Single-cell Sequencing-based Pre-implantation Genetic Testing-M (PGT-M) of the Heterozygous Mutations of PKD1 Gene

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Research

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Abstract

Objectives: Polycystic kidney disease (PKD) is a common autosomal monogenic genetic disease. PKD1 mutation accounts for about 85% of ADPKD patients. Pre-implantation genetic testing-M (PGT-M for monogenic) is an important approach to prevent the transmission of genetic diseases from parents to the offspring.

Design: In this study, We have identified the family linkage and mutation site in embryos with NGS-based SNP phasing and Sanger Sequencing.

Methods: Multiple Annealing and Looping Based Amplification Cycles (MALBAC) method was employed to amplify the whole genome of trophoblast cells. Copy Number Variant (CNV), and single nucleotide polymorphism (SNP) were used to assess the embryo state.

Results: In the eight embryos, Embryo 02 and Embryo 04 were removed from further analysis because of the Multiple chromosomes abnormal (2 of 8, 25%). Embryo 05, Embryo 06, Embryo 07, and Embryo 08 were judged as 46,XN,-15q(q23→qter,~31M,×1,mos*), 45,XN,-16(×1), 47,XN,+2(×3),-7p(pter→p14.3,~35M,×1,mos*), and 46,XN, +16(×3,mos*),-20p(pter→p11.23,~23M,×1,mos*),+22(×3,mos*), respectively (4 of 8, 50%). Meanwhile, Embryo 01 and Embryo 03 were judged as 46, XN (2 of 8, 25%). The results of SNP phasing and Sanger Sequencing suggested that Embryo 01 and Embryo 05 had none of PKD1 gene mutation.

Limitations: Up to now, PGT-M is complicated and expensive. Meanwhile, PGT-M obtains the final diagnosis through invasive manipulation of embryos, which may bring adverse effects on offspring

Conclusion: NGS-based single-cell sequencing combined with CNV, Sanger Sequencing, and SNP phasing is a reliable testing system for PGT-M application. This work presented here would provide a detailed understanding of the NGS-based single-cell sequencing application in ADPKD.

Introduction

Polycystic kidney disease (PKD) is a common autosomal monogenic genetic disease. It is genetically divided into two categories, including dominant inheritance and recessive inheritance. Both types of inheritance can cause bilateral kidney disease [1–3]. Among them, autosomal dominant polycystic kidney disease (ADPKD) is the most common. Three genes including PKD1, PKD2, PKD3 have been identified to be associated with ADPKD. Among them, PKD1 mutation accounts for about 85% of ADPKD patients. PKD2 is located on chromosome 4q21-23, and its mutation is related to about 15% of ADPKD patients. Meanwhile, PKD3 mutations have been proved to be existed in a few families [4]. The main feature of the disease is the continuous formation and expansion of fluid-filled vesicles derived from renal tubular epithelial cells [5]. Fluid-filled vesicles can cause a variety of lesions, including renal tissue damage, renal function changes, hematuria, and proteinuria, which eventually lead to renal failure. In addition to affecting the kidney, ADPKD can also cause extrarenal diseases such as liver cysts, pancreatic

cysts, heart valve disease, colon diverticula, and intracranial aneurysms [6]. ADPKD is a delayed dominant inheritance disease. Patients usually show clinical symptoms at about 40 years of age. However, patients had mostly passed on the disease-causing genes to the offspring [7]. At present, there is no effective treatment for ADPKD [8]. Therefore, it is very important to perform a pre-symptom genetic diagnosis of ADPKD families at risk. In particular, prenatal diagnosis and preimplantation genetic diagnosis of high-risk fetuses or embryos are the keys to controlling their occurrence and development.

