

Identification of genes involved in chronic kidney disease associated with Wilms' tumour gene (WT1) mutation.

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INTRODUCTION

Chronic Kidney Disease (CKD) is a major cause of kidney failure and is increasing worldwide. Glomerular Sclerosis (GS) is one of the most common forms of CKD: Scarring and matrix deposits build up and prevent normal filtration in the glomerulus resulting in a need for dialysis or a kidney transplant. Risk factors for this disease include diabetic nephropathy, premature birth and high blood pressure. In this research I am analysing the expression of genes within the kidney of a mutant mouse [Patek et al.] which has reduced kidney function and develops CKD over a period of time to identify genetic pathways involved in CKD.

AIMS & HYPOTHESIS

Analysis of microarray data from fetal kidneys of four wild type and four mice with mutation in the Wt1 gene to compare gene expression differences occurring before the onset of CKD. At this stage, the mouse model is developing fewer glomeruli. I hypothesise that there will be some genes that contribute to the development of CKD and these genes will be differentially expressed between mutants and controls. I also hypothesise that there may be some genes which are unrelated to kidney disease. I aim to identify some of the contributing genes and eliminate some of the unrelated genes.

IDENTIFICATION OF INTRONS WITHIN THE TOP 150 DIFFERENTIALLY EXPRESSED PROBES

Gene	Positive hit no.	Negative hit no.	Genes that look
Rnf220	6	0	5 possible Algs, G3bp2, Rch, Rnf220, Cank,
Canx	0	5	Digt, Rnf220, Anapc1,
G3bp2	1	4	Copa, Dert1, Ngn7
Digt	0	4	
Hlx	0	4	
...

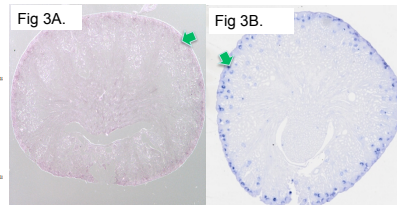


Fig 1. List of the probes with the greatest difference in expression between wild type and mutant. Note: Many are not assigned to a named gene. BLAST [http://www.ncbi.nlm.nih.gov/BLAST/] searching with the probe sequence revealed these to be located within introns.

Fig 3. Of the top intronic probes identified above, only RNF220 had expression data in the GUDMAP database [http://www.gudmap.org/]. RNA specific probes for RNF220 reveal expression in the "nephrogenic zone" at the outer edge of the kidney [Fig 3A, arrow]. RNA expression analysis for Wt1 shows Wt1 expression in the same location [Fig 3B, arrow] – providing more evidence that Wt1 can regulate RNF220.

DESIGN AND TEST PRIMERS

I therefore I decided to investigate further to see if I could find how Wt1 may be affecting expression of Rnf220. I designed PCR primers to specifically look at the expression of RNF220 introns and exons so I could observe if the gene was being truncated or alternatively spliced.

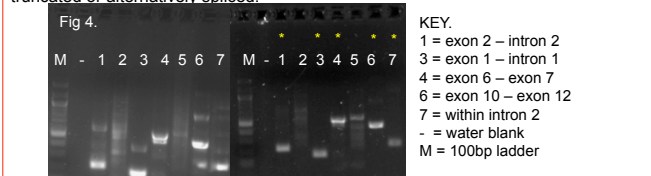


Fig 4. Next I did PCR analysis of mouse genomic DNA to test PCR reaction conditions. DNA was used for the test as it allowed amplification of intron sequences that might be missing from RNA preparations. The PCR on the right was done at higher temperatures with a shorter annealing time which turned out to be more specific. Primer sets 1, 3, 4, 6 & 7 were chosen for further analysis as they gave specific bands and good coverage of RNF220. I have labelled the chosen primers on Fig 4 with a yellow star.



See Fig 5. for representation of RNF220. You can envisage where the primers would anneal.

PCR of Rnf220 introns and exons



Having optimised and identified primers, RNA was reverse transcribed to make cDNA and then PCR amplified to assess RNF220. See RT-PCR gel Fig 6. To test for inclusion of introns, I used primers from exon 6 to exon 7 in RT-PCR on fetal kidney RNA from mutant (17 18 wt, 19,21 mut) and wild type. There is no evidence of DNA contamination in RNA samples as seen by the empty lanes. There are two different forms of RNF220 expressed in both wt and mut (this is OK with ensemble). There is also no evidence of intron inclusion because there are no bands of the same size as genomic DNA.

