Antitumor immunity as the basis for durable disease-free treatment-free survival in patients with metastatic urothelial cancer

Jonathan F. Anker[1], Sumanta K. Pal[2], Seunghee Kim-Schulze[3], Huan Wang[4], Rebecca Halperin[4], Andrew Uzilov[4], Naoko Imai[3], Shingo Eikawa[3], Takuro Saito[5], Robert Sebra[6], Noah M. Hahn[7], Manishkumar Patel[3], Jingjing Qi[3], Hui Xie[3], Nina Bhardwaj*[1], Sacha Gnjatic*[1,3], Matthew D. Galsky*[1]

SUPPLEMENTAL MATERIAL

SUPPLEMENTARY METHODS

HLA Class I and Class II target enrichment

HLA Class I and Class II loci were enriched using PCR amplification of genomic DNA (gDNA) input followed by quantification using the Qubit HS dsDNA kit (Thermofisher Scientific). 200 ng of gDNA was used as input for amplification and added to the Qiagen LongRange PCR mix comprised of 0.4 µl LongRange Enzyme, 2.5 µl LongRange Buffer, 1.25 µl dNTP mix and nuclease free water to achieve a final volume of 24 µl. GenDX NGSgo-AmpX (GenDX) HLA Class I and II amplification primers were used and resuspended in 108 µl of nuclease free water (GenDX). 1.0 µl of HLA Class I and Class II primer was then added per reaction to generate a final reaction volume of 25 µl. The initial denaturation condition performed was at 95°C for 3 minutes for a total of 35 cycles, followed by denaturation at 95°C for 15 seconds, primer annealing at 65°C for 30 seconds, and elongation at 68°C for 5 minutes. A final elongation step was performed after the 35th cycle at 68°C for 10 minutes with a cooling step at 15°C overnight. Once

amplification was completed, the PCR products were quantified using the Qubit HS dsDNA kit (Thermofisher Scientific) and with the Agilent DNA 12000 Bioanalyzer protocol to quantify amplicon length based on the GenDX NGSgo-AmpX kit estimates. (For HLA-A, -B and -C PCR products, sizes were 3 kbp to 4 kbp; DRB1 and DRB3 sizes were 3.7 kbp to 4 kbp; DRB4 and DPB1 contained two expected PCR products, the DRB4 products at 400 bp and 1.5 kb, and the DPB1 products at 5.0 kbp and 5.7 kbp; DRB5 products were 4 kbp; DQA1 products were 5.5 kbp to 5.8 kbp; DQB1 amplicons were quantified to be 3.7 kbp to 4kbp; DPA1 amplicons were 4.7 kbp in length) The amplified PCR products were purified using a bead wash condition of 0.6X volume of AMPure PB Beads (Pacific Biosciences, part no. 100-265-900) and further eluted in 30 µl of elution buffer (Zymo Research).

Single molecule real-time library preparation, DNA sequencing, and HLA typing

Purified PCR products were normalized to equal concentrations and all amplicons were pooled per sample and quantified using the Qubit HS dsDNA protocol (Thermofisher) and the Agilent DNA 12000 Bioanalyzer protocol. 500 ng of pooled product from each sample was then brought through the PacBio Amplicon Template Preparation and Sequencing protocol for SMRTbell library preparation, using the SMRTbell template Prep Kit 1.0 (part no. 100-222-300) using the 0.6X AMPure PB bead size selection condition. SMRTbell libraries for DRB4 amplicons were prepped separately in order to adequately sequence the 400 bp exon 2, using a 1.0X Ampure PB bead size selection followed by each library barcoded individually using SMRTbell Barcoded Adapters (part no. 100-465-900) and multiplexed.

The recommended 20:1 primer:template ratio was used for primer annealing, whereas P6 polymerase binding was performed at a modified 3:1 polymerase to template ratio for DRB4 amplicon SMRTbells and a 10:1 ratio for all other SMRTbells. HLA SMRTbell libraries were immobilized onto SMRT cells at a concentration of 150 pM on-chip for DRB4 amplicons and 70 pM on-chip for all other products. Loading titrations were performed to achieve optimal

sequencing conditions when specific samples required perturbation to achieve optimal loading parameters. SMRT sequencing was then performed on the RSII system. Long Amplicon Analysis pipeline v2 (LAA2) was used to process reads and generate .FASTQ files which were imported into the NGSengine v2.7.0 (GenDX) software for haplotyping and allele annotation, SNP identification, and individual base calling prior to additional analyses.

