SUPPLEMENTAL DIGITAL CONTENT (SDC)

SDC, Methods

Study cohort and renal allograft histology. The for-cause biopsy group consisted of 48 subjects with graft dysfunction and biopsy-confirmed tubulointerstitial fibrosis (Fibrosis biopsy group, N=48) and the protocol biopsy group included 66 subjects with stable allograft function and normal allograft biopsy (Normal biopsy group, N=66).

The biopsy specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin, periodic acid–Schiff, and Masson's trichrome stains. Cryostat or paraffin sections of the for-cause biopsies were examined for C4d deposition with the use of anti-human C4d antibody. In addition to screening for the presence or absence of fibrosis and the grading of fibrosis, the allograft biopsies were also classified using the Banff 07 updated version of Banff 97 diagnostic categories and using the Banff schema 66 allograft biopsies were classified as Normal, and 48 biopsies with fibrosis were classified as IF/TA, no evidence of any specific etiology (N= 30), chronic antibody-mediated rejection (N=6), chronic active T-cell mediated rejection (N=6), and the remaining 6 with fibrosis were also classified as having diabetic nephropathy (N=4) or recurrent glomerular disease (N=2).

The allograft fibrosis biopsies were also scored for concurrent inflammation as indicated by cellular infiltration within non-fibrotic areas of cortical interstitium. Among the 48 patients with allograft fibrosis, 32 biopsies from 32 patients showed no inflammation (inflammation score=0) and 16 biopsies from 16 patients displayed both fibrosis and inflammation. Inflammation was graded as 1 when 10-25% of cortical interstitium was involved (N=8 biopsies), 2 when 26-50% of cortical interstitium was involved N= 2 biopsies) or 3 when greater than 50% cortical interstitium was involved , N= 6 biopsies).

All biopsies were classified by a pathologist (SVS) blinded to the molecular study results.

Urine collection. One hundred and four of the 114 urine specimens for the mRNA profiling study were collected within 24 hours of the biopsy procedure, 8 within 7 days and the remaining 2 specimens within 15 days. These time lines refer to the time intervals between the biopsy procedure and urine specimen collection and not to the time interval between the time the urine was collected and when it was centrifuged to obtain the urine pellet prepared for RNA isolation. In the study reported here, every urine specimen was centrifuged and the cell pellet was prepared within 4 hours of urine collection. RNAlater (50µl) was then added to the urine pellet and stored at -80^{0}C prior to isolation of RNA.

Pre-amplification enhanced real-time quantitative PCR Assay. We designed oligonucleotide primers and fluorogenic probes for the measurement of levels of mRNAs (SDC Table 1). encoding proteins implicated in fibrosis, extracellular matrix accumulation, and/or EMT (TGFß1, integrin ß6 [ITGB6], fibroblast growth factor-2 [FGF2], connective tissue growth factor [CTGF], PAI1, tissue inhibitor of metalloproteinases-1 [TIMP1], fibronectin 1, collagen 1A1, E-cadherin, BMP7 and HGF). We also measured mRNAs for proteins expressed in renal tubular epithelial cells (NKCC2 found on the apical membrane of the thick ascending limb of loop of Henley, and uterine sensitization associated gene 1 [USAG1] expressed in distal collecting tubules), mesenchymal cells (vimentin, FSP1, α-smooth muscle actin [α-SMA]), and effector and/or regulatory T lymphocytes (perforin, granzyme B, CD25, CD103, FoxP3, CTLA4).

PCR analysis was performed by a two-step process, a preamplification step followed by measurement of mRNA with an ABI Prism 7500 Fast detection system. A pre-amplification protocol that allows quantification of these 22 mRNAs from small amounts of cDNA was developed. The pre-amplification reaction for each sample was set up in a 0.2 ml PCR tube with a final reaction volume of 10 µl containing 3.0 µl cDNA (from reverse transcription of 1 µg total

RNA in 100 µl buffer), 1.0 µl 10x buffer, 1.0 µl MgCl2 (25 mM), 0.25 µl 4x dNTP (10 mM each), 0.25 µl Ampli-Taq gold (5 U/µl), 0.15 µl primer mix per gene (50 µM sense and 50 µM antisense primer) and water to final volume of 10 µl. Following vortexing, the PCR was set up using a Veriti thermal cycler (Applied Biosystems) and the 10-cycle PCR reaction profile consisted of an initial hold at 95^{o}C for 10 min, denaturing at 95^{o}C for 15 seconds and primer annealing and extension at 60^{o}C for 1 min. At the end of 10 cycles, 140 µl of TE buffer was added to the PCR reaction and 2.5 µl of diluted PCR amplicons were then used for quantification of mRNA using the real-time quantitative PCR assay.

