A Non-Synonymous Mutation in the Canine Pkd1 Gene Is Associated with Autosomal Dominant Polycystic Kidney Disease in Bull Terriers

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Abstract

Polycystic Kidney Disease is an autosomal dominant disease common in some lines of Bull Terriers (BTPKD). The disease is linked to the canine orthologue of human PKD1 gene, Pkd1, located on CFA06, but no disease-associated mutation has been reported. This study sequenced genomic DNA from two Bull Terriers with BTPKD and two without the disease. A non-synonymous G->A transition mutation in exon 29 of Pkd1 was identified. A TaqMan® SNP Genotyping Assay was designed and demonstrated the heterozygous detection of the mutation in 47 Bull Terriers with BTPKD, but not in 102 Bull Terriers over one year of age and without BTPKD. This missense mutation replaces a glutamic acid residue with a lysine residue in the predicted protein, Polycystin 1. This region of Polycystin 1 is highly conserved between species, and is located in the first cytoplasmic loop of the predicted protein structure, close to the PLAT domain and the second transmembrane region. Thus, this change could alter Polycystin 1 binding or localization. Analytic programs PolyPhen 2, Align GVGD and SIFT predict this mutation to be pathogenic. Thus, BTPKD is associated with a missense mutation in Pkd1, and the application of this mutation specific assay could reduce disease transmission by allowing diagnosis of disease in young animals prior to breeding.

Introduction

Bull Terrier Polycystic Kidney Disease (BTPKD) is an autosomal dominant disease of English Bull Terriers which is characterized by bilateral renal cysts [1,2] and leads to chronic renal failure in middle to old age [1–3]. Cysts can be detected using renal ultrasonography, the currently preferred method of diagnosis for BTPKD. However, this method is expensive and requires an experienced operator, and so a definitive diagnosis, especially early in the disease course, is difficult in some cases [1].

Polycystic Kidney Disease (PKD) has been reported in many species. However, English Bull Terriers, Persian cats, humans and rats suffer from a very similar disease in terms of mode of inheritance, age of onset, clinical signs and renal pathology [1,2,4–6]. Genetic diseases which are similar across species may be caused by mutations in orthologous genes [7].

While, the genetic cause of BTPKD is unknown. Studies in humans have shown that 85–90% of Autosomal Dominant Polycystic Kidney Disease (ADPKD) (OMIM ID: 175900) is caused by mutations in PKD1 (Entrez Gene ID: 5310), with other cases caused by mutations in PKD2 (Entrez Gene ID: 5311). Further, Autosomal Dominant Polycystic Kidney Disease in Persian cats (OMIA ID: 1451) is caused by a premature truncating mutation in the feline Pkd1 orthologue (Entrez Gene ID: 100144606) [8].

In BTPKD, there is strong linkage between the BTPKD phenotype and the region near the canine Pkd1 orthologue (Entrez Gene ID: 606755) on CFA06 [9]. However, no disease-associated mutations were identified in a previous study which sequenced Pkd1 from cDNA made primarily from mRNA from peripheral blood [10]. This may have occurred because the Pkd1 transcript is present in very low copy numbers, and the mutation was not detected. This is supported by the situation in humans and mice where Pkd1/pkd1 expression is greatest in fetal renal tissue, with low levels present in adult tissue [11,12]. Alternatively, the mutation may cause mRNA instability, also making detection of mutations difficult. Further, mutations leading to cryptic splicing, a deletion in part of Pkd1, and chromosomal duplications and rearrangements affecting Pkd1, were not excluded in the previous study. As only coding regions and the 3’ untranslated region (UTR) was sequenced, mutations in intragenic non-coding sequence and the 5’ UTR, non-exonic promoters, enhancers and other regulatory sequences may also not have been detected.

Thus, mutation detection studies on stable genetic material, genomic DNA (gDNA), would be useful to further investigate the role of Pkd1 in BTPKD. This study aimed to screen gDNA from English Bull Terriers of known BTPKD status, for possible mutation/s associated with the disease. This study reports a mutation in Pkd1 that is associated with BTPKD.
Results

Sequencing gDNA revealed a single non-synonymous G to A transition (a change in DNA codon G12 to A12) (NCBI Assay ID: ss316885563) in cDNA nucleotide 9772 in predicted exon 29 of the canine orthologue of Pkd1. This sequence change was present in two BTPKD affected dogs but not in two unaffected dogs. Affected dogs were heterozygous for this sequence change (Figure 1).