Neoantigen prediction pipeline

The novel composite score for neoantigen prediction incorporated the predicted MHC binding affinity (IC50) to cognate HLA, position of the mutation in the short predicted peptide (with or without major anchor motif), Levenshtein distance from the nearest self-epitope, expression in the The Cancer Genome Atlas (TCGA) UC cohort (measured as reads per kilobase of transcript per million reads mapped [RPKM]),[1] variant allelic frequency (VAF), and prior wildtype (WT) protein immunogenicity for IgG reactivity using a database of seromic protein microarray reactivity of sera from multiple cancer types (10,380 protein transcripts, Invitrogen/ThermoFisher's ProtoArrays). The greatest weight was applied to the IC50, scored as a percentile out of a maximum of 500 nM. For point mutations located in a canonical binding position (second or last position for most HLA types), the IC50 of the WT peptide was subtracted from the scoring. Distance from nearest self was normalized by peptide length, favoring epitopes with unique features. TCGA expression and potential for seroreactivity were incorporated as score enhancers if detected.

Each peptide contained up to 25 amino acids to encompass either a point mutation at its center or a new frameshift sequence to allow for natural epitope processing and HLA class II presentation. Up to 20 long polypeptides were synthesized per patient (online supplemental tables S2-S8), with the majority achieving >85% purity (Genscript, Biosynthesis).

The formula for peptide scoring was as follows (subtraction of WT IC50 was only performed for mutations in an anchor position, not for those in a middle position):

*(5000 – (variant IC50 – WT IC50) / 5000) × (peptide length / (peptide length – distance from nearest self – 0.3 × |(9-peptide length)|)) × (frequency of seromic detections/10 + 1) × ((log(TCGA RPKM) + 10) / 10) * VAF*