PGT-M is a reliable detection method used in IVF to select embryos without genetic defects for transplantation [9]. In the past, PGD methods mainly include fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), array comparative genomic hybridization array (aCGH)), and Single nucleotide polymorphism microarray (SNP chip) [10]. With the development of various technologies such as sequential embryo culture, embryo biopsy, embryo vitrification technology, whole-genome amplification (WGA) technology, and next-generation sequencing (NGS), single-cell sequencing Technology has been increasingly applied in PGT-M [11]. Compared with traditional PGT-M methods, single-cell sequencing-based PGT-M has many advantages, including large throughput, fast detection speed, a high degree of automation, high sensitivity and specificity, and low detection costs. Meanwhile, this technology can simultaneously detect embryo chromosomes and pathogenic mutations. Moreover, NGS data with a higher resolution could help to detect abnormalities in the embryo. Therefore, the above advantages have made single-cell sequencing-based PGT-M possible to achieve effective diagnosis in the clinic [12]. For example, Treff et al. Developed a semiconductor-based capture sequencing based on the diagnosis of monogenic diseases in 6 couples of preimplantation embryos. These diseases included 2 cases of cystic fibrosis, Walker-Warburg syndrome, and familial Autonomic dysfunction, X-linked hypophosphatemic rickets, neurofibromas. The results showed that this method is 100% reliable compared with the traditional methods [13]. Fiorentino et al. had compared the chromosome aneuploidy with CGH and NGS detection methods on 190 cases of blastomere WGA products. The results show that NGS is highly consistent with CGH. NGS is more advantageous in terms of cost and accuracy [14]. In addition, Yan et al. had used MASALA to successfully help one case with an autosomal dominant genetic disease and one case with a recessive genetic disease on the X chromosome to produce healthy offspring [15].

In this study, we have employed MALBAC amplification method, Single Nucleotide Polymorphism (SNP), and CNV detection approach to perform the PGT-M for the family with PKD1 mutations. We aim to help the family with the hereditary disease produce a healthy offspring.

Method And Materials

Patients

In this study, a family with a PKD1 mutation (c.4379T > G) was recruited. The husband and wife had been fully informed about the whole experimental process and signed informed consent documents. This study was approved by the Ethics Committee of Heping Hospital Affiliated to Changzhi Medical College.

The 42 years old husband and his mother had been diagnosed with PKD1 mutation (c.4379T > G). The 41 years old wife and husband's father had been diagnosed without PKD1 mutation. Semen was collected by masturbation for testing. Sperm concentration, vitality, and forward motion ratio were analyzed using a computer-assisted sperm quality detection and analysis system (CFT-9202). The diagnosis of azoospermia requires more than 3 centrifugal sediments. Sperm morphology assessment and diagnostic criteria for oligospermia spermatozoa follow the guidelines issued by the WHO in 2010 [16].

PKD1 sequencing with gDNA

In this study, we took 4 ml of venous blood from a patient and placed it in an EDTA anticoagulation tube. Venous blood is stored in a refrigerator at -40 ° C for short periods. DNA was extracted using a commercial blood genomic DNA kit (CWBio, Chian). The specific nested PCR primers of the PKD1 gene were synthesized to amplify the whole PKD1 gene. Subsequently, the long-range PCR amplification product was used as a template for the second round of PCR amplification. Primer sequences and PCR amplification system was followed as mentioned in a previous study[17]. Sanger sequencing analysis of the PKD1 gene amplification product was performed on an ABI 3730 machine. The sequenced PKD1 gene sequence was used to align with the NCBI reference genome sequence.

PGT-M

According to the woman's situation, the long ovulation-promoting protocol was selected [18]. After the mature eggs were taken out, a single sperm was injected with ICSI technology. The blastocyst was cultured (Quinn's Blastocyst Medium, Sage, USA) to 5–6 Days. Subsequently, the embryo biopsy was performed with a laser punch (OCTAX laser shot system MTG, Germany). In brief, blastocysts are cultured to day 3. We first punch A 15 μm holes in the zona pellucida. After the blastocyst trophoblast cells were partially hatched (20 cells) on the 5th to 6th days, 4 to 6 cells were carefully pipetted with a 35 μm inner diameter biopsy needle (Humagen Charlottesville, NC). After the biopsy, the cells were vitrified and frozen. The removed blastomeres were washed three times in PBS and placed in a container containing 2. 5 μl PBS solution of PCR test tube, which was sent to the genetics laboratory for further analysis. After 3 months, a single non-affected euploid embryo was selected for frozen embryo transfer. Luteal support was routinely performed after surgery [19]. After tao weeks, the blood β-hCG is tested to determine whether it is a biochemical pregnancy. B ultrasound was performed after four weeks to determine the clinical pregnancy and gestational sac number. At 22 weeks gestation, we performed an amniocentesis to determine if the fetus carries the mutation site. This case was followed up until the child was born.