Sequence analysis

Thirty-seven allelic variants, including nucleotide substitutions, deletions and insertions, were identified in the 4 dogs whose pkd1 gDNA was sequenced in this study. These variants were in the predicted 5' UTR, 46 exons and 44 introns of Pkd1 (Table 1). Of these 37 variants, the 9772G>A variant was the most likely to be disease-causing, as it was the only variant present in two BTPKD affected dogs but not in two unaffected dogs. Thirty-six other variants were excluded from further analysis as they were less likely to be pathogenic as either; 1) all dogs were homozygous for the variant 2) all dogs were heterozygous for the variant 3) two normal animals and one animal with BTPKD were homozygous for the variant, and the other dog with BTPKD was heterozygous 4) one normal animal was heterozygous for the variant and the other dogs were homozygous for the wild-type allele 5) one dog with BTPKD was heterozygous for the variant and the other dogs were homozygous for the wild-type allele.

Sequencing data was consistent with the presence of 5 haplotype blocks in the 4 dogs analyzed in this study (Table 1). Haplotype 1 was the background haplotype for the G>A mutation in BTPKD. Haplotype 1 and 2 were the most frequent in these 4 dogs.

Alignment of predicted Pkd1 exon 29 sequences in the dog, cat, mouse and human showed 74% homology, with all species having a glutamic residue in the position of the BTPKD mutation or SNP (Figure 2).

Genotyping

All 47 dogs with BTPKD had one copy of the A allele detected by the TaqMan® SNP Genotyping Assay for the BTPKD SNP. None of the 102 dogs without BTPKD had this SNP. Genotyping results were confirmed by sequencing a fragment containing the SNP in an additional 10 Bull Terriers. Results were consistent, with all five BTPKD affected dogs having the SNP but none of the five BTPKD unaffected dogs.

Bioinformatic protein analysis

Bioinformatic protein analysis suggested the BTPKD-associated mutation would change an amino acid in the predicted canine Polycystin 1 protein (NCBI Accession Number: NP_001006651), in a conserved region close to the predicted PLAT (Polycystin 1, Lipoxygenase, Alpha-Toxin) domain and second transmembrane region. The amino acid change, E3258K, would change negatively charged glutamic acid to positively charged lysine. Figure 2, shows these regions are conserved between different mammalian species, with all species having a glutamic residue in the BTPKD-associated mutation location. There was 84% predicted homology between these species’ amino acid sequence in the predicted first cytoplasmic loop of the protein, and 89% in the region of the PLAT domain.

PolyPhen-2, Align GVGD and SIFT were used to predict the effect of the amino acid substitution on the predicted encoded protein (Table 2). PolyPhen-2 classified this mutation as “probably damaging” with a 0.991/1 score. This score indicates the mutation is predicted to affect protein function or structure with a high degree of confidence.

Align GVGD classified the BTPKD substitution as a class C55 change, with a GV score of 0 and GD score of 56.87, and a risk estimate of 2 to 2.5 for this change having a pathogenic effect on the protein (Table 2). This class of sequence changes is the second highest pathogenicity category, of out of the seven classes in the Align GVGD classification system [13].

SIFT also predicted this substitution could affect the protein function (score 0) with this assessment based on an alignment of Polycystin 1 predicted sequences from the dog, cat, human, Rhesus monkey, common chimpanzee, mouse, rat, horse, chicken, and Japanese medaka fish (Japanese rice fish). Amino acid substitutions with scores less than 0.05 are predicted to be deleterious, and those with scores greater than or equal to 0.05 are predicted to be tolerated.

Discussion

This study has found a nucleotide transition in predicted exon 29 of the canine Pkd1 orthologue that is highly likely to cause BTPKD. In this study of 149 dogs, the mutation was only present
Table 1. Nucleotide variants and their location in canine Pkd1 sequence.