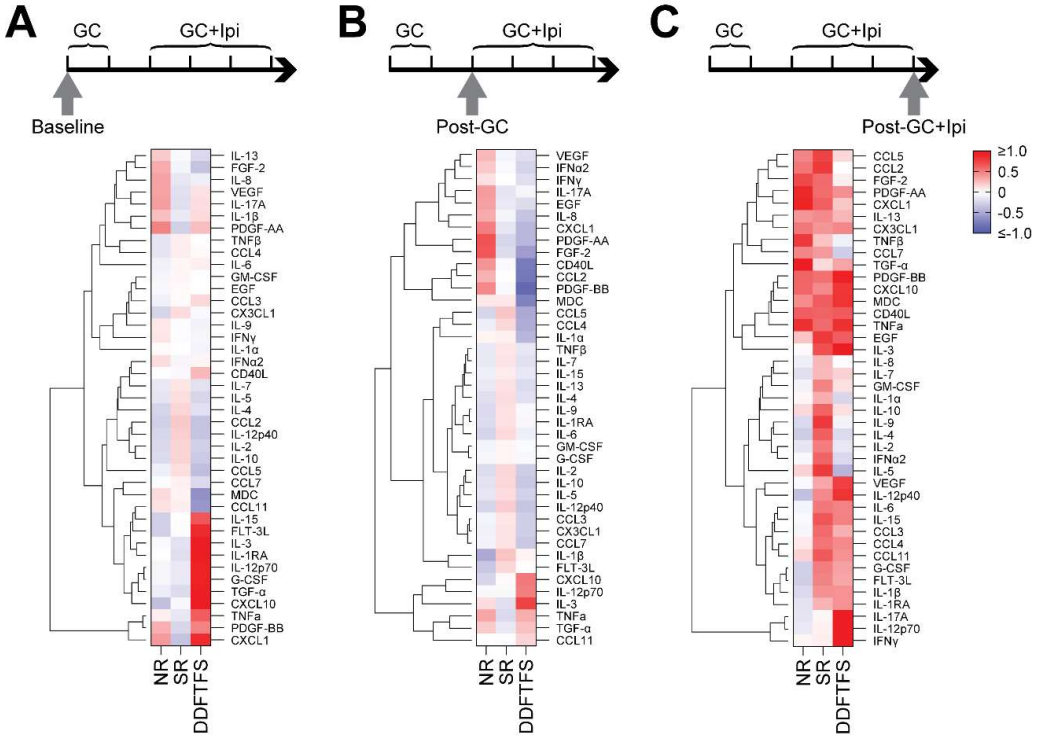
ELISpot

$CD4^+$ and $CD8^+$ T cells were isolated from PBMCs at baseline, cycle 3 day 1, and cycle 6 day 1, using magnetic beads (Invitrogen). 500,000 cells/well were plated in RPMI with 10% human AB serum (complete medium; Sigma-Aldrich) in 96-well round-bottom culture plates (Gibco). Remaining cells were pulsed with cognate pools of up to 20 polypeptides (1 µg/ml) overnight, irradiated, and co-cultured 1:1 with $CD4^+$ and $CD8^+$ T cells in complete medium supplemented with IL-2 (10 IU/ml; Roche) and IL-7 (20 IU/ml; R&D Systems). Remaining PBMCs were used to generate autologous antigen-presenting cells (APCs), which were expanded with recombinant CD40L and IL-21. After 20 ($CD4^+$) or 10 ($CD8^+$) days, T cells were co-cultured for 18 hours in ELISpot 96-well nitrocellulose plates (Millipore Sigma, MSHAS4510) coated with anti-IFN-γ monoclonal antibody (4 µg/ml) at 1:1 and 1:5 ratios with APCs that had been pulsed overnight with peptide subpools of 6-7 peptides or DMSO vehicle as a negative control. Stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin served as a positive control. ELISpot processing and data extraction were performed per manufacturer instructions (CTL ImmunoSpot, Cellular Technology Limited). Reactivity was quantified as the percentage of the well stained, subtracted by that of the negative control and normalized to that of the positive control. A well was considered positive for neoantigen reactivity if spots comprised ≥5% of the well area and the stained area was ≥5-fold increased from that of the respective negative control well.

REFERENCES

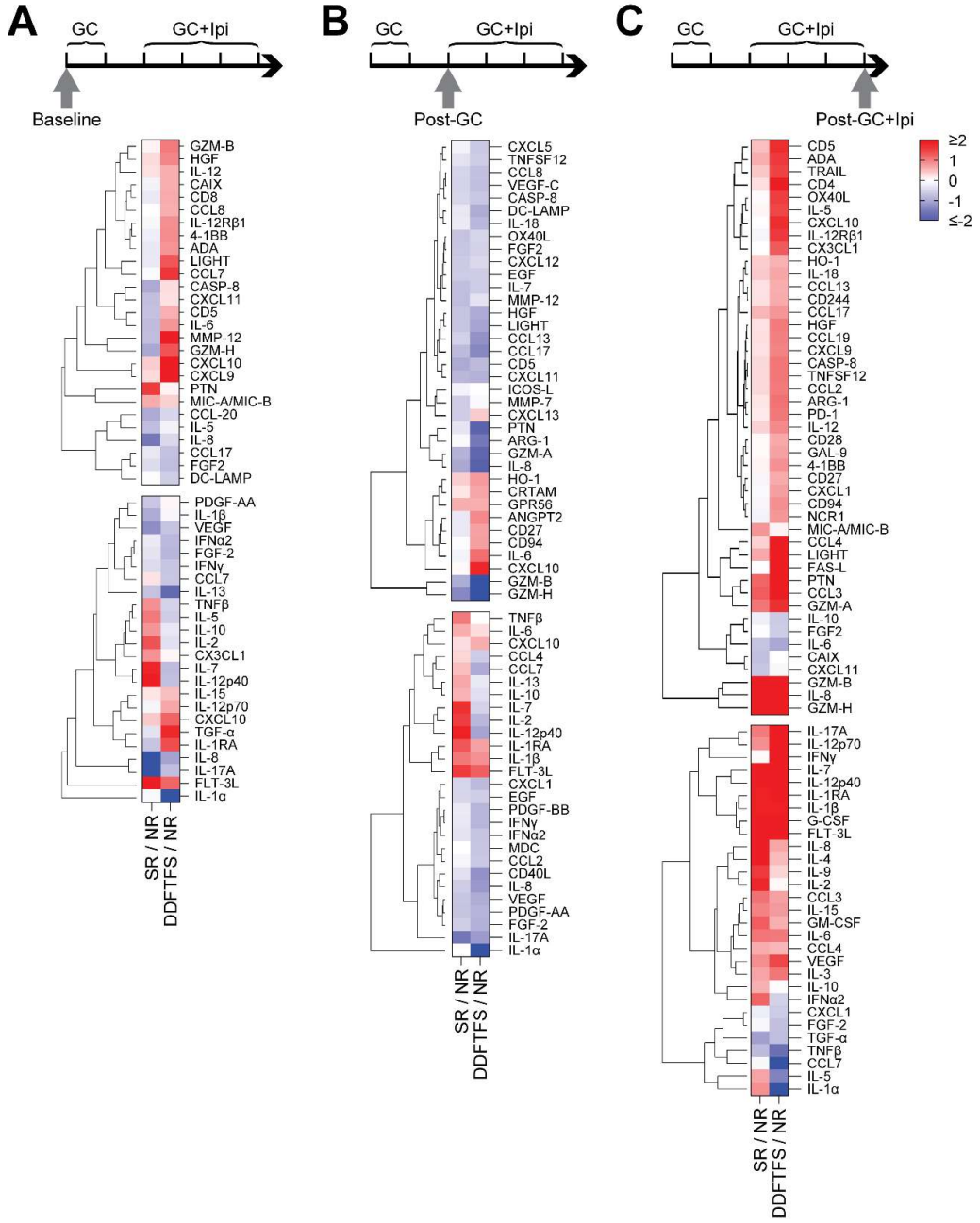
96 1. Network TCGAR. Comprehensive Molecular Characterization of Urothelial Bladder Carcinoma.
97 Nature. 2014;507:315.

