deletion, insertion, substitution or inversion, or can be silent such that there is no change in the reading frame of a polypeptide encoded by the PKD polynucleotide. Pathogenic mutations are those nucleic acid alterations that result in an amino acid change (e.g., a non-silent or non-conservative change) and/or introduces a STOP codon into the nucleotide sequence, or changes nucleotide sequence involved in transcription or translation of the PKD2 nucleotide sequence; for example, a change that results in altered splicing of a PKD2 gene transcript into an mRNA. In table 6.4 the SEQ ID NO 1 shows non sense mutation, Arg stop codon at nucleotide position 309.

An "amino acid alteration" refers to an amino acid modification including a substitution, a frameshift, a deletion, a truncation and an insertion, and/or other modifications relative to the normal PKD amino acid sequence). Thus, a mutation in a PKD gene sequence can result in the expression of a truncated PKD polypeptide, or even a complete loss of expression of the PKD polypeptide.

Nucleotide sequence alterations identified in PKD2 genes can be evaluated for pathogenicity in a number of ways. Mutant PKD nucleotide sequence can be compared to ref seq PKD sequence and the effect of the nucleic acid sequence alterations on amino acid codon(s) assessed. For example, a change in nucleotide sequence that produces a stop codon (e.g., UGA, UAA, UAG) or a frameshift, which generally results in a nonsensical polypeptide and/or also produces a stop codon, or that alters a consensus donor/acceptor splice site would result in a non-functional PKD protein, a truncated PKD protein, or obliterate its expression altogether. These mutations would be expected to be pathogenic and thus correlates with ADPKD.[24]

The above table 6.4 shows two frameshift mutations that are pathogenic in nature. The SEQ ID NO 2 Shows frameshifting after codon 78, which causes a shift in the reading frame of the codons in the mRNA, thus eventually lead to the alteration in the amino acid sequence at protein translation.

The identification of mutations associated with ADPKD provides conclusive diagnostic information, allows the blood relatives of an individual to be pre-symptomatically and inexpensively evaluated for counseling and planning using targeted PKD gene analysis and allows prospective living-related kidney donors to be tested and subsequently accepted or rejected for donation with greater certainty.

CONCLUSION

In our study group of ADPKD patients it found that the mutation occurs in the PKD2 genes. It detects the missense mutation and nonsense mutation from the interpretation of sequencing analysis in Gujarat population. The present mutation analysis of PKD2 gene in Gujarat population with ADPKD may contribute to a better understanding of the genetic diversity between different ethnic groups and enrich the mutation database. Besides, evaluating the pathogenic potential of novel variations should also facilitate the clinical diagnosis and genetic counseling of the disease, particularly through the direct gene approach.

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