TESTING FOR THE CYTOSINE INSERTION IN THE VNTR OF THE MUC1 GENE IN A COHORT OF ITALIAN PATIENTS WITH AUTOSOMAL DOMINANT TUBULOINTERSTITIAL KIDNEY DISEASE

MP - 050

Claudio Musetti[1], Deepak Babu[2], Ileana Fusco[2], Simona Mellone[2], Marco Quaglia[1], Vincenzo Cantaluppi[1], Piero Stratta[1], Mara Giordano[2]

Nephrology and Kidney Translant, Univ. Piemonte Orientale, Novara, Italy
Lab Human Genetics, Univ. Piemonte Orientale, Novara, Italy

claudio.musetti@med.uniupo.it

BACKGROUND

Autosomal dominant tubulointerstitial kidney disease (ADTKD), is a rare genetic disorder characterized by a progressive tubule-interstitial kidney disease, yielding to end stage renal disease (ESRD) between the third to seventh decade of life without extra-renal manifestations, which was first described in 1944 as **medullary cystic kidney disease** (OMIM #174000). A locus on chromosome 1q21, which was the most likely locus associated with the disease, and recently Kirby et al identified an *unusual mutation, undetectable by both traditional Sanger and massively parallel sequencing, i.e. the insertion of a single cytosine in one copy (but a different copy in each patient)* of the repeat unit comprising the extremely long (~1.5–5 kb), GC-rich (>80%) coding variable-number tandem repeat (VNTR) sequence in the coding sequence of the MUC1 gene.

This gene encodes for the **mucin 1 protein**, a heterodimeric type I transmembrane protein, which has a highly glycosylated extracellular subunit with protective and lubricative functions and an intracellular domain with multiple signaling interactions and functions.

METHODS

Genomic DNA of the patients was extracted from the blood samples by standard salting-out method. SNaPshot minisequencing assay was performed for the detection of the single insertion of 'C' creating an eight base stretch of cytosines instead of a seven base stretch (slightly modified from Kirby et al (Nat Genet. 2013) and Ekici et al (Kidney Int. 2014). Briefly, 250 ng of Genomic DNA was overly digested using MwoI (New England Biolabs) which selectively cleaves the wild type sequence (GCCCCCCCAGC) while leaving the stretch with the 'C' insertion (GCCCCCCCCAGC) intact. After the digestion, tailed primers nested within the 60bp repeat units were used to amplify the remaining intact fragments containing the 'C' insertion. The PCR reactions were performed using the Q5 Hot start High fidelity DNA polymerase following the standard protocols of the supplier (New England Biolabs) and the cycling conditions included 98°c for 2 minutes followed by 45 cycles of 98°c for 10 seconds, 67°c for 30 seconds, 72°c for 30 seconds and a hold at 72°c for 2 minutes. PCR products were purified using ExoSAP-IT enzymatic PCR clean up system (Affymetrix). After a second round of MWoI digestion for the enrichment of the sequence with the 'C' insertion repeat, a SNaPshot minisequencing (SNaPshot Multiplex kit, Applied Biosystems) was performed with a 19bp primer designed corresponding to the sequence just upstream of the insertion site. Each reaction was performed with an additional 39 bp SNapshot control primer extending with a 'C' on the complementary DNA strand as an independent SnaPshot control reaction. These conditions were modified with respect to the original report (Ekici et al, 2014) in the initial amount of DNA, that was increased to 250 ng, in PCR thermal cycling profile and in the purification of PCR product, performed with an enzymatic reaction.

Name	Primer Sequence
Muc1-repeat-F	CTGGGAATCGCACCAGCGTGTGGCCCCGGGCTCCACC
Muc1-repeat-R	CGTGGATGAGGAGCCGCAGTGTCCGGGGCCGAGGTGACA
SNaPshot C-insertion primer	CGGGCTCCACCGCCCCCCC
SNaPshot control primer	CGTGGATGAGGAGCCGCAGTGTCCGGGGCCGAGGTGACA

RESULTS

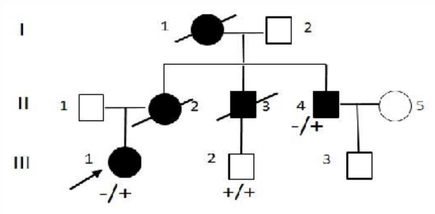
We investigated the presence of this type of MUC1 mutation in *21 unrelated patients affected by **progressive tubulointerstitial kidney disease** without signs of autoimmune or glomerular disease with a clear **autosomal dominant** segregation of renal disease*. Their renal disease was a chronic kidney disease with a **urinary protein < 1 gr/24h, without an active urinary sediment** and with usually (90.5%) one or more **kidney tubule defect**.