98



Supplemental Figure S1

DDFTFS patients demonstrate unique patterns of analyte expression. Heatmaps of average z-scores per response grouping at (A) baseline, (B) post-GC, and (C) post-GC+Ipi with the Milliplex assay. Columns indicate patient grouping by response, rows indicate individual analytes, with unsupervised hierarchical clustering performed on rows. GC, gemcitabine and cisplatin; Ipi, ipilimumab; NR, non-responder; SR, short-responder; DDFTFS, durable disease-free treatment-free survival.



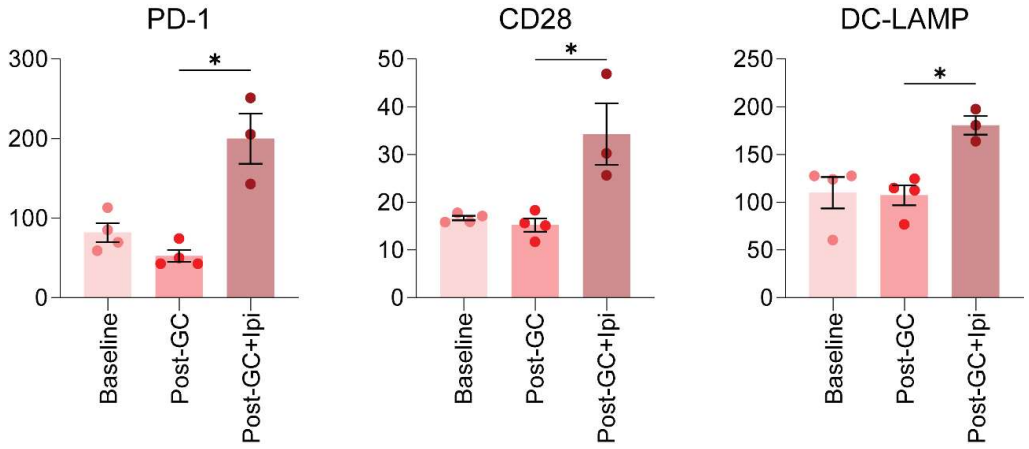
108

109 **Supplemental Figure S2**

110 DDFTFS patients demonstrate a unique immunophenotype normalized to non-responders.

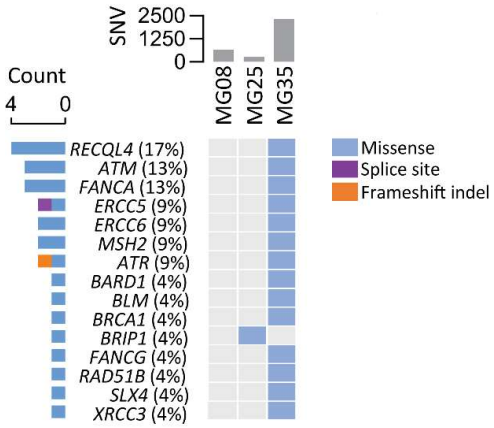
111 Heatmaps of all analytes with a ≥1.5-fold increased or decreased z-score when comparing either

short-responders or DDFTFS patients to non-responders at (A) baseline, (B) post-GC, and (C) post-GC+Ipi. Columns indicate patient grouping normalized to NR values, rows indicating individual analytes, with unsupervised hierarchical clustering performed on rows. Olink assay on top, Milliplex assay on bottom. GC, gemcitabine and cisplatin; Ipi, ipilimumab; NR, non-responder; SR, short-responder; DDFTFS, durable disease-free treatment-free survival.