| Position/Haplotype | Nucleotide Number<sup>1</sup> | Nucleotide in public databases<sup>2</sup> | Dog 1 (affected) | Dog 2 (affected) | Dog 3 (unaffected) | Dog 4 (unaffected) |
|-------------------|-----------------------------|---------------------------|----------------|----------------|----------------|----------------|
| 5' UTR ss31688548A<sup>3</sup> | 171–192 | 22A | 22A/31A | 22A/31A | 22A/31A | 22A/31A |
| 5' UTR ss31688549G | 720 | T | T/C | C/C | C/C | C/C |
| 5' UTR ss316885500 | 1326 | G | G/A | A/A | A/A | A/A |
| Intron 4 ss316885502 | 234 | G or T | G/G | G/G | G/G | G/G |
| Exon 7 ss316885504 | 122 | G | G/G | G/G | G/G | G/T |
| Exon 7 ss316885506 | 206 | G | G/A | G/A | G/A | G/A |
| Intron 7 ss316885508 | 110 | 11C | 10C/11C | 10C/11C | 10C/11C | 10C/11C |
| Intron 8 ss316885512 | 347 | A or G | A/G | G/G | G/G | G/G |
| Intron 9 ss316885514 | 162 | A or G | A/G | G/G | G/G | G/G |
| Intron 11 ss316885519 | 395 | T or G | T/T | T/T | T/T | T/T |
| Exon 12 ss316885521 | 18 | A or G | A/G | G/G | G/G | G/G |
| Intron 12 ss316885523 | 26–29 | ACC or GGG | GGG/GGG | GGG/GGG | GGG/GGG | GGG/GGG |
| Intron 12 ss316885527 | 32 | A or G | G/G | G/G | G/G | G/G |
| Intron 12 ss316885530 | 34–93 | 59 bp present | Del/Del<sup>4</sup> | Del/Del | Del/Del | Del/Del |
| Intron 14 ss316885534 | 171–184 | 11C or 13C | 11C/13C | 11C/13C | 11C/13C | 11C/13C |
| Exon 15 ss316885536 | 1235 | C or G | C/G | C/C | C/C | C/C |
| Exon 15 ss316885539 | 1693 | C or T | C/C | C/C | C/C | C/C |
| Intron 16 ss316885541 | 275 | C or T | C/T | T/T | T/T | T/T |
| Exon 17 ss316885543 | 85–86 | AA or GC | GC/GC | GC/GC | GC/GC | GC/GC |
| Intron 17 ss316885546 | 41 | G or T | G/G | G/G | G/G | G/G |
| Exon 23 ss316885548 | 125 | C | C/T | T/T | T/T | T/T |
| Exon 23 ss316885550 | 518 | T | C/T | T/T | T/T | T/T |
| Intron 23 ss316885552 | 239 | A | A/G | G/G | G/G | G/G |
| Exon 26 ss316885554 | 5 | C or T | C/C | C/C | C/C | C/C |
| Intron 26 ss316885556 | 33 | T or C | T/T | T/T | T/T | T/T |
| Intron 26 ss316885559 | 926 | A or G | A/G | G/G | G/G | G/G |
| Intron 27 ss316885561 | 63–74 | 11C | 10C/11C | 10C/11C | 10C/11C | 10C/11C |
| Exon 29<sup>5</sup> ss316885563 | 42 | G | G/A | G/A | G/G | G/G |
| Intron 30 ss316885565 | 335 | C | C/T | T/T | T/T | T/T |
| Intron 30 ss316885567 | 597 | C | C/T | T/T | T/T | T/T |
| Exon 31 ss316885569 | 28 | G | T/T | T/T | T/T | T/T |
| Intron 37 ss316885571 | 204 | C | C/T | T/T | T/T | T/T |
| Intron 41 ss316885573 | 19 | 6C | 6C/7C | 7C/7C | 7C/7C | 7C/7C |
| Intron 41 ss316885575 | 117 | A or C | C/C | C/C | C/C | C/C |
| Intron 42 ss316885578 | 52 | A | A/G | G/G | G/G | G/G |

Haplotype<sup>6</sup>:

- Haplotype 1: 31A-C-A-G-G-G-10C-G-G-G-T-G-GGG-G-Del-11C-C-C-T-G-C-G-T-T-G-C-T-G-10C-G-G-G-T-T-T-T-7C-C-G
- Haplotype 2: 22A-C-A-G-A-11C-G-G-G-T-G-GGG-G-Del-13C-C-C-T-G-C-G-T-T-G-C-T-G-11C-G-G-G-T-T-T-T-7C-C-G
- Haplotype 3: 22A-C-A-G-T-A-11C-G-G-G-T-G-GGG-G-Del-13C-C-C-T-G-C-G-T-T-G-C-T-G-11C-G-G-G-T-T-T-T-7C-C-G
- Haplotype 4: 22A-T-G-G-G-A-11C-G-A-A-A-T-A-GGG-G-Del-13C-C-C-T-G-C-G-T-T-G-C-T-G-11C-G-G-G-T-T-T-T-7C-C-G
- Haplotype 5: 31A-C-A-G-G-G-10C-G-G-G-T-G-GGG-G-Del-11C-C-C-T-G-C-G-T-T-G-C-T-G-10C-G-G-G-T-T-T-T-7C-C-G

<sup>1</sup>The position of each variant as the number of nucleotide residues from the 5' end of the UTR, intron or exon.

