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Mutagenicity of aristolochic acid in the *lambda/lacZ* transgenic mouse (Muta™Mouse)

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Abstract

Aristolochic acid (AA) is found in a plant that causes urothelial carcinomas in patients with Chinese herb nephropathy (CHN). To evaluate the in vivo mutagenicity of AA, we analysed the mutant frequency (MF) in the *lacZ* and *cII* gene of 10 organs of the *lambda/lacZ* transgenic mouse (Muta™Mouse) after intragastric treatment with AA (15 mg/kg per week × 4). Simultaneously, the clastogenicity of AA was evaluated by the peripheral blood micronucleus assay. The nature of the mutations induced by AA was revealed by the sequence analysis of the *cII* gene, which is also a phenotypically selectable marker in the lambda transgene. MFs in the target organs-forestomach, kidney, and bladder of AA-treated mice were significantly higher than those of control mice (forestomach 33- and 15-fold; kidney 10- and 9-fold; bladder 16- and 31-fold, for the *lacZ* and *cII*, respectively). The MFs in non-target organs, except the colon, showed only slight increases. Sequence analysis of *cII* mutants in target organs revealed that AA induced mainly A:T to T:A transversions whereas G:C to A:T transitions at CpG sites predominated among spontaneous mutations. These results suggested that AA, which is activated by cytochrome P450 and peroxidase to form cyclic nitrenium ions that bind to deoxyadenine, caused the A to T transversions in the target organs of mice. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Aristolochic acid; *cII*; Muta™Mouse; Mutation spectra

1. Introduction

Aristolochic acid (8-methoxy-6-nitrophenanthro [3,4-*d*]-1,3-dioxolo-5-carboxylic acid ; AA) is a mixture of nitrophenanthrene derivatives that are found in several plant species. Plant extracts containing AA have been used since antiquity as drugs in obstetrics and for the treatment of snake bites, festering wounds, and tumors [1,2]. Chinese herb nephropathy (CHN) is a progressive form of renal fibrosis that develops in some patients who take weight-reducing pills containing Chinese herbs. More than 100 CHN cases have been identified, half of whom needed renal transplantation. After these cases of renal failure were first reported in Belgium, similar cases were reported in Spain [3], Japan [4], France [5], the UK [6], and China [7]. Recently, an increasing number of urothelial carcinomas have been reported in CHN patients, suggesting that AA plays a role in their formation. Because of a manufacturing error, one of the herbs in a weight-reducing pill was replaced by *Aristlochia fungchi*, which contains AA. AA, consisting

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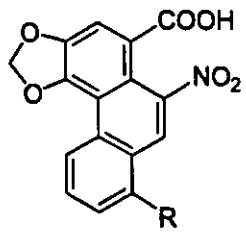

Fig. 1. Structure of aristolochic acid I (AAI; R = OCH_3) and aristolochic acid II (AAII; R = H).

essentially of aristolochic acid I (AAI) and aristolochic acid II (AAII) (Fig. 1), is mutagenic [8–10] and a potent carcinogen in rodents [11–13] and human [14,15]. AAI and AAII are activated by reduction of the nitro group to a nitrenium ion, which reacts with DNA at the exocyclic amino groups of adenine and guanine (Fig. 2); this binding occurs at the C7 position of the corresponding aristolactams, the major metabolites of AA. Forestomach and kidney are target organs in AA induced carcinogenesis in rats and mice.

Specific AA-DNA adducts were detected in urothelial tissues of CHN patients by the ^{32}P-postlabeling method [15]. This unambiguously showed that all CHN patients analyzed so far had indeed ingested AA [16,17]. Schmeiser et al. reported that AA-induced carcinogenesis in rodents was initiated by the activation of the Ha-*ras* gene by a specific A:T to T:A transversion mutation at the adenine in the middle of codon 61 (CAA) [18]. In a further study, they demonstrated by polymerase arrest that both the adenines in the codon are AA binding sites [19]. These results suggest that AA can cause for urinary bladder tumors in human.