Supplemental Figure S3

PD-1, CD28, and DC-LAMP increased among DDFTFS patients post-GC+Ipi. Scatter bar plots of individual analytes with significantly altered levels when comparing DDFTFS patients at different timepoints, with data displayed as mean ± SEM. Y-axis is transformed NPX values. * P < 0.05. GC, gemcitabine and cisplatin; Ipi, ipilimumab.



Supplemental Figure S4

Heterogeneity in DDR alterations among DDFTFS patients. Percentage and type of alterations among the entire cohort indicated on the left per gene. Total SNV count per DDFTFS patient indicated on top. SNV, single nucleotide variant; DDR, DNA damage response; DDFTFS, durable disease-free treatment-free survival.

Group		Patient ID	Best response	Time from treatment initiation to death or censor (months)	Time from treatment initiation to last follow up if alive (months)
DDFTFS No evidence of disease and >2 years off all treatment at time of last follow-up N=4		MG08	CR		84.2
		MG25	CR		95.0
		MG35	CR		90.9
		MG36	CR		60.3
Non-DDFTFS N=32	**Short-Responder** Best response CR or PR with recurrence of disease N=21	MG01	PR	6.9	
		MG02	CR	34.8	
		MG03	PR	10.6	
		MG04	PR	7.3	
		MG06	PR	26.6	
		MG10	PR	9.5	
		MG11	CR	18.4	
		MG13	CR	27.3	
		MG14	PR	10.4	
		MG17	PR	12.2	
		MG18	PR	12.3	
		MG19	PR	10.1	
		MG21	PR	2.8	
		MG22	PR	13.8	
		MG23	PR	15.0	
		MG26	PR	16.5	
		MG29	PR	23.5	
		MG30	PR	5.6	
		MG32	CR	26.9	
		MG34	PR	37.4	
		MG37	PR	37.4	
	Non-Responder Best response PD or SD with recurrence of disease N=11	MG05	SD	5.0	
		MG07	SD	27.7	
		MG09	SD	10.6	
		MG12	SD	6.5	
		MG15	SD	9.8	
		MG16	SD	10.6	
		MG20	SD	14.5	
		MG27	PD	4.8	
		MG28	SD	13.9	
		MG31	SD	22.5	
		MG33	SD	6.5	

Supplemental Table S1

Patient classifications by response. DDFTFS and non-DDFTFS (short-responders and non-responders) criteria and sample size listed, with best clinical response and time from treatment initiation to death/censor or last follow up if alive. DDFTFS, durable disease-free treatment-free survival; NR, non-responder; SR, short-responder; SD, stable disease; PD, progressive disease; PR, partial response; CR, complete response.

MG02		
Gene	**Variant**	**Peptide**
TMEM185B	p.D277H	KGGNHWWFGIRRHFCQFLLEIFPFL
CLSTN1	p.L68V	DPPLIALDKDAPVRFAESFEVTVTK
PARN	p.G469D	KTSDLYQLFSAFDNIQISWIDDTSA
MXRA8	p.S121L	ELSASAFDDGNFLLLIRAVEETDAG
MCAM	p.Q356H	SDVRVSPAAPERHEGSSLTLTCEAE
PARP10	p.A19V	HLMPRPRVAMAEVEAGVAVEVRGLP
MAP1S	p.A66T	DEQLKVFVSRHSTTFSSIVKGQRSL
JADE2	p.D475N	YRRLKLFTHLRQNLKRVRNLCYMVT
CORIN	p.T155M	THSQCQMLPYHAMLTPLLSVVRNME
ADCY2	p.S57F	PLIVFLLLIVMGFCLALLAVFFALG
TAGLN	p.W55S	GRPDRGRLGFQVSLKNGVILSKLVN
SEPN1	p.V180A	ETMTKSKDGFLGASRLALSGLRNWT
KIF1C	p.P408L	SVRGALPAVSSPLAPVSPSSPTTHN
SFRP1	p.H267Y	DCPCHQLDNLSHYFLIMGRKVKSQY
PIK3C2B	p.S399C	LQEALTFTCNCSCTVDLLIYQTLCY

Supplemental Table S2

Top ranked neoantigen predictions and peptide sequences synthesized for MG02.