Haplotype linkage analysis of embryo

Sixty high-frequency SNP loci were selected within the 2M region of upstream and downstream of the PKD1 gene (47 Kb). Ion AmpliSeq Designer (https://www.ampliseq.com/) was used to design multiple PCR primers. 20 ng MALBAC whole-genome amplification product was used as the template. PCR thermal cycles were listed as following: 95℃ for 10 min, 35 cycles of 95℃ for 30 s, 58℃ for 30 s, 72℃

for 45 s, then 72℃ for 5 min, and 4℃ forever. The 20 ul PCR amplification system was listed as following: 5 ul 2x Goldstar Master Mix (CWBio, Chian), 1 ul Primer mixture (10 pmol), 30 ng DNA template, and 3 ul ddH_2O. After ligation, purification, library amplification, and illumina HiSeq 2500 sequencing platform. We had employed the bioinformatics method to analyze the effective SNP sites (upstream and downstream ≥ 2) in this family to establish a genetic linkage relationship (if the proband is a heterozygous AB site, the father is AA, and the mother is AB. Then the B site is the mother's effective site, the A site is the father's effective site. If the proband is a heterozygous site AB, the father is AB, and the mother is AA, then the B site is the father's effective site, and the A site is the mother's effective site). In this study, we used the linkage relationship to judge the pathogenicity of the embryo.

Sanger sequencing analysis of mutation site of embryo

We designed specific primers for the pathogenic site of the family PKD1 gene. 50 ng MALBAC whole genome amplification product was used as DNA template. PCR amplification was performed on the pathogenic site. The amplification procedure and system were followed as mentioned in the method section of this study. After examination of PCR products with 1% agarose gel electrophoresis, ABI373 was used to directly sequence the amplified PCR products.

Copy number variant analysis of embryo

In this study, 120 ng of MALBAC whole-genome amplification product was digested by enzyme digestion. The digested DNA was treated with ligation, purification, and library PCR amplification. The final library was sequenced with low-depth (0.03X of the whole genome) on illumine Hiseq 2500 sequencing platform. Copy number variation analysis of 24 chromosomes was performed using the previous method [20].

Results

Patients and IVF

In this study, Fig. 1 suggested that the family linkage of this PKD1 family. The proband was 42 years male who had been diagnosed with a heterozygous mutation of the PKD1 gene (c.4379T > G). The proband’ wife and father were diagnosed without the PKD1 gene mutation. Meanwhile, the proband’s mother was diagnosed with the same heterozygous mutation of the PKD1 gene (c.4379T > G). In this study, we have performed the pedigree validation and mutation site validation to confirm the mutation allele and the genetic relationship between three family members. Figure 2 showed that the mutation site validation results, which was consistent with the previous diagnosis outcomes. Meanwhile, an analysis of 60 SNPs located upstream and downstream of the mutation sites suggested that the husband’s father and mother who provided the sample were the biological parents of the proband in this study (data not are shown). Further examination of the couple revealed no other organic or hormonal diseases that can affect fertility. After the long-protocol treatment, 13 mature eggs were obtained. Intracytoplasmic sperm injection (ICSI) was performed 4 h after egg retrieval. Among them, 10 eggs were fertilized normally. In the

end, eight blastocysts were cultured to the 5th or 6th day. Therefore, eight blastocysts were biopsied on day 5 post-insemination. The biopsied trophoblast cells were transferred into 200uL polymerase chain reaction (PCR) tubes with 5.0 μL of lysis buffer (Yikon Genomics) for WGA analysis.

CNV

In this study, eight samples were amplified successfully. CNV analysis of eight samples suggested that Embryo 02 and Embryo 04 were judged as Multiple chromosomes abnormal (2 of 8, 25%). Therefore, the subsequent SNP linkage analysis and mutation site analyses will not be performed in those three Embryos. Embryo 05, Embryo 06, Embryo 07, and Embryo 08 were judged as 46,XN,-15q(q23→qter,~31M,×1,mos*), 45,XN,-16(× 1), 47,XN,+2(× 3),-7p(pter→p14.3,~35M,×1,mos*), and 46,XN, + 16(× 3,mos*),-20p(pter→p11.23,~23M,×1,mos*),+22(× 3,mos*), respectively (4 of 8, 50%). Meanwhile, Embryo 01 and Embryo 03 were judged as 46, XN (2 of 8, 25%) (Table 1, Supplementary Fig. 1, Supplementary Fig. 2, Supplementary Fig. 3, Supplementary Fig. 4, Supplementary Fig. 5, Supplementary Fig. 6, Supplementary Fig. 7, and Supplementary Fig. 8). Based on the uncertainty of chromosome mosaic judgment, we have carried out SNP linkage analysis and mutation site analyses to Embryo 01, Embryo 03, Embryo 05, Embryo 06, Embryo 07, and Embryo 08.