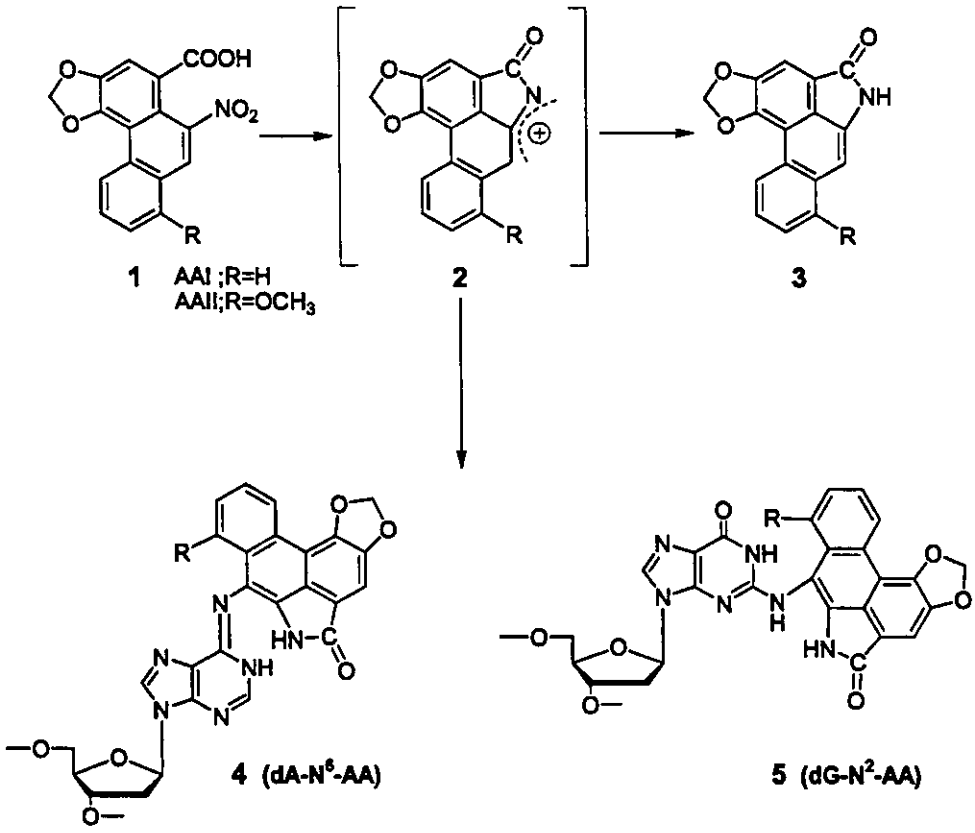

Fig. 2. Proposed mechanism of DNA adduct formation by AAI (1, R = OCH_3) or AAII (1, R = H) after reductive activation. The four major AA adducts formed are dA-AAI (4, R = OCH_3), dA-AAII (4, R = H), dG-AAI (5, R = OCH_3), and dG-AAII (5, R = H).

The transgenic mouse mutation assay is a powerful tool for studying chemical mutagenesis in vivo. Because in vitro experiments often do not reflect the metabolism or distribution of test compounds, data from transgenic mouse assays have more relevance in studies mechanism of initiation of carcinogenesis. Many carcinogens induce mutations in the transgene of their target organs, which demonstrates the relevance of the assay [20]. In addition, molecular analyses of induced mutations revealed chemical-specific mutation spectra. A database containing large number of *lacI* mutations obtained from Big Blue® transgenic mouse assays is now available [21], but fewer sequence analyses have been done with Muta™Mouse, mainly because of the labor required to find mutations in a 3 kb region. The much smaller *cII* target gene (294 bp), which was also incorporated into the *lambda/lacI* transgenic mouse (Big Blue®), facilitates molecular analysis in Muta™Mouse [22].

In the present study, we analyzed the mutagenicity of target and non-target tissues in transgenic mice to investigate whether mutation induction is necessary for the initiation of carcinogesis. In addition, we characterized the mutation spectrum induced by AA to elucidate the molecular nature and mechanisms of AA mutagenesis.

