

Supplementary Appendix for

Urinary mRNA for the diagnosis of renal allograft rejection: the issue of normalization

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SUPPLEMENTARY MATERIAL AND METHODS

Method for absolute quantification of mRNAs and miRNAs

RNA levels will be calculated using a previously published standard curve method (Kawai T, et al. N Engl J Med 2008; 358:353–361). The standard curve is established using PCR generated 73bp mouse Bak amplicon as the standard. The Bak amplicon is generated in a PCR reaction using Veriti® thermal cycler and with 3 µl cDNA and 22 µl of dNTP, 10X PCR buffer, Taq DNA polymerase, and Bak specific oligonucleotide primer pair (Sense primer: 5' CCCACATCTGGAGCAGAGTCA 3' [192-212]; Antisense primer: 5' CAGATGCCATTTTCAGGTCTTG 3' [264-242], Accession Number Y13231). The PCR product is separated by electrophoresis with a 2% agarose gel and the amplicon size (73bp) is confirmed using a DNA size standard of pUC mix marker 8 (Crystalgen, Plainview, NY). The Bak amplicon is isolated and purified from the gel with QIAquick gel extraction kit (Qiagen). The absolute quantity of the purified amplicon is measured by A260 and converted to the number of copies using the molecular weight of DNA. The Bak amplicon is diluted to the concentration of 10^7 copies/µl (stock solution). When a standard curve is to be established for the real-time quantitative PCR assay, the stock solution is diluted over 6 orders of magnitude (1000000, 100000, 10000, 1000, 100, 10 copies per 1 µl) (work solution). 2.5 µl of the work solution is added to duplicate wells and amplified with Bak specific primer pair and Bak specific fluorogenic TaqMan probe (5' FAM CAGGTGACAAGTGACGGTGGTCTCCA TAMRA 3' [215-240]). The threshold cycles (C_T) is then plotted versus the log of the initial amount of the Bak amplicon to develop the standard curve.

Supplemental Table 1: Patient characteristics

Variables	Whole population (n=108)	Acute Rejection (n=52)	No rejection (n=56)	P Value^a
<i>Recipients characteristics</i>				
Male, n (%)	67(62)	31(60)	36(64)	0.69
Age at biopsy, mean±SD, yr	47±16	45±17	49±16	0.21
Ethnicity				0.38
White, n (%)	57(53)	28(55)	29(51)	
Black, n (%)	27(25)	10(20)	17(30)	
Other, n (%)	23(21)	13(25)	10(18)	
Cause of end stage renal disease, n (%)				0.02
Glomerulonephritis	23(21)	12(23)	11(20)	
Diabetes	13(12)	1(2)	12(21)	
Cystic/hereditary/congenital	12(11)	6(12)	6(11)	
Hypertension	20(19)	10(19)	10(18)	
Miscellaneous conditions	22(20)	15(29)	7(13)	
Etiology uncertain	18(17)	8(15)	10(18)	
<i>Immunosuppressive regimen</i>				
Induction therapy				0.34
IL-R antagonist, n (%)	50(46)	22(48)	25(45)	
Thymoglobulin®, n (%)	51(47)	25(42)	29(52)	
<i>Transplant variables</i>				
Donor age, mean±SD, yr	55±17	54±18	56±16	0.43
Deceased donor, n (%)	82(76)	40(77)	42(75)	0.83
Retransplantation, n (%)	11(10)	7(13)	4(7)	0.35
Cold ischemia time, mean±SD, hr	16±9	15±9	16±10	0.66
Type of rejection, n (%)				
TCMR	11 (21)	11(21)	0 (0)	
ABMR	28 (54)	28 (54)	0 (0)	
Mixed	9 (17)	9(17)	0 (0)	
Borderline	3 (6)	3(6)	0 (0)	

^aRejection vs no rejection, Student's t-test for quantitative variables and two-sided exact Fisher's test for categorical data.