Transcript levels (copy number/µg total RNA) were calculated by a standard curve method and all analyses of mRNA copy numbers statistically controlled for the copy number of the reference gene 18S ribosomal RNA (rRNA).

Advantage of the LOESS Model. LOESS (locally weighted scatterplot smoothing) is a powerful tool to elucidate the potentially non-linear relationship between two variables since it has the advantage of fitting segments of data without pre-specifying a specific, usually linear, global function. Importantly, a threshold effect at which the risk for an outcome increases can be ascertained.

Definition of Parsimonious Model. A parsimonious model is a model that contains the fewest number of predictor variables for a given outcome, without compromising the model's prediction accuracy. In essence, it balances the trade-off between simplicity (simpler is better) and the incremental increase in prediction accuracy that is obtained by including more predictors in a model. Our analyses of levels of 22 mRNAs measured in our study showed that the diagnostic accuracy of the 4-gene model (vimentin, 18S, NKCC2 and E-cadherin) is not significantly improved by inclusion of the levels of any or all

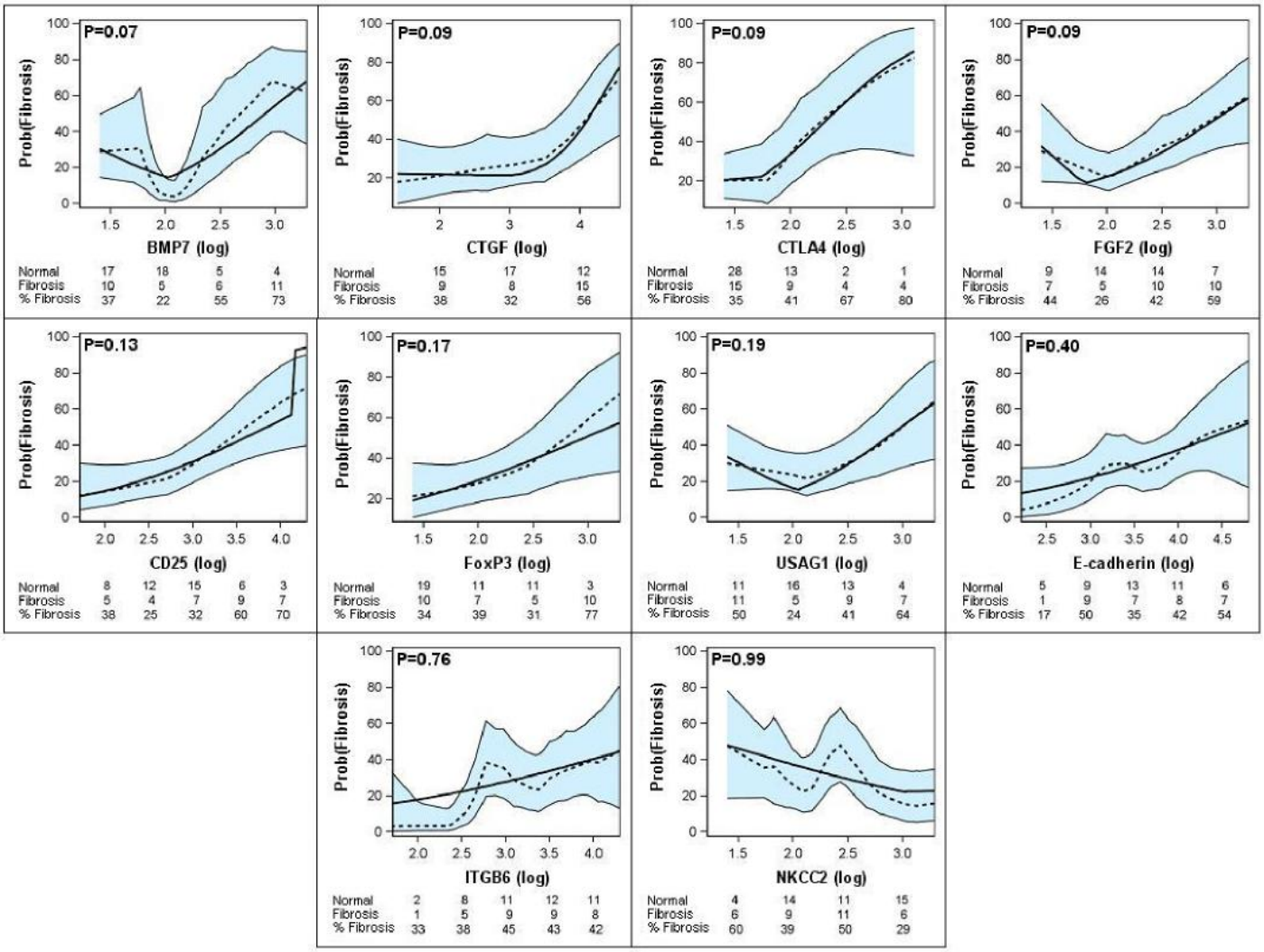
of the remaining 18 mRNAs we measured. Thus, the 4-gene model is the parsimonious model in this study.