Table 1
Summary of the CNV, mutation site, and SNP linkage analysis in all eight samples

Sample	CNV	Pathogenic sites and SNP test results
		c.4379T > G (Paternal)
01	46,XN	Nonmutation
02	Multiple chromosome abnormal	Multiple chromosome abnormal
03	46, XN	Mutation
04	Multiple chromosome abnormal	Multiple chromosome abnormal
05	46,XN,-15q(q23→qter,~31M,×1,mos*)	Nonmutation
06	45,XN,-16(× 1)	Mutation
07	47,XN,+2(× 3),-7p(pter→p14.3,~35M,×1,mos*)	Mutation
08	46,XN, + 16(× 3,mos*),-20p(pter→p11.23,~23M,×1,mos*),+22(× 3,mos*)	Mutation

Mutation site and SNP lineage analysis

In this study, we have performed SNP linkage analysis and mutation site analyses to each Embryo after CNV examination. For Embryo 01, six available SNP sites were obtained from the 60 SNP sites tested.

SNP linkage analysis of Embryo 01 suggested that this embryo carried none of the mutation, which is consistent with the results of the mutation site examination (Supplementary Fig. 1). For Embryo 3, six available SNP sites were obtained from the 60 SNP sites tested. SNP linkage analysis of Embryo 03 suggested that this embryo carried the PKD1 mutation, which is consistent with the results of the mutation site examination (Supplementary Fig. 3). Furthermore, SNP lineage analysis of Embryo 05 suggested that this embryo carried none of the mutation, which is consistent with the results of the mutation site examination (Supplementary Fig. 5). Meanwhile, SNP lineage analysis of Embryo 06 suggested that this embryo carried the mutation, which is consistent with the results of the mutation site examination (Supplementary Fig. 6). It was notable that this embryo lacked a chromosome 16. Therefore, the number of available SNPs sites was less than other embryo. Moreover, SNP lineage analysis of Embryo 07 suggested that this embryo carried the mutation, which is consistent with the results of the mutation site examination (Supplementary Fig. 7). In addition, SNP lineage analysis of Embryo 08 suggested that this embryo carried the mutation, which is adapted to the results of the mutation site examination (Supplementary Fig. 8). Ultimately, we have summarized the CNV, mutation sites, and SNP lineage analysis of Embryo 01, Embryo 02, Embryo 03, Embryo 04, Embryo 05, Embryo 06, Embryo 07, and Embryo 08 in Table 1. Based on the clinical characteristics, Embryo 01 at blastocyst 5 stages were transferred into the uterus of the patient. A healthy baby was born 38 weeks after transplantation. Sanger sequencing result of the new-born baby showed that it carried without the PKD1 mutation.

Discussion

It is estimated that there are about 12. 50,000 ADPKD patients. Approximately 50% of ADPKD patients require kidney replacement therapy. Therefore, the cost of polycystic kidney-related treatments in the world can reach tens of billions of dollars each year. Among them, ADPKD-related renal dysfunction accounts for about 10% of the causes of renal failure [21]. ADPKD can cause multiple organ dysfunctions and complications, such as liver, pancreas, spleen cysts, intracranial aneurysms, and so on. There is currently no effective cure for ADPKD. Patients have long been under a heavy psychological burden and are prone to severe mental illness. Therefore ADPKD has become a global public health burden [22].

There is genetic heterogeneity in the disease-causing genes of ADPKD. There are currently two main mutation genes known to cause the disease. ADPKD patients with PKD1 gene mutations account for about 85–90%. At the same time, PKD2 genes and unknown mutations account for about 10–15% of the remaining [23]. A previous study had performed genetic screening of 148 ADPKD patients and revealed that 118 patients had mutations in the PKD1 gene in China [24]. Patients with PKD1 mutations have more severe disease and symptoms that appear earlier than those with PKD2 mutations [25]. In this study, we have identified a family with PKD1 c.4379T > G mutation, which had been judged as likely pathogenic in PKDB(https://pkdb.mayo.edu/cgi-bin/v2_display_mutations.cgi?apkd_mode=PROD). In this clinical trial, we had identified this mutation was a pathogenic site in this family.