2. Materials and methods

2.1. Chemicals

Aristolochic acid (AA, CAS No. 10190-99-5) was purchased from Sigma and dissolved in saline (10 mg/ml). The purity of AA was 96% (AAI: 56%, AAII: 40%). Phenyl-β-D-galactoside (P-gal) was also purchased from Sigma.

2.2. Animals and treatments

Male Muta™Mice (7–8-week-old, ca. 25 g body weight) supplied by Covance Research Products (PA, USA) were acclimatized for 1 week before use. AA solution (10 ml/kg) was intragastrically injected into groups of four mice at a dose of 15 mg/kg (1/4 of the LD_{50} in mice [11]) once a week for 4 weeks. Olive oil was administrated to four control mice at the same time and in the same manner.

2.3. Peripheral blood micronucleus assay

Forty-eight hours after of the first injection, peripheral blood (5 μl) was collected without anticoagulant from a tail blood vessel, placed on an acridine orange-coated glass slide, covered with a cover slip, and supravitally stained [23]. One thousand reticulocytes per animal were analyzed by fluorescence microscopy within a few days of slide preparation, and the number of cells with micronuclei was recorded.

2.4. Tissue collection

Mice were killed by cervical dislocation 7 days after the final treatment. Liver, bone marrow, urinary bladder, kidney, colon, lung, forestomach, glandular stomach, spleen, and testis were removed, quickly frozen in liquid nitrogen, and stored in a deep freezer at −80 °C until analysis.

2.5. DNA isolation and in vitro packaging

The isolation of total genomic DNA from tissue samples was carried out by the standard phenol/chloroform method (Stratagene manual, 1994). Briefly, homogenized tissues were incubated with RNase and proteinase K, and genomic DNA was extracted using a phenol/chloroform mixture and chloroform. The DNA was precipitated with ethanol and dissolved in TE-4 buffer (10 mM Tris at pH 8.0 containing 4 mM EDTA).

2.6. In vitro packaging and determinations of the lacZ and cII MF

The *lacZ* transgene, integrated into the lambda phage vector (lambda gt10), was recovered by in vitro packaging reactions. The DNA solution (5 μl) adjusted to 0.4 mg DNA/ml was gently mixed with the Transpack packaging extract (Stratagene, La Jolla, CA, USA) and incubated at 37 °C for 3 h. *LacZ*-positive selection was performed according to the manufacturer's manual (Corning Hazleton, 1996). Briefly, the phage solution was adsorbed to 2 ml of *E. coli C* (*lac*$^-$, *galE*$^-$) at room temperature for 20–30 min. For the titration, 1 ml of phage–*E. coli* solution, appropriately diluted, was mixed with 23 ml of

1/4 LB top agar (containing 10 mM $MgSO_4$). Then, 6 ml of the mixture was plated onto each of four Petri dishes (9 cm), containing 6 ml of bottom agar. The remaining phage–*E. coli* solution was mixed with 22 ml of 25% LB top agar containing P-gal (3 mg/ml) and plated in the same manner. The plates were incubated overnight at 37 °C. Positive selection for *cII* mutants was performed according to Jakubczac et al. [24] with a slight modification. Briefly, the phage solution was adsorbed to 1 ml of *E. coli* G1225 (*hfl*$^-$) at room temperature for 20–30 min. For the titration, appropriately diluted phage solution was mixed with 200 μl of *E. coli* G1225. The phage–*E. coli* solution was mixed with 14 ml (for selection) and 6 ml (for titration) LB top agar (containing 10 mM $MgSO_4$), and plated onto five and two Petri dishes (9 cm), respectively, containing 10 ml of bottom agar. The plates were incubated for 48 h at 25 °C for the selection of *cII* mutants or at 37 °C for the titer of total phages. Wild type phage, recovered from Muta^{TM}Mouse, has a *cI*$^-$ phenotype, which permits plaque formation with the *hfl*$^-$ strain at 37 °C but not at 25 °C. The mutant frequency (MF) was calculated as MF = total plaques on selection plates/(total plaques on titer plates × dilution factor).

