Supporting Information

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SI Materials and Methods

Pyrosequencing. To analyze point-mutations in *FGFR3* (epithelial form FGFR3 IIIb) and *HRAS* genes, pyrosequencing assays were designed using PSQ Assay Design software v.1.0.6 (Table S3). Gradient PCR was used to optimize annealing temperatures for each assay individually to avoid nonspecific amplification and to achieve the highest PCR product yield. The optimal annealing temperatures were 66 °C for *FGFR3* codon 373, 65 °C for *HRAS* codon 61 and 59 °C for *HRAS* codons 12 and 13. HotStarTaq Plus Master Mix DNA Polymerase kit (Qiagen) was used for PCR amplification as recommended by the manufacturer, with 20–40 ng of DNA per reaction. Before PCR amplification, DNA polymerase was activated by incubation at 95 °C for 5 min. PCR amplification was performed as follows: 94 °C for 45 s (denaturation), optimal annealing temperature for 45 s, 72 °C for 1 min (elongation), for 35 cycles. After incubation at 72 °C for 10 min, products were stored at 4 °C. The purity of the PCR products was evaluated with an e-Gene capillary electrophoresis system.

Pyrosequencing was performed using a Pyromark MD system (Qiagen) according to the manufacturer's protocol. Briefly, 5–10 μL of PCR was incubated with streptavidin-Sepharose beads in binding buffer in a total volume of 80 μL in a 96-well plate. Incubation was performed on rotating platform (1,400 rpm, room temperature) for 10 min. Binding buffer was aspirated with a Vacuum Prep Tool, and the beads were washed consecutively in 70% (vol/vol) ethanol (5 s), 0.2 M NaOH (5 s), and 1× washing buffer (5 s), and then transferred into a 96-well pyrosequencing plate containing 12 μL of a 0.33 μM solution of sequencing primer (Table S3) in 1× annealing buffer. The plate was incubated at 8 °C for 2 min and then slowly cooled for 10 min at room temperature. The allele quantification mode was used for the pyrosequencing reaction, which allows for accurate allele quantification from ∼5% to ∼95%. The data were analyzed using PyroMark MD software v.1.0.

MS Identification of Aristolactam-DNA Adducts. Renal cortical DNA (74 μg in 5 mM bis-Tris buffer, pH 7.1) was digested with DNase I, nuclease P1, alkaline phosphatase, and spleen phosphodiesterase for 18 h at 37 °C, followed by solid-phase extraction of the deoxyadenosine (dA)-aristolactam (AL) adducts (1). Isotopically labeled [$^{15}N_3$]-dA-AL-II was used as an internal standard to estimate the dA-AL-I and dA-AL-II adduct levels, as both adducts displayed comparable ionization efficiencies. The internal standard was added to DNA before enzyme digestion at a level of 4 pg, or 3.2 adducts per 10^8 DNA bases.

The chemical identities and quantification of dA-AL adducts were determined using the Velos linear quadrupole ion trap mass spectrometer (Thermo Fisher) interfaced with an 1100 Series capillary LC system (Agilent Technologies) equipped with an Aquasil C18 column (0.32 mm i.d. × 250 mm, 5-μm particle size; Thermo Fisher). The flow rate was set to 5 μL/min, using mixtures of (A) 9:1 H_2O/CH_3CN (high-purity Burdick and Jackson solvents) with 0.01% HCO_2H and (B) 95:1 $CH_3CN:H_2O$ containing 0.01% HCO_2H. The adducts were resolved with a linear gradient starting at 25% B and reaching 100% B at 20 min.

MS analysis was conducted by electrospray ionization in the positive ionization mode. The capillary temperature was 300 °C and the temperature of the heated electrospray ionization source was set at 150 °C. The sheath gas setting was 11 and the auxiliary gas flow was set at 5 units. Helium was used as the collision damping gas in the ion trap, set at a pressure of 1 mTorr. The automatic gain control settings were full MS target 30,000 and MS^n target 10,000. One microscan was used for data acquisition, and the maximum injection time was 10 ms. The acquisition of the data was done with a single segment that contained four different scan events. The MS/MS^2 scan mode was used to monitor the loss of deoxyribose from the protonated DNA adducts $[M+H-116]^+$. The aglycone adduct $[BH_2]^+$ underwent fragmentation at the MS^3 scan stage to obtain full product ion spectra. The following transitions were monitored: dA-AL-I, m/z 543 → 427 → 150–500; dA-AL-II, m/z 513 → 397 → 150–500; and [$^{15}N_3$]-dA-AL-II m/z 516 → 400 → 150–500. The normalized collision energy and isolation widths were set, respectively, at 25% and m/z = 3 for MS/MS^2, and 35% and m/z = 1 for MS/MS^3. The activation Q was 0.35, and the activation time 10 ms for both MS/MS^2 and MS/MS^3 scan modes.