ADPKD has been reported to cause azoospermia, severe oligozoospermia, and weak spermatozoa in patients. Among them, weak spermatozoa are the most common, accounting for about 40% of ADPKD

patients [26–27]. In this study, there were no significant abnormalities in sperm parameters. ADPKD causes abnormal sperm parameters that may form reproductive tract cysts and/or obstruction of seminal tract emptying. Up to now, there is currently no effective treatment for ADPKD related abnormal sperm parameters [28]. Previous studies have shown that patients with ADPKD combined with male infertility can achieve better clinical pregnancy rates and live births by applying ICSI [29]. However, most couples are still very concerned about whether the offspring will continue to develop the disease in clinical work and genetic counseling. Therefore, PGT-M is an ideal method to block the inheritance of this disease.

PGT-M mainly refers to the analysis of the genetic material of embryos formed by *in vitro* fertilization. This method can diagnose whether the embryo has certain genetic abnormalities. An embryo with no genetic disease is selected for implantation in the uterine cavity to obtain a normal fetal diagnosis [30]. PGT-M avoids the huge economic and spiritual pressure caused by selective abortion. Meanwhile, PGT-M also solves the problem of fertility in some patients with chromosomal abnormalities or monogenic diseases, which significantly reduces the number of births of children with genetic diseases. However, there are still many problems with this technology in actual clinical applications. For example, the operation is complicated and expensive. Meanwhile, this technology cannot be popularized on a large scale. In addition, PGT-M obtains the final diagnosis through invasive manipulation of embryos, which may bring adverse effects on offspring [31]. Therefore, PGT-M requires long-term follow-up of large samples in the future.

Conclusion

In summary, we demonstrated that NGS-based single-cell sequencing combined with CNV, Sanger Sequencing, and SNP phasing is a reliable testing system for PGT-M application. Meanwhile, PGT-M can effectively prevent births in patients with PKD1 mutation. This work would provide a detailed understanding of the NGS-based single-cell sequencing application in ADPKD.

Declarations

Ethics statement

The Research Ethics Committee of the Heping Hospital Affiliated to Changzhi Medical College approved the collection of tissue samples for research (Hep-2018-023).

Authors’ contributions

Na Li and Cong Xiu Miao for data analysis and writing of the manuscript. HuiLing Bi, Dan Li, and Min Li for the discussion of and Hui Miao for discussion and comments on an earlier version of the manuscript. All authors read and approved the final manuscript.

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Conflicts of interest

All authors declare no conflict of interest.

Informed consent statement

Not applicable.