MG16		
Gene	**Variant**	**Peptide**
SLC12A7	p.T256M	AAAMLHNMRVYGMCTLVLMALVVFV
NAA35	p.Y524fs	LYSMHEYYYIYWLSL
STEAP2	p.P380L	LGLLSLLAVTSILSVSNALNWREFS
CMTM3	p.I141N	SKAAGVFGFFATNVFATDFYLIFND
CARD11	p.V171M	LEDEKKQMTLTRMELLTFQERYYKM
MFAP5	p.R158Q	LPPRRLRRSNYFQLPPCENVDLQRP
DHRS4	p.Q54H	DGIGFAIARRLAHDGAHVVVSSRKQ
MROH1	p.G421S	AVVQVISAMAHHSYLEQPGGEAMIE
CROCC	p.R1729W	VEESEGALRDKVWGLTEALAQSSAS
HN1L	p.S44L	LSGQGPWAPLQRLASTRLVSGGHEA
SYVN1	p.L110del	DFSPRFVALFTLLFLKCFHWLAEDR
LRRC48	p.K69E	RIDNLWQFENLRELQLDNNIIEKIE
TP53	p.R273C	SGNLLGRNSFEVCVCACPGRDRRTE
HJURP	p.V188M	RVTPLPSLASPAMPAPGYCSRISRK
SOGA2	p.V586M	VEEEANILGRKIMELEVENRGLKAE

Supplemental Table S3

Top ranked neoantigen predictions and peptide sequences synthesized for MG16.

MG18		
Gene	**Variant**	**Peptide**
MIB2	p.V131fs	VQVGMRVGARRGLEVGPAGRRRGRR
MIB2	p.V131fs	DTRPHSGRAVGPGHAHQLPRRLPGR
MIB2	p.V131fs	RLPGRARPAAVRQRPDRRPAPQHHL
SMYD5	p.L60F	IFVERPLVAAQFFWNALYRYRACDH
ARL3	p.T147fs	EIAEGLNLHPRPSLADPVLLSSHRR
HDAC1	p.H39Y	GHPMKPHRIRMTYNLLLNYGLYRKM
IRX5	p.R269fs	RARCLRVPAGPRLSIRRLRRRLLRC
G2E3	p.E141fs	RSYHFPCGLQRMYFPVYWQFCVILL
CYP4F11	p.D43N	LARVLAWTYTFYNNCRRLQCFPQPP
SNX3	p.E75Q	KESTVRRRYSDFQWLRSELERESKV
GTF2IRD1	p.S114L	EGRVVRRVLTVALRALCPTGGPPWK
ZFP36L2	p.P207L	ELCRTFHTIGFCLYGPRCHFIHNAD
STAP2	p.G173R	EAQLLLERYPECRNLLLRPSGDGAD
VPS16	p.E642Q	GSFHIRASYAAEQRIEGRVAALQTA
ATP5SL	p.E59K	ILQFLTNYFYDVKALRDYLLQREMY
GP6	p.S184Y	AAHSGTYRCYSFYSRDPYLWSAPSD
CLMN	p.P735S	PHDLFYFPHYEVSLAAVLEAYVEDP
TBC1D20	p.D357H	FRGLLRPEDRTKHVLTKPRTNRFVK
KDM6A	p.E168K	VLYVDPSFCRAKKIHLRLGLMFKVN
TRA2A	p.R269I	RRRSPSPYYSRYISRSRSRSYSPRR
CCR4	p.L146F	DRYLAIVHAVFSFRARTLTYGVITS
ARID4A	p.E1066fs	ASGTCSIIVQERRVRRGQVMEIVD
ARL3	p.T147fs	KSRRMEMQELREPNSVLKNTNLLLS

Supplemental Table S4

Top ranked neoantigen predictions and peptide sequences synthesized for MG18.