TCMR, T cell-mediated rejection; ABMR, antibody-mediated rejection

Supplemental Table 2: Banff scores (mean \pm SD) and percent of patients with histology scores >0 on biopsy

Elementary lesions	Whole population (n=108)	Rejection (n=52)	No rejection (n=56)	P value ^a
Glomerulitis (g)				
% with g score >0	28.7%	57.7%	1.8%	<0.0001
Mean g score	0.60 ± 1.03	1.23 ± 1.20	0.02 ± 0.13	<0.0001
Interstitial infiltrate (i)				
% with i score >0	31.5%	57.7%	7.1%	<0.0001
Mean i score	0.59 ± 0.97	1.12 ± 1.11	0.11 ± 0.41	<0.0001
Tubulitis (t)				
% with t score >0	31.5%	63.5%	1.8%	<0.0001
Mean t score	0.69 ± 1.13	1.40 ± 1.27	0.04 ± 0.27	<0.0001
Vasculitis (v)				
% with v score >0	6.8%	14.3%	0.0%	0.0043
Mean v score	0.12 ± 0.49	0.24 ± 0.69	0.00 ± 0.00	0.0170.
Allograft glomerulopathy (cg)				
% with cg score >0	11.1%	23.1%	0.0%	<0.0001
Mean cg score	0.19 ± 0.63	0.40 ± 0.87	0.00 ± 0	0.0015
Interstitial fibrosis (ci)				
% with ci score >0	64.8%	79.6%	51.8%	0.0040
Mean ci score	1.40 ± 1.20	1.65 ± 1.09	1.18 ± 1.25	0.0404
Tubular atrophy (ct)				
% with ct score >0	63.8%	77.6%	51.8%	0.0081
Mean ct score	1.36 ± 1.20	1.63 ± 1.11	1.13 ± 1.24	0.0290
Chronic vascular changes (cv)				
% with cv score >0	69.4%	78.3%	61.5%	0.0832
Mean cv score	1.37 ± 1.11	1.59 ± 1.09	1.17 ± 1.20	0.0642
Mesangial matrix increase (mm)				
% with mm score >0	31.5%	44.2%	19.6%	0.0073
Mean mm score	0.48 ± 0.83	0.62 ± 0.82	0.36 ± 0.82	0.1047
Peritubularcapillaritis (ptc)				
% with ptc score >0	41.1%	82.4%	3.6%	<0.0001
Mean ptc score	0.75 ± 0.99	1.53 ± 0.92	0.04 ± 0.19	<0.0001
Arteriolar hyalinosis (ah)				
% with ah score >0	64.2%	70.6%	58.2%	0.2256
Mean ah score	1.41 ± 1.15	1.33 ± 1.18	1.05 ± 1.10	0.1055
C4d staining				
% with C4d score >0	28.9%	43.5%	15.7%	0.0034
Mean C4d score	0.52 ± 0.93	0.85 ± 1.13	0.22 ± 0.54	0.0010

^a Rejection vs no rejection, Student's t-test for quantitative variables and two sided exact Fisher's test for categorical data

Supplemental Table 3: Oligonucleotide primers and probes used for the quantification of RNAs

Gene	Sequence	Location
IP-10	Sens: 5' TGTCCACGTGTTGAGATCATTG 3'	235-256
	Antisense: 5' GGCCTTCGATTCTGGATTCA 3'	309-290
	Probe: 5' FAM TACAATGAAAAAGAAGGGTGAGAA MGB 3'	258-281
CD3ε	Sense: 5' AAGAAATGGGTGGTATTACACAGACA 3'	131-156
	Antisense: 5' TGCCATAGTATTTTCAGATCCAGGAT 3'	233-209
	Probe: 5' FAM CCATCTCTGGAACCACAGTAATATTGACATGCCTAMRA 3'	170-202
18S rRNA	Sens: 5' GCCCGAAGCGTTTACTTTGA 3'	929-948
	Antisense: 5' TCCATTATTCCTAGCTGCGGTATC 3'	1009-986
	Probe: 5' FAM AAAGCAGGCCCGAGCCGCC TAMRA 3'	965-983
GAPDH	Sens: 5' CCACATCGCTCAGACACCAT 3'	85-104
	Antisense: 5' TGACCAGGCGCCAATA 3'	157-141
	Probe: 5' FAMAGTCAACGGATTTGGTC MGB3'	123-139
HPRT	TaqMan® Gene Expression Assays Hs02800695_m1 (Life Technologies)	
UPK1A	TaqMan® Gene Expression Assays Hs01086736_m1 (Life Technologies)	