<sup>2</sup>Expected nucleotide residues based on the sequence available in the public databases. These nucleotides are considered as normal alleles, as they are not likely to cause the disease (refer to discussion). Where two nucleotides are recorded, there were two nucleotide variants reported for the relevant position in different databases.

<sup>3</sup>NCBI Assay ID available at Single Nucleotide Polymorphism database (dbSNP).

<sup>4</sup>Del; Deletion of 59 bp.

<sup>5</sup>Mutation segregating with BTPKD. G is the wild type allele, and A the mutant allele.

<sup>6</sup>Predicted haplotypes observed in the dogs (the nucleotide in bold indicates the BTPKD SNP):

- Haplotype 1: 31A-C-A-G-G-G-10C-G-G-G-T-G-GGG-G-Del-11C-C-C-T-G-C-G-T-T-G-C-T-G-10C-G-G-G-T-T-T-T-7C-C-G
- Haplotype 2: 22A-C-A-G-A-11C-G-G-G-T-G-GGG-G-Del-13C-C-C-T-G-C-G-T-T-G-C-T-G-11C-G-G-G-T-T-T-T-7C-C-G
- Haplotype 3: 22A-C-A-G-T-A-11C-G-G-G-T-G-GGG-G-Del-13C-C-C-T-G-C-G-T-T-G-C-T-G-11C-G-G-G-T-T-T-T-7C-C-G
- Haplotype 4: 22A-T-G-G-G-A-11C-G-A-A-A-T-A-GGG-G-Del-13C-C-C-T-G-C-G-T-T-G-C-T-G-11C-G-G-G-T-T-T-T-7C-C-G
- Haplotype 5: 31A-C-A-G-G-G-10C-G-G-G-T-G-GGG-G-Del-11C-C-C-T-G-C-G-T-T-G-C-T-G-10C-G-G-G-T-T-T-T-7C-C-G

<sup>7</sup>Hap; Haplotype.

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A Mutation Causing Polycystic Kidney Disease

**A**

Dog mutant
GCGAGGCCGCCCTGCCGCGGTTCCCGCGGCTCTGGCCTGAGCTGAGCCGCCGCTCTCTCTCTCTCT

Dog wild type
GCGAGGCCGCCCTGCCGCGGTTCCCGCGGCTCTGGCCTGAGCTGAGCCGCCGCTCTCTCTCTCTCT

Cat
GGCAAGGGCCGCTGAGCTGAGCCGCCGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT

Mouse
ATGAAAGCTGAGCTGAGCTGAGCCGCCGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT

Human
GCGAGGCCGCCCTGCCGCGGTTCCCGCGGCTCTGGCCTGAGCTGAGCCGCCGCTCTCTCTCTCTCTCTCTCTCT

**B**

Dog mutant
TTGACAAACACACATCTGCGCTCTCACTATGGAGACACCCGCCCTCGCAGGCCTCTCACTCTCTCT

Dog wild type
TTGACAAACACACATCTGCGCTCTCACTATGGAGACACCCGCCCTCGCAGGCCTCTCACTCTCTCT

Cat
TTGACAAACACACATCTGCGCTCTCACTATGGAGACACCCGCCCTCGCAGGCCTCTCACTCTCTCT

Mouse
TTGACAAACACACATCTGCGCTCTCACTATGGAGACACCCGCCCTCGCAGGCCTCTCACTCTCTCT

Human
TTGACAAACACACATCTGCGCTCTCACTATGGAGACACCCGCCCTCGCAGGCCTCTCACTCTCTCT

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Dog mutant
GTTACGCTGTTGGAGAAGACACCGGCCCTCCAGGTTGGTTCCCAGGGGCTCGCGGATTGCCCT

Dog wild type
GTTACGCTGTTGGAGAAGACACCGGCCCTCCAGGTTGGTTCCCAGGGGCTCGCGGATTGCCCT

Cat
GTTACGCTGTTGGAGAAGACACCGGCCCTCCAGGTTGGTTCCCAGGGGCTCGCGGATTGCCCT

Mouse
GTTACGCTGTTGGAGAAGACACCGGCCCTCCAGGTTGGTTCCCAGGGGCTCGCGGATTGCCCT

Human
GTTACGCTGTTGGAGAAGACACCGGCCCTCCAGGTTGGTTCCCAGGGGCTCGCGGATTGCCCT

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Dog mutant
CTCCTCTGCTC

Dog wild type
CTCCTCTGCTC

Cat
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Mouse
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Human
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Dog mutant
YIVLLTCICVCLTYTVMAVILRKLDQLDVSRTVFIIFCGKGLFKYEILVKTGWRGSGT