SDC, Table S1. Oligonucleotide primers and probes used in kinetic quantitative polymerase chain reaction assays for the quantification of mRNAs.

Gene	Accession number	Sequence	Location
Vimentin	NM_003380.2	Sense: 5' TCAGAGAGAGGAAGCCGAAAAC 3'	706-727
		Antisense: 5' CCAGAGACGCATTGTCAACATC 3'	770-749
		Probe: 5' FAM CCCTGCAATCTTTCAGAC MGB 3'	729-746
HGF	BC063485	Sense: 5' CAAATGTCAGCCCTGGAGTTC 3'	526-546
		Antisense: 5' CTGTAGGTCTTTACCCCGATAGCT 3'	604-581
		Probe: 5' FAM ATGATACCACACGAACACAGCTTTTTGCC TAMARA 3'	548-576
α-SMA	NM_001613	Sense: 5' TGGGACGACATGGAAAAGATC 3'	288-308
		Antisense: 5' CAGGGTGGGATGCTCTTCAG 3'	365-346
		Probe: 5' FAM CCACTCTTTCTACAATGAGCTTCGTGTTGCC TAMRA 3'	314-344
Fibronectin 1	XM_055254	Sense: 5' GAAAGTACACCTGTTGTCATTCAACA 3'	2582-2607
		Antisense: 5' ACCTTCACGTCTGTCACTTCCA 3'	2688-2666
		Probe: 5' FAM CCACTGGCACCCCACGCTCA TAMRA 3'	2613-2632
PAI1	NM_000602.1	Sense: 5' AATCAGACGGCAGCACTGTCT 3'	716-736
		Antisense: 5' GGGCGTGGTGAACTCAGTATAGT 3'	792-770
		Probe: 5' FAM TGTGCCCATGATGGC MGB 3'	738-752
Perforin	M28393	Sense: 5' GGACCAGTACAGCTTCAGCACTG 3'	492-514
		Antisense: 5' GCCCTCTTGAAGTCAGGGTG 3'	587-568
		Probe: 5' FAM TGCCGCTTCTACAGTTTCCATGTGGTACAC TAMRA 3'	526-555
TGFß1	NM_000660	Sense: 5' GCGTGCTAATGGTGGAAACC 3'	1170-1189
		Antisense: 5' CGGAGCTCTGATGTGTTGAAGA 3'	1263-1242
		Probe: 5' FAM ACAACGAAATCTATGACAAGTTCAAGCAGAGTACACA TAMRA 3'	1191-1227
TIMP1	NM003254	Sense: 5' GACGGCCTTCTGCAATTCC 3'	288-306
		Antisense: 5' GTATAAGGTGGTCTGGTTGACTTCTG 3'	366-341
		Probe: 5' FAM AGGGCCAAGTTCGTGG MGB 3'	319-334
Granzyme B	J04071	Sense: 5' GCGAATCTGACTTACGCCATTATT 3'	534-557
		Antisense: 5' CAAGAGGGCCTCCAGAGTCC 3'	638-619
		Probe: 5' FAM CCCACGCACAACTCAATGGTACTGTCG TAMRA 3'	559-585
FSP1	CR450345.1	Sense: 5' AGGAGCTGCTGACCCGG 3'	104-120
		Antisense: 5' GCTTCATCTGTCCTTTTCCCC 3'	158-138
		Probe: 5' FAM CTGCCCAGCTTCT MGB 3'	124-136
CD103	XM_008508	Sense: 5' CGTGCTCAGCTCCCTTCTG 3'	211-229
		Antisense: 5' CCTGGTGTCCTCTTGGTTCTG 3'	297-277
		Probe: 5' FAM ACCAAGACCCCAGCACCAACCATACCT TAMRA 3'	231-257
Collagen 1A1	NM_000088.3	Sense: 5' CCAGAAGAACTGGTACATCAGCAA3'	4050-4073
		Antisense: 5' CGCCATACTCGAACTGGAATC3'	4144-4124
		Probe: 5' FAM ACAAGAGGCATGTCTGG MGB 3'	4085-4101
BMP7	NM_001719.1	Sense: 5' GCTTCGTCAACCTCGTGGAA 3'	526-545
		Antisense: 5' CAAACCGGAACTCTCGATGGT 3'	597-577
		Probe: 5' FAM ATGACAAGGAATTCTTCCACCCACGCTAC TAMRA 3'	547-575