All pts tested negative for mutations in other genes associated to ADTKD (UMOD, HNF1B, and PAX2).

2 of the 21 patients (9.5%) carried the MUC1 C insertion in the VNTR. The comparisons between affected patients from the two mutated families (n=8, also including deceased affected patients) and negative probands (n=19) showed that ADTKD-MUC1 developed ESRD more frequently (p=0.033) and at a younger age (p=0.011).

	ADTKD-MUC1 patients (n=8)	MUC1 negative probands (n=19)	p
Male	4/8 (50%)	11/19 (57.9%)	0.516
Age at disease onset	43.5 (42-45.8)	47.5 (38.3-59.3)	**0.233**
Age > 50 years	0/8 (0%)	10/19 (52.6%)	**0.025**
Patients with ESRD	7/8 (87.5%)	7/19 (36.8%)	**0.033**
Age at ESRD	44.5 (42.5-51.3)	65 (63-68)	**0.011**
Creatinine (mg/dL)	3.5 (1.8-6.6)	1.2 (0.79-1.6)	**0.005**
Urin. Proteins (g/24h)	0.2 (0.2-0.3)	0.15 (0.11-0.3)	0.221
Hematuria	3/4 (75%)	8/18 (44.4%)	0.293
Uric Acid	8.0 (6.2-9.6)	5.6 (4.8-6.8)	*0.075*
Potassium (mEq/L)	4.5 (3.8-5.1)	4.2 (3.9-4.4)	0.212
Hypertension	7/8 (87.5%)	10/19 (52.6%)	*0.099*
Urine density (g/L)	1010 (1009-1011)	1011 (1008-1015)	0.419
Kidney tubule defect			
Ren. Tub. acidosis	0/5	4/15 (26.7%)	0.282
Low Ur. Uric ac.	1/4 (25%)	4/17 (23.5%)	0.696
Low ser. phosphate	0/5	6/19 (31.6%)	0.202
Glycosuria	0/5	2/19 (10.5%)	0.620
HypoKaliemia	1/5 (20%)	3/19 (15.8%)	0.635
HypoMagnesemia	0/3	1/15 (6.7%)	0.833
Other	0/4	2/19 (10.5%)	0.676

Family 1



	III-1	II-4	II-3	II-2	I-1
Age first symptoms	44	39	43	33	48
Age at ESRD	44	41	43	33	49
Age at kidney transplant	53	46	44	-	-
Age / Living status	56 – living	61 – living	47 – Dec.	33 – Dec.	49 – Dec.
Creatinine (mg/dL)	18.2	3.5	6.6	n/a	n/a
Uric Acid	10.1	5.4	6.5	n/a	n/a
Potassium (mEq/L)	6.2	4.5	5.1	n/a	n/a
Urinary Proteins	0.3 g/24h	0.2 g/24h	0.5 g/24h	n/a	n/a
Hematuria	10-20 RBC/hpf	No	5-10 RBC/hpf	n/a	n/a
Renal outcome	ESRD	ESRD	ESRD	ESRD	ESRD
Renal US	Small atrophic kidneys. No cysts.	Small hyper-echoic kidneys, no cysts	n/a	n/a	n/a
Hypertension?	No	Yes (3 drugs)	Yes (2 drugs)	Yes	Yes

The **proband (III-1)** was diagnosed with ESRD of unknown origin at the age of 44 (Table 2). She underwent a deceased donor KTx in 2012 without complications. She had a thyroid multinodular goiter, requiring thyroidectomy (2010), a double-component monoclonal gammopathy of undetermined significance MGUS (IgG and IgM) and breast microcystic adenosis (2011), without malignant degeneration (latest biopsy in 2015).

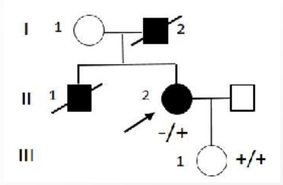
No detailed clinical information were available for her **grandmother (I-1)** except that she died at 49 (1961) with ESRD.

The **proband's mother (II-2)** died at 33 (1964) with ESRD. She was hypertensive and had maintained a residual diuresis until ESRD. No other further clinical information were available.

The living **maternal uncle (II-4)** was first diagnosed with CKD stage 4 in 1993 at the age of 39, without proteinuria or hematuria. He developed ESRD in 1995 and received a deceased donor kidney transplant in 2000. He suffers from a moderate COPD, but he was a smoker until KTx. His transplant was complicated by a BK virus associated nephropathy (2001), and he developed an IgG MGUS (2009) and a colloido-cystic thyroid goiter (2010). In 2010 he was found with a non-invasive, low-grade (G2) *urothelial carcinoma* of the bladder (stage pT1) treated with endoscopical resection and local chemotherapy. The genetic analysis revealed the presence of the MUC1 mutation in this subject.