Dog wild type
YIVLLTCICVCLTYTVMAVILRKLDQLDVSRTVFIIFCGKGLFKYEILVKTGWRGSGT

Cat
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Mouse
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Human
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Dog mutant
TAIVGIMLYGEGRHGLDGRARFNSLDVFQIAITPHSLGSVRKLRVWHDNKLSPA

Dog wild type
TAIVGIMLYGEGRHGLDGRARFNSLDVFQIAITPHSLGSVRKLRVWHDNKLSPA

Cat
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Mouse
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Human
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Dog mutant
WFLQHIIVRDLQANRSTFFLNDWLSVTEANGGLVEKELAASEEAALRRSLRLVALQ

Dog wild type
WFLQHIIVRDLQANRSTFFLNDWLSVTEANGGLVEKELAASEEAALRRSLRLVALQ

Cat
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Mouse
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Human
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Dog mutant
RGFDKHIWLSWDRPPRSRFRVQRATCCILLVLFLGAGNANVYVG

Dog wild type
RGFDKHIWLSWDRPPRSRFRVQRATCCILLVLFLGAGNANVYVG

Cat
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Mouse
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Human
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in 47 affected animals, and likely to be pathogenic based on the protein bioinformatic evidence. All BTPKD dogs were heterozygous for this sequence change, consistent with the findings in mice and cats that animals homozygous for Pkd1 mutations die embryonically [14–16].

In this study more than 27 kb of the canine Pkd1 orthologue was sequenced, however some regions were excluded (Figure S1). Only the 399 bp at the 3’ end of intron 1 was sequenced, as the rest of the predicted intron is a GC-rich area of more than 14 kb, and the sequence of the 5’ splicing site was unknown at the time of the study. Similarly, a region of 600 bp in predicted intron 30 was excluded from sequencing as the sequence was unknown at the time of the study. Finally, 79 bp of sequence in 3’ untranslated region extending from nucleotides 903 to 981 could not be sequenced, possibly due to the formation of hairpin or other secondary structures.

Thirty-six other nucleotide variants were found in canine Pkd1. However, these variants were less likely to cause the disease, not only due to inconsistencies with the mode of inheritance of BTPKD, but also 19 of these variants were present in sequence in public databases where, for example, NCBI sequence contained one allele and Ensembl a second. These database sequences were considered likely to be non-disease-associated variants as Pkd1 sequence in Ensembl is from Boxer gDNA, a breed not reported with PKD. Similarly, the sequence in NCBI is from Martin-Darby Canine Kidney Epithelial Cells (MDCK) from a Cocker Spaniel, another breed not reported with PKD. However, some of these variants could still play a role in disease, and further studies could investigate this possibility.

The BTPKD mutation was not detected in a previous study which sequenced Pkd1 from mRNA extracted from white blood cells of BTPKD affected and normal animals [10]. The samples used in the current study were from the same animals used in this previous study, suggesting the mutant mRNA may be unstable and degrade rapidly [17]. Expression analysis, using methods such as quantitative PCR, to measure Polycystin 1 expression in affected tissues would have been useful to investigate this. However, suitable kidney tissue samples were not available for such analysis.

The exon 29 SNP that segregated with the BTPKD phenotype is predicted to result in an amino acid change in the predicted protein, Polycystin 1, replacing a negatively charged glutamic acid residue with a positively charged lysine residue. Charge reversal amino acid substitutions may have deleterious effects on proteins.

Table 2. Prediction of pathogenicity of the predicted amino acid change in BTPKD as determined by PolyPhen-2, Align GVGD and SIFT.

| Prediction Tool | Prediction data |
|-----------------|-----------------|
| PolyPhen-2      | Score 0.991     |
|                 | Sensitivity 0.60|
|                 | Specificity 0.96|
| Align GVGD      | GV 0.00         |
|                 | GD 56.87        |
|                 | Prediction Probably damaging substitution¹ |
| SIFT            | Sequences at position³ 10 |
|                 | Median sequence conservation 3.45 |
|                 | Score⁴ 0.00     |
|                 | Prediction Affects protein function |

¹Probably damaging, i.e., it is predicted to affect protein function or structure with a high degree of confidence.
²Substitution classification in Align GVGD: GD > =5S+Tan[100(GV-2.0)]= >Class C55.
³Number of aligned sequences at the position of the substitution.
⁴SIFT scores range from 0 to 1. The amino acid substitution is predicted to be damaging if the score is < =0.05, and tolerated if the score is >0.05.