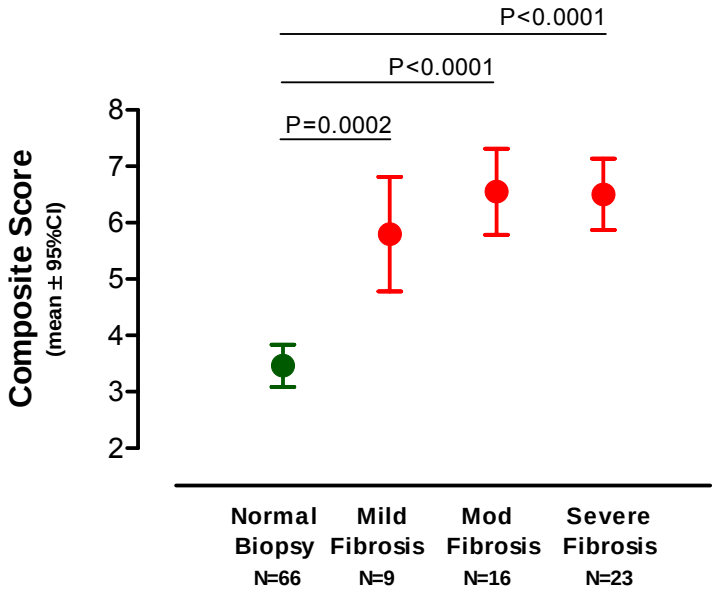
CTLA4	BC074893	Sense: 5’ CGCCATACTACCTGGGCATAG 3’	441-461
		Antisense: 5 GATCCAGAGGAGGAAGTCAGAATC 3’	529-506
		Probe: 5’ FAM CAGATTTATGTAATTGATCCAGAACCGTGCCC TAMRA 3’	473-504
CTGF	NM_001901	Sense: 5’ TGTGTGACGAGCCCAAGGA 3’	639-657
		Antisense: 5’ TAGTTGGGTCTGGGCCAAAC 3’	725-706
		Probe: 5’ FAM CCTGCCCTCGCGGCTTACCG TAMRA 3’	674-693
FGF2	NM_002006.3	Sense: 5’ CCGACGGCCGAGTTGAC 3’	601-617
		Antisense: 5’ TAACGGTTAGCACACACTCCTTTG 3’	712-689
		Probe: 5’ FAM ACCCTCACATCAAGCTACAACTTCAAGCAGAA TAMRA 3’	637-668
CD25	NM_000417	Sense: 5’ GACTGCTCACGTTCATCATGGT 3’	185-206
		Antisense: 5’ AATGTGGCGTGTGGGATCTC 3’	266-247
		Probe: 5' FAM AGAGCTCTGTGACGATGACCCGCC TAMRA 3'	222-245
FoxP3	NM_014009	Sense: 5’ GAGAAGCTGAGTGCCATGCA 3’	939-958
		Antisense: 5 GGAGCCCTTGTCGGATGAT 3’	1025-1007
		Probe: 5’ FAM TGCCATTTTCCCAGCCAGGTGG TAMRA 3’	962-983
USAG1	NM_015464	Sense: 5' TGGAGGCAGGCATTTCAGTAA 3'	364-366
		Antisense: 5' TTCCCGGCAACCCACTT 3'	412-396
		Probe: 5' FAM CCCGAGTGTTCCGATCCAGTCCAGT TAMRA 3'	392-368
NKCC2	BC040138.2	Sense: 5’ TCACGAGCAACTCGCAAAGA 3’	588-607
		Antisense: 5’ TCCCATCACCGTTAGCAACTC 3’	658-638
		Probe: 5’ FAM TGTGGCAGTCACCCCAAGTTCAGC TAMRA 3’	609-632
ITGB6	NM_000888.3	Sense: 5’ GGATTGAACTGCTTTGCCTGTT 3’	21-42
		Antisense: 5’ GGCACAGCCACCTTGTACGT 3’	69-88
		Probe: 5’ FAM TTTCTATTTCTAGGAAGGAATG MGB 3’	44-65
E-cadherin	XM_007840	Sense: 5' TGAGTGTCCCCCGGTATCTTC 3'	2469-2489
		Antisense: 5' CAGCCGCTTTCAGATTTTCAT 3'	2549-2529
		Probe: 5' FAM CCTGCCAATCCCGATGAAATTGGAAAT TAMRA 3'	2495-2521
18S rRNA	K03432	Sense: 5' GCCCGAAGCGTTTACTTTGA 3'	929-948
		Antisense: 5' TCCATTATTCCTAGCTGCGGTATC 3'	1009-986
		Probe: 5’ FAM AAAGCAGGCCCGAGCCGCC TAMRA 3’	965-983