Supplemental Table 4: experimental conditions, total quantity of obtained RNA and copy numbers of IP10, 18S, GAPDH, HPRT and UPK RNAs in three in vitro experiments mimicking clinical situations.

	Condition 1	Condition 2	Condition 3
Cell mixture (n)			
Unstimulated PBMCs	1.0E+5	0	0
Stimulated PBMCs	0	1.0E+5	1.0E+5
Urothelial cells	1.0E+6	1.0E+6	1.0E+6
Tubular epithelial cells	0	0	1.0E+5
Quantity of purified RNA (μg)	21.1	25.4	43.9
RNA copy number (copies/μg RNA)			
IP-10 mRNA	3,06E+02	2,33E+04	8,05E+03
18S rRNA	1,33E+10	1,69E+10	1,21E+10
GAPDH mRNA	7,80E+06	9,98E+06	1,62E+07
HPRT mRNA	2,18E+05	2,71E+05	2,25E+05
UPK mRNA	2,30E+03	3,30E+03	1,23E+03

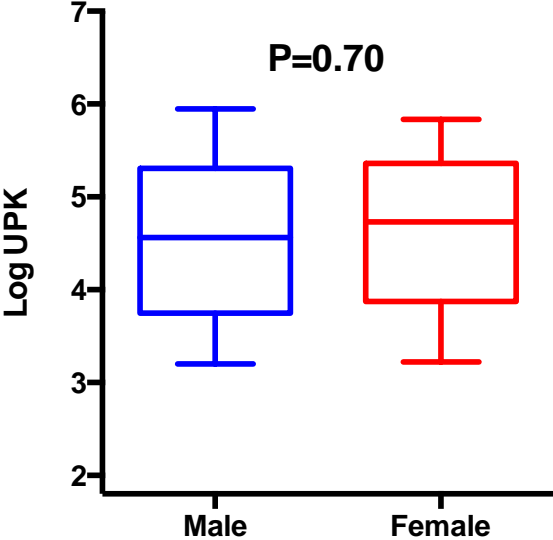
SupplementalTable 5: Discrimination of acute rejection by the urinary biomarkers. The C statistics, sensitivity, and specificity are shown.

Biomarker	ROC-based discrimination measures			
	AUC (95% CI)	AUC P value	Sensitivity	Specificity
IP-10 mRNA				
Absolute copy number	0.76 (0.66-0.86)	0.000008	0.69	0.71
18S-normalized	0.73 (0.63-0.84)	0.00004	0.60	0.80
GAPDH-normalized	0.75 (0.65-0.85)	0.00002	0.73	0.69
HPRT-normalized	0.70 (0.59-0.81) ^a	0.0004	0.63	0.73
UPK-normalized	0.73 (0.63-0.84)	0.00004	0.69	0.76
CD3ϵ mRNA				
Absolute copy number	0.67 (0.56-0.78)	0.002	0.68	0.67
18S-normalized	0.64 (0.53-0.75)	0.009	0.70	0.65
GAPDH-normalized	0.69 (0.58-0.80)	0.0006	0.66	0.71
HPRT-normalized	0.56 (0.44-0.68) ^{b, c}	0.2	0.53	0.64
UPK-normalized	0.62 (0.50-0.73)	0.02	0.63	0.63

^aP=0.03 versus GAPDH normalization; ^bP=0.04 versus absolute copy number; ^cP=0.00009 versus GAPDH normalization

ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval.

Supplemental Figure 1:



Supplemental Figure 2