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effect of this mutation on the cellular location and function of Polycystin 1 by expressing both the wild type and the mutant proteins in Madin-Darby Canine Kidney (MDCK) cells.

Identification of the mutation that is likely to cause BTPKD supports the first cytoplasmic loop of Polycystin 1 being important in the protein’s function. Little is known about this protein region in humans or other species, and further studies on the effect of this mutation on the structure and function of Polycystin 1 could increase understanding about the protein.

Studies in ADPKD and PKD mice models have shown that Polycystin 1 is present in high levels in fetal kidney tissues, but only in low levels in adult tissue [11,12]. While cystogenesis does occur in some cases in vitro, a second somatic PKD1/Pkd1 mutation appears to be required in a cell for a cyst to form from clonal expansion of this cell [14,15,28,29], and as at least some humans with ADPKD develop cysts in adulthood [30,31], it is likely that the mutation continues to have a clinically significant effect beyond the fetal stage. Other factors besides age of the patient are also likely to play a role in cystogenesis including level of Polycystin 1 expression, and penetration of pathogenic alleles [15,32–34].

The pathogenicity prediction tools, PolyPhen-2, Align GVGD and SIFT also supported this amino acid substitution being pathogenic. PolyPhen-2 is a software tool that predicts the possible impact of amino acid substitutions on the structure and function of proteins using biophysical and evolutionary comparisons. Align GVGD is a program that combines the analysis of the biophysical characteristics of amino acids and protein multiple sequence alignments to predict the effect of mutations on the encoded proteins. Finally, SIFT produces predictions about mutation pathogenicity using amino acid sequences which are conserved among different species, using multiple sequence alignments. The prediction results obtained using these tools are in agreement with each other, and suggest that this mutation is in a conserved region of Polycystin 1 and has a high potential to affect the structure and function of the protein.

Further support for the pathogenicity of the Pkd1 SNP found in the BTPKD-affected dogs in this study comes from studies in other species. In humans, mutations in PKD1 are responsible for up to 85–90% of ADPKD cases. Mutations are found anywhere in the gene, and include nonsense, missense, frame shift, splicing, deletions and insertions (http://www.hgmd.cf.ac.uk/ac/index.php). Around 50% of the mutations found in PKD1 in humans are classified as missense/nonsense mutations, and around half of these are missense mutations. While the missense mutation associated with BTPKD in this study has not been reported in ADPKD, 10 missense mutations in PKD1 producing amino acid changes in the first cytoplasmic loop of the protein have been reported [34–40], of which six were shown to segregate with the disease phenotype [35,38,40]. The closest of these missense mutations to the BTPKD SNP is a R3247H mutation, five amino acids upstream of the equivalent position of the BTPKD mutation in human Polycystin 1. This mutation produces a conservative substitution of arginine to histidine.

Three further disease-related glutamic acid to lysine changes have been reported in ADPKD [34,36,38,40]. One of these E to K changes in ADPKD in the PKD1 gene is an E3604K mutation that has been classified as likely to be pathogenic [38]. Rossetti et al (2001) have also reported that a putative missense mutation in position 2771 segregated with the disease in four unrelated families and was not observed in 230 normal cases, supporting its role in the disease. Rossetti et al (2002) have also reported the same amino acid change at amino acid position 1811 in ADPKD patients. While, they were not able to test whether this change segregated with the disease due to lack of samples [40], this amino acid change has also been reported to be pathogenic in other proteins as it changes their function [41,42]. Thus, mutations which are similar to that in BTPKD are pathogenic and so also likely to be disease causing in ADPKD.

Interestingly, feline PKD is due to C>A transition in predicted exon 29 of canine Pkd1, resulting in a stop codon at the equivalent position of 3204 in human Polycystin 1 [8]. This mutation is located in the equivalent region to the first transmembrane domain of human Polycystin 1, only 32 amino acids downstream of the BTPKD mutation. Feline PKD is another similar disease to ADPKD and BTPKD, providing further support for the BTPKD-associated mutation’s causative role in the disease.