1. Goodenough AK, Schut HA, Turesky RJ (2007) Novel LC-ESI/$MS/MS^{(n)}$ method for the characterization and quantification of 2′-deoxyguanosine adducts of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by 2-D linear quadrupole ion trap mass spectrometry. *Chem Res Toxicol* 20:263–276.

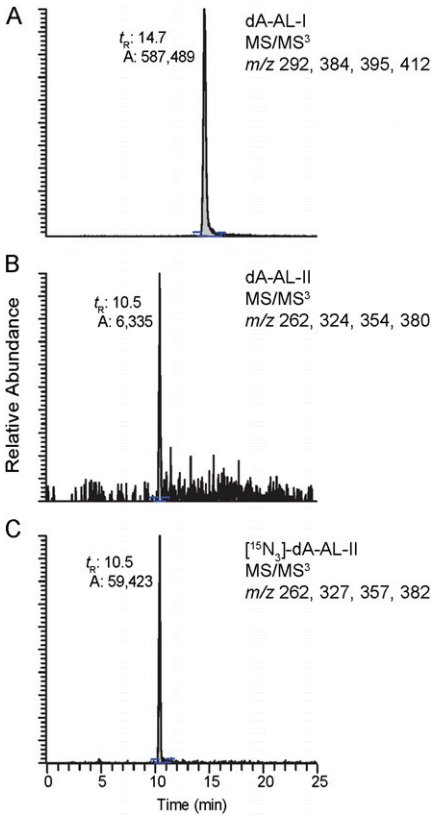

Fig. S1. MS characterization of DNA-AL adducts in the renal cortex of a Taiwanese subject with upper urinary tract urothelial cell carcinoma (UUC). Ion chromatograms were reconstructed from liquid chromatography electrospray ionization/multistage mass spectrometry (LC-ESI/MS/MS^3) analyses of dA-AL adducts. (*A*) dA-AL-I, identified in renal cortex; (*B*) dA-AL-II, identified in renal cortex; and (*C*) internal standard [$^{15}N_3$]-dA-AL-II spiked into renal cortical DNA at a level of 3.2 adducts per 10^8 DNA bases. The chromatograms were reconstructed with the four principal fragment ions observed in the spectra of the MS^3 scan stage (Fig. 3). The dA-AL-I level was estimated at 316 adducts/10^9 bases (based on total ion counts of dA-AL-I to [$^{15}N_3$]-dA-AL-II total ion counts), and the amount of dA-AL-II was estimated at 3.4 adducts/10^9 bases.

Table S1. *TP53* mutations detected in Taiwan UUC samples (individual data)