MG25		
Gene	**Variant**	**Peptide**
OTOP1	p.L77del	IVFVAGLLLLAWAVHAAGV
OTOR	p.C37Y	ASKKLCADDEYVYTISLASAQ
MTHFR	p.E594K	TNAPKLQPNAVTWGI
TLL1	p.E496Q	CVWKITVSQSYHVGLTFQ
RIC8A	p.T514K	GMSPRGHLKSLQDAMCETM
ITGB5	p.R662T	CHSLCTDEVITWVDT
KDR	p.T139I	VSDQHGVVYIIENKNKTVVI
MYO1C	p.T1002M	VVLQSDHVIEMLTKTALSA
TGFB1I1	p.S359W	GPILDNYIWALSALWHP
TP53-1	p.N268fs	NLLGRTALRCVFVPVL
TP53-2	p.N268fs	TGAQRKRISARKGSLTTSCPQGAL
TP53-3	p.N268fs	ALSEHCPTTPAPLPSQRRNHWMENI
TP53-4	p.N268fs	RNHWMENISPFRSVGVSASRC
TP53-5	p.PDDIEQW46del	SQAMDDLMLSFTEDPG
TMEM66	p.P60S	YTTSRRLDSIPQLKCV
ASPM	p.F2233L	YWAMKERNIQLQRYNKLRHSV
PCOLCE2	p.N244S	IVSERSELLIQFL
NMUR2	p.R153C	YVAILHPFCAKLQSTRRRA
SNX14	p.K328R	FAEPRNRKPSVLKLEL
DAPK1	p.T78S	KEIQHPNVISLHEVYENKT
RPS4Y1	p.R148H	LVTHDARTIHYPDPVIKV
CAD	p.D1469H	KLVRLPGLIHVHVHL
MYO3A	p.Q366P	LEKCYSRDPIYVYVG
SETD3	p.S458F	KSVLKNHDLFVRAKMAIKL

Supplemental Table S5

Top ranked neoantigen predictions and peptide sequences synthesized for MG25.

MG31		
Gene	**Variant**	**Peptide**
DNAJC3	p.S13L	MVAPGSVTSRLGLVFPFLLVLVDLQ
MSMO1	p.A187T	EFQAPFGMEAEYTHPLETLILGTGF
ACSL3	p.E657K	WEELCNSCEMENKVLKVLSEAAISA
PLTP	p.S139L	YWFFYDGGYINALAEGVSIRTGLEL
PIGU	p.L207F	YPLTLFVPGLLYFLQRQYIPVKMKS
ABTB1	p.Q374H	PGLKRLCGRSLAHMLDEDTVVGVWR
LTN1	p.K279N	FYRVVTCSLLALNRLLCLLPDNELD
LETM1	p.D174N	KHYYHGFRLLWINTKIAARMLWRIL
ENKD1	p.Q293H	ENQRLETLTKLLHSQSQLLRELVLL
NAP1L2	p.D435E	EVNDAIYDKIIYENWMAAIEEVKAC
PEX11G	p.R48C	VEQCPARSEVGTCLLVVSTQLSHCR
ZZEF1	p.S1899F	RQRLIQPYIHNYFWLLFAALALYSA
MPHOSPH9	p.P476L	LPNALDDRISFSLDSVLEPSMSSPS
PVR	p.P129L	DEGNYTCLFVTFLQGSRSVDIWLRV
WNT6	p.P59L	LAGRQAELCQAELEVVAELARGARL

Supplemental Table S6

Top ranked neoantigen predictions and peptide sequences synthesized for MG31.

MG32		
Gene	**Variant**	**Peptide**
CNOT1	p.H1506P	LSVIIFFFVYIWPWALPLILNNHHI
DCAF7	p.D243N	KQDPNYLATMAMNGMEVVILDVRVP
TAPT1	p.L407F	SDSVARRMGFIPFPLAVLLIRVVTS
LONP1	p.S692L	ALCGLDESKAKLLSDVLTLLIKQYC
RP11-766F14.2	p.Q1575L	PPLPFTLQGAQPLVLCFSPPSMPAP
TMEM52B	p.H143Y	SLDTLPGYEEALYMSRFTVAMCGQK
LINGO1	p.N390fs	SRARSSRTSLMCYCPTTSPAAAPAS
LINGO1	p.N390fs	PESTWSQPRAMGGSQSSLMARWRCA
EPN3	p.P293L	EKEVRSWQGDGSLMANGAGAVVHHQ
POLQ	p.S679L	YRFFCLWEKLPTLMKRVAELVGVEE
KCNK1	p.S9L	MLQSLAGSLCVRLVERHRSAW
ADCY3	p.D1018N	ERERWQHLADLANFALAMKDTLTNI
GPR176	p.T189M	VFAVTNVADIYAMSTCTEVWSNSLG
FREM2	p.M327I	NTAPKPSFVAMMIMEVDQFVLTALT
HIATL2	p.V117I	DVWGRKPFLLGTIFFTCFPIPLMRI
IPO5	p.P554A	EEKFVPYYDLFMASLKHIVENAVQK
EHBP1L1	p.R1380W	QALEQEQRQIDGWAAEVEMQLRSLM
TNPO3	p.958HSCTVPVTQECLF (stop-loss)	CWALRDFTRLFRHSCTVPVTQECLF
TCTN1	p.S345L	YTDAGEVTKADLLFVLGTVSSVVVP
TLX1	p.S228L	LEKRFHRQKYLALAERAALAKALKM
PNPLA5	p.T218A	ELNVFNFSFQISAENFFLGLICLIP

Supplemental Table S7

Top ranked neoantigen predictions and peptide sequences synthesized for MG32.