Development of the TaqMan® SNP Genotyping Assay is a useful molecular genetic diagnostic test to diagnose BTPKD. This test is favoured for detecting single nucleotide polymorphisms in large-scale population mutation screening, as it is accurate, sensitive, specific, rapid and cost effective [43]. Such a diagnostic test would allow breeders to diagnose disease prior to breeding, and thus prevent breeding of affected animals. It would also allow the diagnosis of animals prior to development of clinical disease, and so aid early treatment. Currently, ultrasonography is the preferred method of diagnosis for BTPKD, as it is sensitive, noninvasive, and quick, if performed by a highly skilled operator [30,31]. However it is costly and the presence of cysts is nonspecific for BTPKD. Ultrasonography may also not detect disease in dogs with late enlargement of renal cysts [1]. In addition, where less than 3 cysts are detected, or the cysts are present only in one kidney, definitive diagnosis requires retesting, pedigree inspection and possibly test mating. Moreover, variation in the echogenicity of normal kidneys in Bull Terriers can also make it difficult for ultrasonography to give a definitive diagnosis [1]. Thus, this accurate, easy and inexpensive molecular genetic diagnostic test will be useful to detect BTPKD at any age, from a sample of blood, hair or a buccal swab.

All cases of reported BTPKD to date in Australia descend from two affected half-siblings, making it very likely that there is only one disease-causing mutation in the Australian Bull Terrier population. Interestingly, these half-sibs were recently descended from an imported animal, suggesting the disease may well be international and due to this one mutation. Thus, a mutation-based diagnostic test is desirable in this disease, as these tests are accurate, sensitive, specific and inexpensive. They also avoid some of the inaccuracies of a linkage-based diagnostic test, which detect and analyze the presence of a genetic marker located near the disease locus. Despite this, due to differences in the clinical presentation, inheritance and pathology of PKD in other canine breeds, it is unlikely the same mutation is the cause of PKD in the Cairn Terrier [44] or West Highland White Terrier [45].

In summary, a G>A transition at nucleotide 9772 in CDNA in predicted exon 29 of canine Pkd1 segregates with the BTPKD phenotype and results in a predicted change of a glutamic acid to a lysine residue at amino acid number 3258 of the predicted canine Polycystin 1 protein. A TaqMan® SNP Genotyping Assay has been developed to identify this mutation.

**Materials and Methods**

**Ethics Statement**

The University of Queensland, Animal Ethics Committee, has approved this study.

**Selection of dogs and DNA extraction**

Diagnosis of BTPKD was established using ultrasound as described previously [1]. Unaffected Bull Terriers were over one
Amplification of Pkd1

Canine Pkd1 sequence was obtained from databases NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org). At the time of the study, the gene sequence was integrated into the canine genome map with the exception of missing sequence from predicted introns 1 and 30.

For PCR amplification canine Pkd1 was divided into 22 fragments 500–2000 bp in size (Table S1). Intron 1 was not amplified as it was 14 kb, GC-rich and the 5′ sequence was unknown. However, 399 bp at the 3′ end of this intron was amplified to investigate 3′ splicing sites for intron 1. Primers were designed in predicted exonic sequence where possible using Primer 3 version 0.4.0 (http://frodo.wi.mit.edu/), and analyzed using OligoAnalyzer version 3.1 (http://www.idtdna.com/analyzer/applications/oligoanalyzer/) (Table S1).

Table fragments were amplified in polymerase chain reactions (PCR)s containing 1× PCR Buffer containing 0.3 mM dNTPs and 1.5–2.5 mM MgSO4 (QIAGEN, Hilden, Germany), 0.5–1 μM forward and reverse primers, 0.5 U HotStar HiFidelity DNA Polymerase (QIAGEN, Hilden, Germany), 0–1× Q solution, and 10–40 ng DNA in a 10 μl reaction volume. Standard thermocycling conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min; and one cycle of 72°C for 10 mins (Table S1).

To confirm the size of the PCR products, samples were stained using Blue/Orange 6 × loading dye (Promega, Madison, USA) and electrophoresed (BIO-RAD, Hercules, USA) on a 1.5% agarose gel (Promega, Madison, USA) using 1× TAE Buffer (Promega, Madison, USA), 10000× SYBR® Safe DNA gel stain (Invitrogen Eugene, Oregon, USA) and size standard Lambda DNA/ EcoRHIindIII (Promega, Madison, USA), and visualized using a transilluminator (BIO-RAD, Hercules, USA).