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Figures

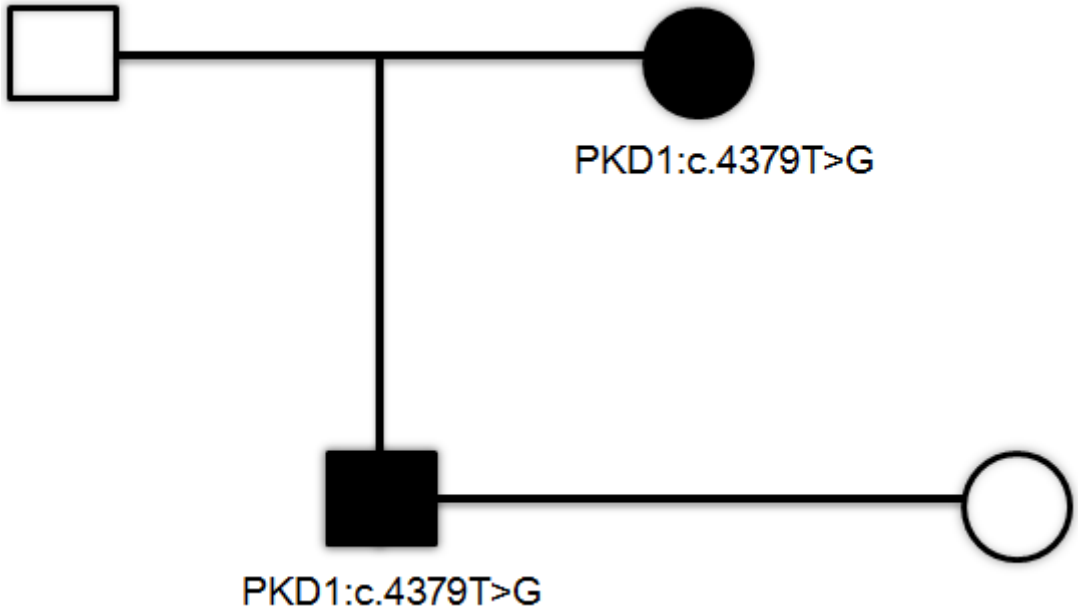


Figure 1

Family linkage and mutation sites in this hereditary deafness family

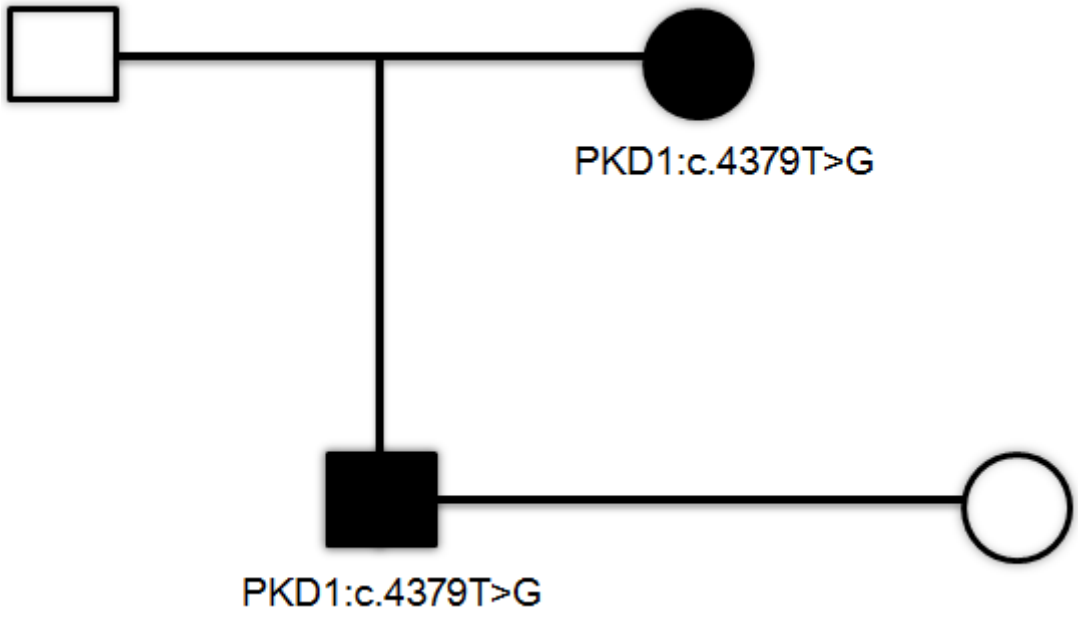
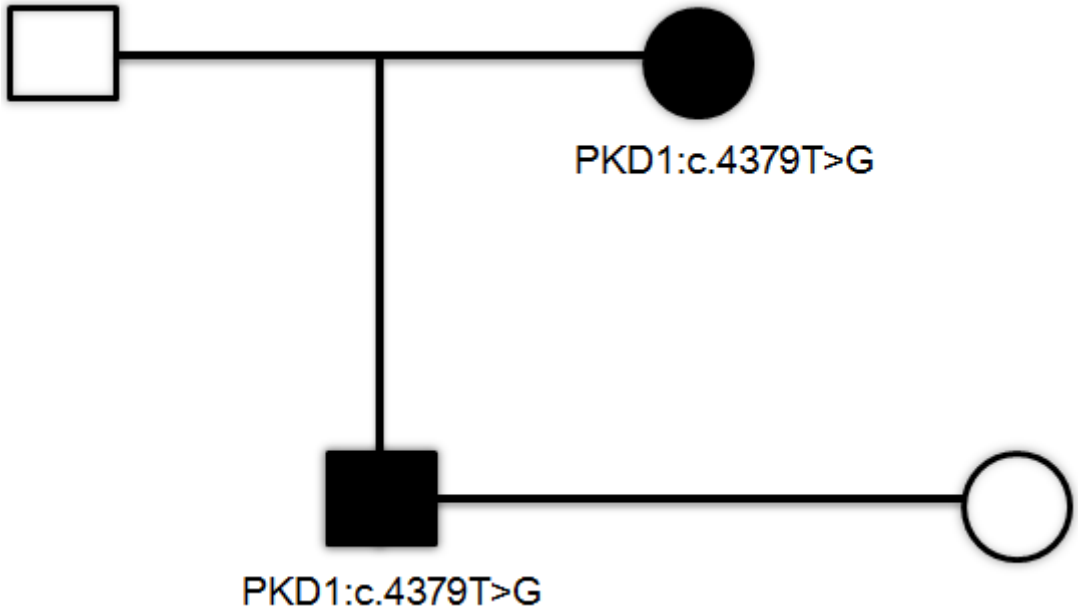