The other proband's deceased **uncle (II-3)** developed ESRD as the first manifestation of his kidney disease (1984) and underwent kidney transplantation at the age of 44 (1985). He later developed a *pancreatic carcinoma* from which he died at the age of 47.

Family 2



Pt. ID in Figure 1	II-2	II-1	I-2
Age at first symptoms	49	45	43
Age at ESRD	-	58	45
Age at kidney transplant	-	59	-
Age / Living status	64 – Living	65 – deceased	45 – deceased
Creatinine (mg/dL)	1.59	1.8	n/a
Uric Acid	9.4	n/a	n/a
Potassium (mEq/L)	3.2	n/a	n/a
Urinary Proteins	< 0.15 g/24h	0.2 g/24h	n/a
Hematuria	10-20 cells/HPF	n/a	n/a
Renal outcome	CKD st. IV	ESRD	ESRD
Renal US	One cortical cyst (24 mm), hyperechoic parenchima, uneven profile	Bilateral small hyperechoic kidneys. Left pyelectasis (grade 1)	n/a
Hypertension?	Yes (3 drugs)	Yes	Yes

The **proband (II-2)** underwent a kidney biopsy in 2003 at the age of 49 to investigate renal failure (eGFR=38 ml/min/1.73m2) with isolated microhematuria: it revealed few sclerotic glomerular lesions and chronic tubule-interstitial nephropathy with a negative immunofluorescence and normal ultrastructural analysis. She was hypertensive since 2000 and had high levels of uric acid (> 9 mg/dL) and low potassium levels requiring oral supplementation. In 2007 she was diagnosed with carcinoma of the cervix and underwent a radical hystero-oophorectomy, with no signs of relapse to date. She had never smoked and had no known clinical risk factors for *cervical carcinoma*, even if she was never vaccinated for human papillomavirus (HPV). Moreover, the DNA of high risk HPV (type 16, 18, 35 and 41 were tested) was not detected within the neoplastic tissue.

Her **brother (II-1)** developed a non-invasive *bladder carcinoma* in 1986 (38 years), which underwent endoscopic resection. In 1993, he was found with hypertension and asymptomatic ESRD, which progressed to ESRD by 2006 (58 years), when he started peritoneal dialysis. He underwent a deceased donor kidney transplantation in 2007, but developed a chronic antibody-mediated rejection in 2012. He died in 2013 of sudden death.

Their **father (I-2)** died at 45 of cerebrovascular accident and was affected by severe kidney disease (no further clinical records are available).

DISCUSSION

The cytosine insertion in the MUC1-VNTR has been found in 4/10 (40%) families with ADTKD of European ancestry (Eckici et al, 2014) and 16/19 (84%) selected families with UMOD- and REN-negative ADTKD (Bleyer et al, 2014).

However, most of the previously reported pedigrees consisted of large families with a high chance to find a full penetrance mutation, while 40% of our families were composed by only two affected members. Indeed, in the smaller pedigrees we cannot rule out other genetic kidney disease with incomplete penetrance which would not be the case of ADTKD-MUC1.

Moreover, it should be noted that ADTKD-MUC1 leads to ESRD in almost all cases and at a young age: we might therefore *speculate that families with a clearly AD transmission of kidney disease characterized by **full penetrance** and **ESRD by the age of 60** -particularly if confirmed in a **large family tree**- have a **higher chance** to carry this type of MUC1 mutation.*

Unexpectedly, we found an apparent co-occurrence of malignancies with ADTKD-MUC1 in both the reported families, which can indeed be explained by other risk factors, like smoking and kidney transplant. However *this apparent association with epithelial malignancies, benign epithelial degenerations and MGUS seem to represent the most common extra-renal findings* and should deserve further attention. Indeed, as the observed mutation produces an aberrant protein with still unknown signaling properties, this type of MUC1 mutation might deserve further research investigating its associated extra-renal phenotype.

This is the first study reporting this type of MUC1 mutation as the cause of ADTKD in Italian families, showing that the ***genetic test** proposed by Kirby et al and replicated by Eckici et al is indeed **highly reproducible***. The affected family members showed a "classical" ADTKD renal phenotype but further studies will be welcome to better define the criteria for genetic testing and possibly investigating unexpected extra renal characteristics. The hypothesis that an aberrant MUC1 product may impair intracellular signal transduction is consistent with previous literature and might explain this observation.

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