Patient no.	Codon/AA change	Mutation	Patient no.	Codon/AA change	Mutation
1	Int 4	A→T	42	N131Y	AAC→TAC
	Int 7	A→T		N288S	AAT→AGT
2	K164X	AAG→TAG		Int 8	A→T
	Int 8	A→T	43	Int 8	A→T
4	P98L	CCT→CTT		R209X	AGA→TGA
	Y220C	TAT→TGT	44	R209X	AGA→TGA
6	240 or 241	ΔT	47	S183X	TCA→TGA
7	R248Q	CGG→CAG	48	G325X	GGA→TGA
8	G187S	GGT→AGT		Int 6	A→T
	H214R	CAT→CGT	49	I195T	ATC→ACC
	L383F	CTC→TTC	52	R249W	AGG→TGG
10	S183X	TCA→TGA	53	Int 7	A→T
12	I195T	ATC→ACC	56	K132M	AAG→ATG
13	Int 6	A→T	61	D259V	GAC→GTC
	Int 7	A→T	63	R249W	AGG→TGG
15	N131Y	AAC→TAC	64	N131Y	AAC→TAC
16	V172F	GTT→TTT	65	Int 3	A→T
17	Q104L	CAG→CTG		R249W	AGG→TGG
20	E204X	GAG→TAG	69	E326K	GAA→AAA
21	V31I	GTT→ATT	70	E171Q	GAG→CAG
23	G244D	GGC→GAC		E224X	GAG→TAG
24	R248L	CGG→CTG		M246L	ATG→TTG
25	Q331X	CAG→TAG		K319N	AAG→AAT
27	V31I	GTT→ATT	74	E271V	GAG→GTG
	G244D	GGC→GAC	78	H214R	CAT→CGT
28	Int 8	A→T		Y220C	TAT→TGT
29	M340I	ATG→ATA	79	H214Y	CAT→TAT
30	Int 6	A→T	80	Int 6	A→T
31	P47P	CCG→CCA	81	K291X	AAG→TAG
32	Q38L	CAA→CTA	82	R282W	CGG→TGG
34	Int 8	A→T	83	Int 9	A→T
36	E258Q	GAA→CAA	87	N239I	AAC→ATC
	Int 7	A→G	89	V143E	GTG→GAG
37	R196X	CGA→TGA	93	Y205C	TAT→TGT
	Q375X	CAG→TAG	94	K120M	AAG→ATG
38	I195F	ATC→TTC		Int 8	A→T
39	R209X	AGA→TGA	95	P87P	CCA→CCT
	K320X	AAG→TAG		K292X	AAA→TAA
40	E286V	GAA→GTA		Int 5	A→T
41	Y163C	TAC→TGC	102	R280S	AGA→AGT
103	I162F	ATC→TTC	145	R280S	AGA→AGT
105	I162F	ATC→TTC	149	P152S	CCG→TCG
	R273C	CGT→TGT	152	Q136L	CAA→CTA
108	E294X	GAG→TAG	153	R280T	AGA→ACA
112	N131Y	AAC→TAC	154	K164X	AAG→TAG
	Int 3	A→T	157	R248L	CGG→CTG
113	Int 8	A→T	159	R283H	CGC→CAC
114	K305X	AAG→TAG	161	R174W	AGG→TGG
115	Y220H	TAT→CAT		Int 2	A→T
116	Q331X	CAG→TAG	162	R202C	CGT→TGT
122	K132X	AAG→TAG	163	Int 5	A→T
131	Int 10	A→T	164	L111L	CTG→CTT
134	K305X	AAG→TAG		E180X	GAG→TAG
135	E198X	GAA→TAA	167	R158L	CGC→CTC
141	K120M	AAG→ATG		R267L	CGG→CTG
	R249W	AGG→TGG		L350L	CTC→CTG
144	E346X	GAG→TAG			

A→T and T→A transversions are indicated by green and yellow backgrounds, respectively. AA, amino acid.

Table S2. Individual data for *HRAS* and *FGFR3* mutations

Patient no.	*TP53*	*HRAS* Codon 61 CAG	*FGFR3* Codon 373 AGT
15	**A > T**	**A > T**	
22	WT	**A > T**	
26	WT	**A > T**	
52	**A > T**	**A > T**	
60	WT	**A > T**	
74	**A > T**	**A > T**	
86	WT	**A > T**	
31	G > A		**A > T**
33	WT		**A > T**
40	**A > T**		**A > T**
41	A > G		**A > T**
88	WT		**A > T**
123	WT		**A > T**
Mutational frequency		**4.7% (A > T)** (7/150)	**4.0% (A > T)** (6/150)
COSMIC Mut. Freq.		**1.1%(A > T)** (12/1123)	**0.78% (A > T)** (28/3574)

DNA obtained from upper urinary tract tumors from 150 Taiwanese subjects was available for pyrosequencing analysis of *HRAS* and *FGFR3* mutations. A > T mutations are in boldface type and shaded in green; G > A and A > G mutations are shaded in orange and yellow, respectively. COSMIC, Catalogue Of Somatic Mutations In Cancer, http://www.sanger.ac.uk/genetics/CGP/cosmic using transitional cell carcinoma as a search term.

Table S3. Primers used for pyrosequencing

Assay no.	Forward primer	Reverse primer	Sequencing primer	Codons and nucleotide positions analyzed
1	TTGCAGCCGAGGAGGAGC	Bio-GGGAGCCC AGGCCTTTCTT	GAGGCTGACGAGGCG	*FGFR3* 373 (nt 1117)
2	GGGAGACGTGCCTGTTGGA	Bio-GCGCATGT ACTGGTCCCG	TCCTGGATACCGCCG	*HRAS* 61 (nt 182)
3	CTGAGGAGCGATGACGGAATAT	Bio-GGATGGTC AGCGCACTCTT	TGGTGGTGGTGGGCG	*HRAS* 12 (nts 34, 35) *HRAS* 13 (nts 36, 37)

Bio, biotin was added to the 5′-end of the reverse PCR primers in each pair to facilitate the isolation of a single DNA strand from the PCR product for pyrosequencing; nt, nucleotide position.