Sequencing of Pkd1

PCR products were purified using MinElute PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced bidirectionally using forward, reverse and internal sequencing primers. The standard 20 μl sequencing reaction contained 10–100 ng purified DNA, 1 μl Big Dye Terminator version 3.1 (Applied Biosystems, Foster City, USA), 3.5 μl BigDye Sequencing Buffer (Applied Biosystems, Foster City, USA) and 3.2 pmol sequencing primer. Standard sequencing conditions were 94°C for 5 mins, followed by 30 cycles of 94°C for 10 sec, 50°C for 30 sec, and 60°C for 2 mins (Table S1). Unincorporated dye terminators were removed using an ethanol/EDTA/sodium acetate precipitation method, according to the BigDye® Terminator v3.1 Cycle Sequencing Kit protocol (Applied Biosystems, Foster City, USA), the pellet diluted and run on a 3130X/Genetic Analyzer (Applied Biosystems, Foster City, USA).

Sequence analysis

Sequences were analyzed and assembled using ChromasPro, version 1.33 (Technelysium Pty Ltd, Queensland, Australia) and Sequencher, version 4.7 (Gene Codes Corporation, Ann Arbor, USA). Consensus sequence was compared with those in public databases NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi and Ensembl http://www.ensembl.org/Multi/blastview and from BTKPD affected and unaffected animals.

Bioinformatic protein analysis

The translate tool http://au.expasy.org/tools/dna.html from the ExPASy Proteomics Server was used to predict the change in amino acid residue caused by the mutation. In order to predict the location and effect of the amino acid change on the predicted canine Polycystin 1 structure, it was compared to the well-annotated human Polycystin 1. Thus, the predicted sequence of canine Polycystin 1 in normal dogs and BTKPD dogs was compared with that of Polycystin 1 from humans by using the following databases and tools: UniProt http://www.uniprot.org from European Bioinformatics Institute (EMBL-EBI), Protein http://www.ncbi.nlm.nih.gov/protein and Conserved Domain Database http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml from National Centre for Biotechnology Information (NCBI). ClustalW2 http://www.ebi.ac.uk/Tools/clustalw2/index.html from EMBL-EBI and COBALT http://www.ncbi.nlm.nih.gov/tools/colab/ from NCBI were used to align canine Polycystin 1 sequence with orthologous sequence in other species. Tools including PolyPhen-2 http://genetics.bwh.harvard.edu/pph2/dokuwiki/start, Align GVGD http://agvgd.iarc.fr/ and SIFT http://sift.jcvi.org/ were used to predict the affect of the predicted altered amino acid on the encoded protein.

Genotyping

The BTKPD-associated SNP was investigated using TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, USA) in a total of 47 affected and 102 unaffected Bull Terriers from a large pedigree in which BTKPD was segregating. All the animals were more than one year old at the time of the diagnosis to ensure the diagnosis was correct [1]. A Custom TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, USA) using specific PCR primers (sequence of forward primer TCTGTCCGTCCGTCCCT, and reverse primer GCCAGATGTGCTTGTCAAAGAAG), and probes (probe sequence to detect the wild type allele was TGGGCGAGGTGCAG labelled with VIC® fluorescent dye, and probe sequence to detect the mutant allele was TGGCGCCAAGTGCAAG labelled with FAM™ fluorescent dye). Final concentrations of 1× TaqMan® SNP Genotyping Assay Mix, 1× TaqMan® Genotyping Master Mix and 10 ng canine gDNA were mixed in a 25 μl total volume on a 96-well plate, and the assay performed using a 7500 Real Time PCR System (Applied Biosystems, Foster City, USA). The results were analyzed using 7500 Software Version 2.0.4 (Applied Biosystems, Foster City, USA). Fragment 19 was amplified and sequenced from gDNA from five Bull Terriers with BTKPD and five Bull Terriers without BTKPD (Table S1), to confirm SNP genotyping and sequencing results.

Supporting Information

Figure S1 Canine Pkd1 gene. This figure shows the canine Pkd1 gene from the 5′ untranslated region (UTR) to the 3′ untranslated region. The solid boxes represent the 46 predicted exons. The regions in 5′ UTR, intron 1 and intron 30 marked with clear ovals are regions of the gene that were not sequenced in this study.

Table S1 Conditions used for amplification and sequencing of Canine Pkd1 gene. This table shows sequences of primers, and PCR amplification and sequencing reaction conditions used for sequencing canine Pkd1 from genomic DNA. (DOC)
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Author Contributions

Conceived and designed the experiments: PG CAO MKT DLD. Performed the experiments: PG. Analyzed the data: PG CAO MKT. Wrote the paper: CAO MKT RAS.