MG35		
Gene	**Variant**	**Peptide**
APOBEC1	p.S97F	TWFLSWSPCWECFQAIREFLSRHP
ZFHX3	p.E209K	TFHIASSFGKWFKGPDQAFPN
SCN3A	p.E71K	KNLPFIYGDIPPKMVSEPLEDLDP
ART1	p.G180D	PRCHQVFRDVHGLRFRPA
FA2H	p.G30W	RRLAAGACWVRRWARLYDLSSFVRH
PAK7	p.D517N	VDMYSSYLVGNELWVVMEFLEG
TNN	p.S479F	QAVIDKYVVRYTFADGDTKEMAVHK
AGPAT5	p.F65L	RLYCVYQSMVLFLFENYTGVQILLY
MMP9	p.F581L	SVFEERLSKKLFLFSGRQVWVYTG
SLC6A6	p.P253T	TGKVVYFTATFTFAMLLVLLVRGL
ATP8B1	p.S925Y	EGMQAVMSSDYYFAQFRYLQRLLL
PIEZO1	p.S217L	VTLLALAGIAHPLALSSVYLLLFLA
FAM120C	p.S583F	EASLGDGEPHIPFLLSMSTRNHMDI
PAK2	p.D317H	VNFLDSYLVGHELFVVMEYLAGG
VWF	p.A1098fs	CSCESIGDCVLLRHHCCLCPRVCPA
USP24	p.S2411L	LEVMFALRELTGLLLALIEMVV
TRAM1	p.M165I	WRAYPHNLMTFQIKFFYISQLAY
KIAA0922	p.Q385E	KACLFSSVAEGYFRMDSSATQ
PICALM	p.Q521E	KPTVASENQNLPVAKLPP
DHX9	p.E358Q	GPLAFATPQQISMDLKNELMY
MACF1	p.S3200F	KTKETKHQIFSSNECKEKSYQE
MSN	p.Q48R	TIGLREVWFFGLRYQDTKGFSTWLK
HDAC9	p.K1002N	NMNAVISLQNIIEIQSKYWKSV
ZMYM6	p.S282L	IPPYALGKSLRPLAEMIET
HES1	p.F238L	GQFAFLIPNGALAHSGPVIPVYTS

Supplemental Table S8

Top ranked neoantigen predictions and peptide sequences synthesized for MG35.

Characteristic	DDFTFS (N=4)	Non-DDFTFS (N=32)
Age, median (range)	66.5 (64-72)	62 (36-82)
Sex, N (%)		
Male	4 (100)	32 (78.1)
Female	0 (0)	4 (21.9)
Baseline neutrophil-lymphocyte ratio, median (range)	4.5 (2.2-7.3)	3.0 (1.3-7.0)
Mutation count, median (range)	664 (268-2324)	639 (273-2102)
Primary Tumor, N (%)		
Bladder	3 (75)	25 (78.1)
Renal Pelvis	0 (0)	7 (21.9)
Ureter	1 (25)	0 (0)
Karnofsky performance status, N (%)		
100%	2 (50)	7 (21.9)
90%	2 (50)	12 (43.8)
80%	0 (0)	11 (34.4)
ECOG performance-status score, N (%)		
0	2 (50)	7 (21.9)
1	2 (50)	25 (78.1)
Site of metastatic disease, N (%)		
Lymph node/soft tissue only	3 (75)	17 (53.1)
Visceral	1 (25)	13 (40.6)
Liver	1 (25)	6 (18.8)
Lung	1 (25)	9 (28.1)
Bone	0 (0)	10 (31.3)
Prior treatment, N (%)		
Systemic chemotherapy	0 (0)	5 (15.6)
Cystectomy or nephroureterectomy	3 (75)	9 (28.1)
Radiation therapy	0 (0)	2 (6.25)

168

169 **Supplemental Table S9**

170 Demographic and clinical characteristics of DDFTFS and non-DDFTFS patients. DDFTFS,
171 durable disease-free treatment-free survival; ECOG, Eastern Cooperative Oncology Group.

172