2.7. Sequencing of mutants

The entire lambda *cII* region was amplified directly from mutant plaques by Taq DNA polymerase (Takara Shuzo, Tokyo Japan) with primers P1, 5′-AAAAAGGGCATCAAATTAAACC-3′, and P2, 5′-CCGAAGTTGAGTATTTTTGCTGT-3′. Amplification was done by the Minicycler PTC-150-25 (MJ Research, Inc., MA, USA) with an initial heating step at 95 °C for 5 min followed by 30 cycles of denaturing at 95 °C for 20 s, annealing at 53 °C for 30 s, and extension at 72 °C for 40 s, followed by a 10 min incubation at 72 °C. A 446 bp PCR product was purified with a microspin column (Amersham Pharmacia, Tokyo, Japan) before being used for a sequencing reaction with the Ampli Taq cycle sequencing kit (PE Biosystems, Tokyo, Japan). The sequencing reaction was done by Minicycler PTC-150-25 with 25 cycles of denaturing at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 4 min, with the primer P1. The reaction product was purified by ethanol precipitation and analyzed by the ABI PRISM^{TM} 310 Genetic Analyzer (PE Biosystems, Tokyo, Japan).

3. Results

3.1. Micronucleus induction in peripheral blood

The mean frequency of micronucleated reticulocyts (MNRETs) in the AA-treated group (0.18%) was not statistically different from that in the control group (0.13%) (data not shown). This is similar to the value reported for other strains of mice [25].

3.2. Mutant frequency of lacZ and cII genes

The results of the *lacZ* and *cII* MF analyses are shown in Table 1. Organ specificity of mutation induction was clearly demonstrated. The MFs in the AA target organs of AA-treated were significantly higher than in those of the control mice (forestomach, 33- and 15-fold, kidney, 10- and 9-fold, bladder, 16- and 31-fold, for *lacZ* and *cII*, respectively). Colon, which is a non-target organ, also showed large increases in *lacZ* and *cII* MF, but other non-target organs showed only slight increases.

3.3. cII mutation spectrum

Twenty-eight, twenty, and twenty-three AA-induced mutants in kidney, bladder, and forestomach, respectively, were subjected to sequence analysis, together with the 34 spontaneous mutants in kidney. The mutation spectra are summarized in Table 2. Spontaneous mutations consisted mainly of base substitutions (25 of 34 mutations). Among them, G:C to A:T transitions at CpG sites (14 of 25 transitions) predominated. AA-induced mutations also consisted mainly of base substitutions (24 of 28 mutations in the kidney, 18 of 21 in the bladder, and 22 of 23 in the forestomach). The predominant changes induced by AA were a reduction of G:C to A:T transitions at CpG sites and an increase of A:T to T:A transversions (14 of 28 transversions in the kidney, 7 of 21 in the bladder, and 12 of 23 in the forestomach). A:T to T:A transversions were rare in the control (5 of 34 mutations). The distribution of mutations in the kidney is shown in Fig. 3. Mutations were distributed over all the genes and no apparent hot spot were observed, except for C to T transitions at some CpG sites and a deletion/insertion at six runs of G and A for spontaneous mutations.

Table 1
Mutant frequency in the *lacZ* and *cII* genes from various organs of Muta™Mouse treated with AA

