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**The Opdc missense mutation of Pax2 has a milder than loss-of-function phenotype**

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Introduction/ Objectives

Papillorenal syndrome (renal-coloboma syndrome or RCS) is an autosomal dominant disease characterized by optic disc and retinal abnormalities which are associated with renal malformations. The severity of the disease varies with some cases manifesting kidney defects without eye disorders while others show hearing loss and cerebellar abnormalities. The syndrome is associated with mutations in the paired domain of the pax2 gene.

Pax2 is one of the 9 paired box genes (PAX) that has a conserved domain that contains two subdomains each with 3 α-helices connected by a linker region. This domain is what binds target DNA sequences even though the subdomains are structurally independent and do not bind DNA equally. Loss of function mutations in Pax2 genes occur from termination, splice site mutation and insertions or deletions.

This paper identifies and describes an N-ethyl-N-nitrosourea (ENU) induced mouse mutation, Opdc, in which an I40T missense mutation in the first α-helix of Pax2 causes a milder than loss-of-function phenotype. The phenotypes resulting from this missense mutation were compared with the wild type phenotypes. The DNA-binding properties, transcriptional activation and transcription of downstream genes by the Opdc mutants were compared with the wild type gene. Their results on certain genetic backgrounds of the heterozygous Opdc mutation suggest that Opdc could be a candidate gene for screening patients with colobomas without renal defects.

Experimental approach and results

1. **Opdc (Optic disc coloboma) is a point mutation in the *Pax2* gene**

The exons of *Pax2* and flanking regions were amplified by PCR and sequenced from ENU induced Opdc mutants, BALB/cAnN parental strain and two strains used to map the mutation, C3H/HeH and C57BL/6. An I40T missense mutation in exon 2 was located in the first α-helix of the N-terminal subdomain of the DNA binding paired domain. Secondary structure prediction using JPRED suggested that the position of the mutation did not disrupt the helical structure and a 3D model showed that the same position does not interact with DNA directly.

1. **The heterozygous Opdc phenotype is milder than *Pax2* loss-of-function and is background dependent**

C57BL/6J mice were crossed with Opdc for 9 generations and only 2/3 of the expected heterozygotes survived to weaning compared to crossing into the C3H background for >4 generations and there was no detectable loss of heterozygotes. Kidney weights were measured at E18.5 from Opdc heterozygote embryos and their corresponding wild –types. On the C3H background, there was no significant difference in the kidney weight between wild type and Opdc heterozygotes. On the C57BL/6J background, Opdc Heterozygous kidneys were 71.5 % the size of the wild-type (Table 2). In addition, the C57BL/6J background showed reduced glomeruli number in mutants compared to wild-type even though the difference was marginal. There was no difference in glomeruli count between the mutant and wild-type on the C3H background (Table 3). Unilateral renal agenesis was noted in 1/21 heterozygotes on the C57BL/6J background compared to 0/13 on the C3H background.

1. **Homozygous Opdc phenotype**

Homozygous mutant alleles of Pax2 are embryonically lethal or suffer neonatal death and this may be attributed to kidney agenesis. Opdc homozygous is also embryonically lethal. Opdc heterozygote animals were crossed and 100 offspring from the intercrosses were genotyped at weaning to obtain 61 heterozygotes, 39 wild-type and 0 homozygotes. Embryos from Pax2 Opdc heterozygotes were recovered at various developmental stages and were used for analyses. MRI scans at E15.5 of the homozygous embryos showed kidney agenesis compared to heterozygous and wild-type kidneys (Fig 2). Homozygous mutant eyes appear malformed compared to the heterozygous and wild type eyes. Histological sections through the eyes of E15.5 homozygotes showed retina expansion down the optic stalk. The same abnormality is exhibited by the heterozygous mutant though it is less severe (Fig 3B). The semicircular canals and cochlear of the homozygous mutants appear grossly dilated and deformed compared to the wild type based on 3D scans (Fig 3A). To check for cerebellar formation, Opdc mutation was crossed into each of the two backgrounds and the heterozygotes of each were mated to produce homozygous and wild type offspring. Both the homozygous and heterozygous mutants had a cerebellum present indicating that the Opdc mutation does not affect cerebellar formation (Fig 3C). 6/20 homozygous mutants on the C57BL/6J background had exencephaly while 20/21 on C3H background showed the phenotype. Only 1/47 heterozygotes on the C57BL/6J background showed exencephaly while 4/34 showed the phenotype (data not shown). The Opdc phenotypes are less severe than the loss-of-function Pax2 null alleles.