Figure 1

Family linkage and mutation sites in this hereditary deafness family



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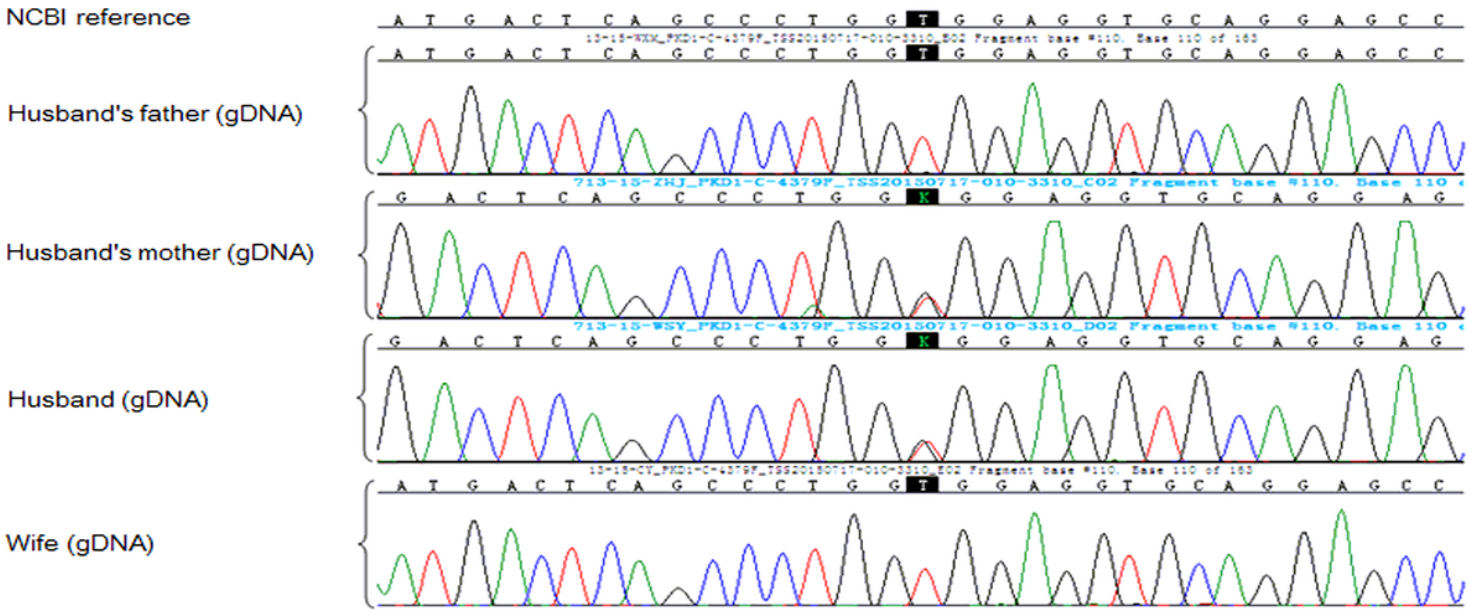
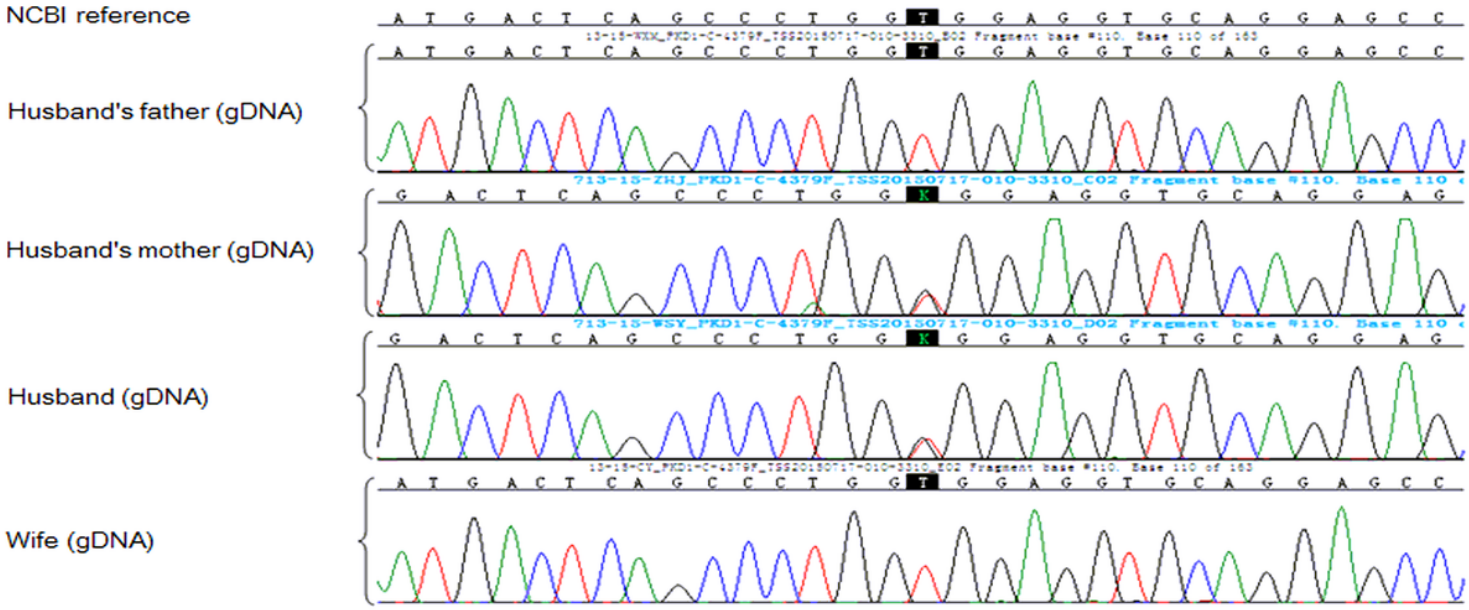