Fig 7. To further test for inclusion of introns, I used primers located within intron 2 (primer set 7) in RT-PCR on fetal kidney RNA from mutant and wild type. Empty lanes means there is no evidence of DNA contamination in RNA samples but there is some evidence of expression of the intron sequence in samples 17 and 21. However this does not vary with genotype. I have discovered RNF220 does have retained introns but this does not seem to be 100% associated with the mutations. Therefore it is unlikely to be involved in CKD predisposition. But more samples may reveal some association.

EFFECTS OF LOW NEPHRON NUMBER IN OLD MICE

In addition to the analysis of fetal kidney gene expression in a mouse model of CKD, an additional mouse mutant with reduced nephron number was analysed. Very old (15 months) mice were examined to look for the long-term effects of reduced nephron number.

- DNA-PCR was used to detect the mutation in five mice of the same age and litter [Fig. 8].
- Urine dipsticks (Combur3, Roche) were used to detect any excessive protein within the urine that could indicate CKD [Fig. 9].
- Kidneys were sectioned and stained with haematoxylin/eosin to detect any pathological changes that could indicate CKD [Fig. 10]

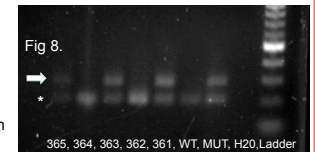


Fig 8. PCR mutation detection. The normal copy of the Wt1 gene appears in all samples (marked with *). The mutated copy is seen in 3 mice (arrow). WT and MUT are control samples.

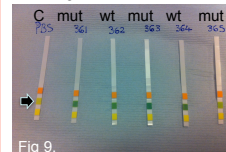


Fig 9. Urine dipstick analysis. Compared with the saline control (PBS, C), all mice show evidence of protein in their urine (green band, arrow). There is no correlation with genotype, eg, compare 364 and 365. We conclude that the presence of protein in the urine is a normal feature of aging in mice and not diagnostic for CKD.

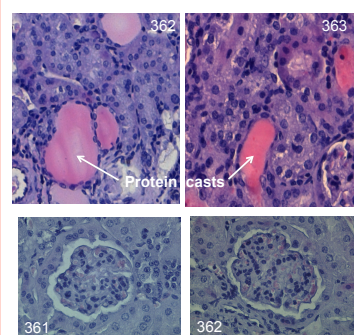


Figure 10. Representative kidney sections stained with haematoxylin/eosin (X20 magnification). Both wild type (362) and mutant (363) kidneys had large "protein casts" that are due to protein leaking from the glomerulus during filtration. This is a sign of a failing kidney but seems to be a normal feature of mouse aging.

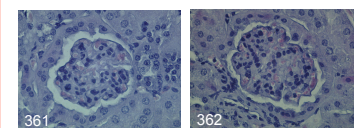


Figure 11. High magnification (X40) images of representative glomeruli from wild type (362) and mutant (361) kidney sections. Pathological assessment (A. Ahmad) showed no differences between mutant and wild type glomeruli.

All of the kidneys showed some evidence of disease but the mutant kidneys were not more diseased than the wild type. This is most probably due to the age of the mice. In the future it would be interesting to prepare kidney slides to compare the histology over a period of time.

DISCUSSION

RNF220 is a gene expressed in developing glomeruli, like Wt1. RNF220 isoforms are not affected by Wt1 mutation. RNF introns are expressed in fetal kidney but not related to Wt1 mutation. Old mice with a milder mutation in Wt1 do not have increased disease symptoms compare to wild type.

CONCLUSION

Careful validation of microarray data is necessary to determine the significance of any gene expression changes. At the level of individual probes, RNF220 is unlikely to be a target of Wt1 but analysis of additional mice may reveal a trend. Aged mice show significant signs of kidney disease even in the absence of Wt1 mutation – again more mice may reveal trends plus looking at larger numbers of younger mice may provide evidence of a more rapid onset, for example, in mutant mice.