SDC, Figure S1. Predicted probability of fibrosis as a function of urinary cell mRNA copy number in the Discovery set, for LOESS model and piece-wise linear logistic regression model, after controlling for 18S rRNA copy number.



SDC, Figure S1. Urine samples were collected from 32 renal transplant recipients with graft dysfunction and biopsy-confirmed fibrosis and 44 recipients with stable allograft function and normal allograft biopsy, and levels of mRNA in urinary cells were measured with the use of pre-amplification enhanced kinetic quantitative PCR assays. Figure shows the predicted probability of fibrosis (Y-axis), controlling for 18S rRNA, as a function of individual $\log_{10}$-transformed mRNA copy numbers for ten of the twenty-two genes (X-axis). Each plot shows the LOESS model's predicted probabilities (dotted line), their 95% confidence interval (shaded area) and the logistic regression model's predicted probabilities (solid line). Adjusted P-value for each parametric model is shown. The number of stable patients, number of fibrosis patients, and percentage of fibrosis patients within categories of the mRNA measure appear in each plot.

SDC, Figure S2. Mean level (and 95%CI) of the 4-gene composite score by Banff diagnostic category.



SDC, Figure 2. Mean level (and 95%CI) of the 4-gene composite score by fibrosis grade. Kidney allograft biopsies were classified as normal, mild fibrosis (grade I, <25% of cortical area), moderate (grade II, 26-50% of cortical area), or severe (grade III, >50% of cortical area). The mean (and 95%CI) composite scores derived from urinary cell vimentin, NKCC2 and E-cadherin mRNA levels and 18S rRNA level were significantly different across the four groups ($P<0.0001$, one-way ANOVA). Pair-wise comparisons revealed that the mean composite score of normal biopsies was significantly different from those of mild fibrosis ($P=0.0002$, Tukey's honestly significant differences criterion), moderate fibrosis ($P<0.0001$) and severe fibrosis ($P<0.0001$). Within the fibrosis group however the mean composite scores were not significantly different (mild vs. moderate [$P=0.64$], mild vs. severe [$P=0.65$] and moderate vs. severe [$P=0.99$]). Values under each biopsy diagnosis show the number of kidney graft recipients from whom urine samples were collected for the measurement of urinary cell mRNA.