Organ	Treatment	ID	*lacZ*				*cII*			
			Total plaques	Mutants	MF × 10^6	Mean ± S.D.	Total plaques	Mutants	MF	Mean ± S.D.
Kidney	Control	11	1132000	131	115.7		1856400	90	48.5	
		12	226000	16	70.8		411600	14	34.0	
		13	156500	14	89.5		249600	13	52.1	
		14	1326000	66	49.8		1747200	77	44.1	
		Total	2840500	227	79.9	81 ± 24	4264800	194	45.5	45 ± 6.8
	AA	51	170500	120	703.8		321300	116	361.0	
		52	347000	262	755.0		300000	142	473.3	
		53	916000	1040	1135.4		1550400	668	430.9	
		54	1546000	1249	807.9		1412400	853	603.9	
		Total	2979500	2671	896.5	851 ± 169	3584100	1779	496.4	467 ± 88
Bladder	Control	11	677500	67	98.9		1032000	30	29.1	
		12	667500	16	24.0		1380000	38	27.5	
		13	315000	18	57.1		711000	17	23.9	
		Total	1660000	101	60.8	60 ± 31	3123000	85	27.2	27 ± 2.2
	AA	51	1290000	1694	1313.2		1062000	1341	1262.7	
		52	820000	711	867.1		813000	556	683.9	
		53	155000	139	896.8		147750	98	663.3	
		Total	2265000	2544	1123.2	1026 ± 204	2022750	1995	986.3	870 ± 278
Forestomach	Control	11	505000	16	31.7		1107000	92	83.1	
		12	1380000	50	36.2		2370000	77	32.5	
		13	775000	21	27.1		1284000	25	19.5	
		14	1102500	42	38.1		1818000	56	30.8	
		Total	3762500	129	34.3	33 ± 4.3	6579000	250	38.0	41 ± 25
	AA	51	1480000	1516	1024.3		1956000	933	477.0	
		52	748750	1056	1410.4		1416000	948	669.5	
		53	1712500	2282	1332.6		2319000	1763	760.2	
		54	1862500	1397	750.1		2034000	1512	743.4	
		Total	5803750	6251	1077.1	1129 ± 262	7725000	5156	667.4	663 ± 112
Glandular stomach	Control	11	697500	33	47.3		1614000	61	37.8	
		12	507500	27	53.2		1035000	20	19.3	
		13	520000	20	38.5		843000	13	15.4	
		14	840000	36	42.9		1548000	87	56.2	
		Total	2565000	116	45.2	45 ± 5.5	5040000	181	35.9	32 ± 16
	AA	51	648750	90	138.7		1303500	70	53.7	
		52	1042500	165	158.3		1293000	82	63.4	
		53	1096250	187	170.6		1716000	96	55.9	
		54	378750	36	95.0		588000	27	45.9	
		Total	3166250	478	151.0	141 ± 29	4900500	275	56.1	55 ± 6.2
Lung	Control	11	2102500	90	42.8		3936000	191	48.5	
		12	1037500	101	97.3		2799000	108	38.6	
		13	1432500	122	85.2		4059000	134	33.0	
		14	1670000	87	52.1		2709000	148	54.6	
		Total	6242500	400	64.1	69 ± 23	13503000	581	43.0	44 ± 8.4

Table 1 (*Continued*)

Organ	Treatment	ID	*lacZ*				*cII*			
			Total plaques	Mutants	MF × 10^6	Mean ± S.D.	Total plaques	Mutants	MF	Mean ± S.D.
	AA	51	327500	37	113.0		681000	47	69.0	
		52	917500	140	152.6		1512000	113	74.7	
		53	2877500	357	124.1		4366500	345	79.0	
		54	2885000	250	86.7		3060000	202	66.0	
		Total	7007500	784	111.9	119 ± 24	9619500	707	73.5	72 ± 5
Colon	Control	11	300000	16	53.3		1074000	50	46.6	
		12	880000	66	75.0		2745000	70	25.5	
		13	383750	35	91.2		1434000	46	32.1	
		14	1405000	84	59.8		2478000	132	53.3	
		Total	2968750	201	67.7	70 ± 15	7731000	298	38.5	39 ± 11
	AA	51	725000	311	429.0		2247000	263	117.0	
		52	372500	177	475.2		1248000	152	121.8	
		53	545000	296	543.1		1203000	205	170.4	
		54	410000	417	1017.1		1122000	341	303.9	
		Total	2052500	1201	585.1	616 ± 235	5820000	961	165.1	178 ± 75
Liver	Control	11	415000	13	31.3		1413000	40	28.3	
		12	585000	30	51.3		1650000	38	23.0	
		13	711250	63	88.6		4050000	75	18.5	
		14	942500	64	67.9		3498000	101	28.9	
		Total	2653750	170	64.1	60 ± 21	10611000	254	23.9	25 ± 4.2
	AA	51	2972500	168	56.5		3327000	114	34.3	
		52	5125000	126	24.6		1788000	74	41.4	
		53	380000	41	107.9		613500	28	45.6	
		54	2682500	153	57.0		3543000	129	36.4	
		Total	11160000	488	43.7	62 ± 30	9271500	345	37.2	39 ± 4.4
Bone marrow	Control	11	802000	51	63.6		1420800	41	28.9	
		12	719000	73	101.5		1038000	41	39.5	
		13	1346250	65	48.3		1929000	30	15.6	
		14	1006250	55	54.7		1356000	42	31.0	
		Total	3873500	244	63.0	67 ± 21	5743800	154	26.8	29 ± 8.6
	AA	51	634000	75	118.3		655200	31	47.3	
		52	1307000	180	137.7		1756800	100	56.9	
		53	252500	48	190.1		630000	35	55.6	
		54	830000	85	102.4		1125000	40	35.6	
		Total	3023500	388	128.3	137 ± 33	4167000	206	49.4	49 ± 8.5
Spleen	Control	11	1487500	76	51.1		2400000	91	37.9	
		12	1065000	51	47.9		2226000	34	15.3	
		13	1502500	96	63.9		1920000	70	36.5	
		14	1520000	69	45.4		2352000	66	28.1	
		Total	5575000	292	52.4	52 ± 7.1	8898000	261	29.3	29 ± 9