1. **DNA-binding properties of *Pax2Opdc***

His-tagged recombinant Pax2 proteins were produced from wild-type and Opdc mutant and these were used to bind to a number of known Pax2 binding sites (Fig 4E). Pax2Con which is a high affinity binding site was used first to bind both proteins and both showed a shift in the gel. A less optimal site, e5, was used next and only the wild-type bound to it indicating that the Opdc mutation might prevent its binding to suboptimal sites (Fig 4A). Competition bandshift analyses to check for binding strength to Pax2Con showed that the Opdc mutant was disrupted at lower concentrations indicating that it was not tightly bound compared to the wild-type (Fig 4B). Binding sites upstream of Pax2, Pax6 and Pax6 retinal enhancer were used and wild-type protein bound to all sites. Opdc bound only to Pax2 and weakly to Pax6 and not at all to the retinal enhancer (Fig 4C). Even though Opdc binds most target sites, binding is variable and reduced at some sites.

1. **Transcriptional activation by Opdc mutant *Pax2***

Two Pax2 isoforms were cloned into CMV and transfected into mouse NIH 3T3 fibroblasts cells. The fibroblasts were also co-transfected with a plasmid each containing a Pax2 promoter binding site and a luciferase reporter gene downstream of the sites. Fig 5 shows that wild-type Pax2 strongly activates the luciferase gene with each of the three promoters compared to the mutant Pax2 (see P-values).

1. **Transcription of downstream genes in *Pax2Opdc/+* mutants**

The levels of *Wt1* and *Ret*, two Pax2 target genes were analyzed in vivo in the C3H and C57BL/6J backgrounds. Their expression in kidneys of E18.5 wild-type and heterozygous Opdc and 1Neu lines were determined by RT-PCR. Figure 6 shows that there was no significant variation between the lines and wild type and heterozygotes (Fig 6A and B).

**Conclusion:**

The heterozygous Opdc mutation phenotype is milder than Pax2 loss-of-function mutation and is background dependent. This genetic background change may be due to strain dependent modifier genes which affect penetrance, expressivity and dominance. Opdc homozygous mutants showed variable penetrance with genetic backgrounds where some backgrounds showed only 30% exencephaly while others showed a 95% increase in penetrance of the same phenotype. In addition, haploinsufficiency caused by the mutation may be attributed to the defective phenotypes.

The Opdc mutant protein is able to bind target DNA sites and transactivate reporter genes with reduced efficiency compared to the wild-type. The I40T mutation is a change from a hydrophobic amino acid to a hydrophilic amino acid but this does not alter the binding of the protein to the target DNA. The mutation is in the first α-helix of the N-terminal and the side chain of the isoleucine extends towards the third α-helix which binds to the DNA. Replacing Isoleucine with Threonine may cause the third α-helix-DNA binding to be destabilized. However, since the C-terminal also binds DNA and the two sub-domains are structurally independent, the reduced ability of the mutant Opdc protein to bind some DNA targets and transactivate luciferase under some promoters may be mediated by the C-terminal.

Some Pax2Opdc heterozygous mutants show coloboma defects without kidney disease making Pax2 a candidate gene for screening humans with the same phenotype.

**Future research/Critique**

A larger number of patients should be screened for mutations in Pax2 since previous screens on a small number of patients with optic nerve coloboma and other eye defects did not show a Pax2 mutation. In addition, more patients with unusual but related phenotypes to RCS should also be screened to get the full scope of the diseases that characterize Pax2 mutations. Investigation of the strain specific modifier genes would shed light on the differences in phenotypes based on genetic backgrounds. Genetic modifiers responsible for suppressing some defects may be used in gene targeting to see if defects are suppressed on the more susceptible backgrounds. Pax6 is also crucial for eye development and therefore should also be investigated especially in the cases where patients have coloboma defects but do not show any Pax2 mutations.

Loss of function phenotype images would have been informative for comparison with Opdc mutants. Activation by the CMV construct alone was mentioned and considered but not explained. Relative RNA increase in transcript measurements showed counter results to previous research- no explanations why.