Figure 2

Sanger sequencing analysis the mutation site of all family members



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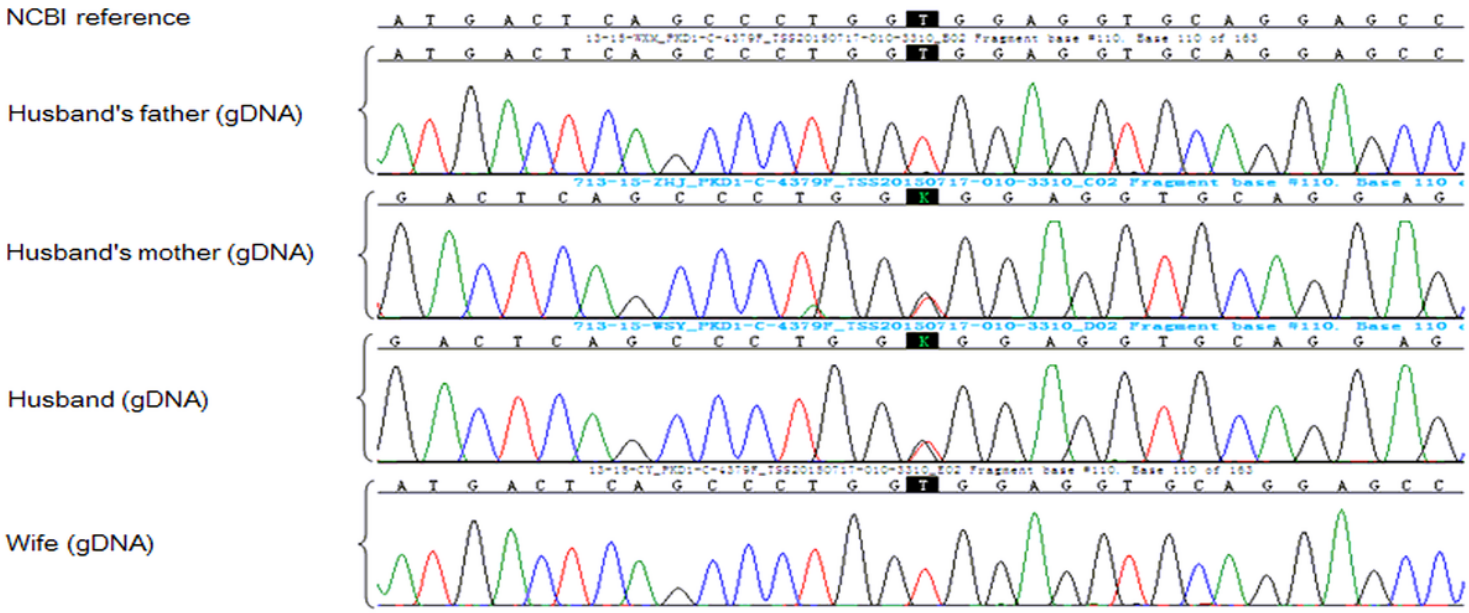

Figure 2

Sanger sequencing analysis the mutation site of all family members

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