Table 1 (*Continued*)

Organ	Treatment	ID	*lacZ*				*cII*			
			Total plaques	Mutants	MF × 10^6	Mean ± S.D.	Total plaques	Mutants	MF	Mean ± S.D.
	AA	51	2607500	264	101.2		3420000	182	53.2	
		52	1615000	261	161.6		2694000	179	66.4	
		53	1028750	126	122.5		1656000	80	48.3	
		54	756250	123	162.6		1617000	48	29.7	
		Total	6007500	774	128.8	137 ± 26	9387000	489	52.1	49 ± 13
Testis	Control	11	638750	15	23.5		936000	14	15.0	
		12	938750	36	38.3		1010250	4	4.0	
		13	447500	8	17.9		1362000	16	11.7	
		14	863750	14	16.2		1362000	23	16.9	
		Total	2888750	73	25.3	24 ± 8.7	4670250	57	12.2	12 ± 4.9
	AA	51	393750	23	58.4		495000	8	16.2	
		52	451250	14	31.0		993000	27	27.2	
		53	712500	21	29.5		1308000	9	6.9	
		54	601250	18	29.9		1023000	10	9.8	
		Total	2158750	76	35.2	37 ± 12	3819000	54	14.1	15 ± 7.8

Table 2
Spontaneous and AA-induced mutation spectra in the kidney, forestomach, and bladder of Muta^{TM}Mouse

Mutation class	Kidney		Bladder (AA (%))	Forestomach (AA (%))
	Control (%)	AA (%)		
Total	34 (100)	28 (100)	21 (100)	23 (100)
Base substitution	25 (74)	24 (86)	19 (90)	22 (96)
Transitions	15 (44)	8 (29)	8 (38)	6 (26)
G:C to A:T	14 (41)	7 (24)	7 (33)	6 (26)
At CpG sites	11 (32)	1 (4)	4 (19)	4 (17)
A:T to G:C	1 (3)	1 (4)	1 (5)	0 (0)
Transversions	10 (29)	16 (57)	11 (52)	16 (70)
A:T to T:A	5 (15)	14 (50)	7 (33)	12 (52)
A:T to C:G	0 (0)	1 (4)	0 (0)	1 (4)
G:C to T:A	5 (15)	1 (4)	3 (14)	3 (13)
G:C to C:G	0 (0)	0 (0)	1 (5)	0 (0)
−1 Frameshifts	4 (12)	1 (4)	0 (0)	1 (4)
+1 Frameshifts	3 (9)	1 (4)	1 (5)	0 (0)
Deletion	0 (0)	0 (0)	0 (0)	0 (0)
Insertion	0 (0)	0 (0)	0 (0)	0 (0)
Complex	2 (6)	2 (7)	1 (5)	0 (0)
MF ($\times 10^{-6}$)	45.5	496.4	986.3	667.4

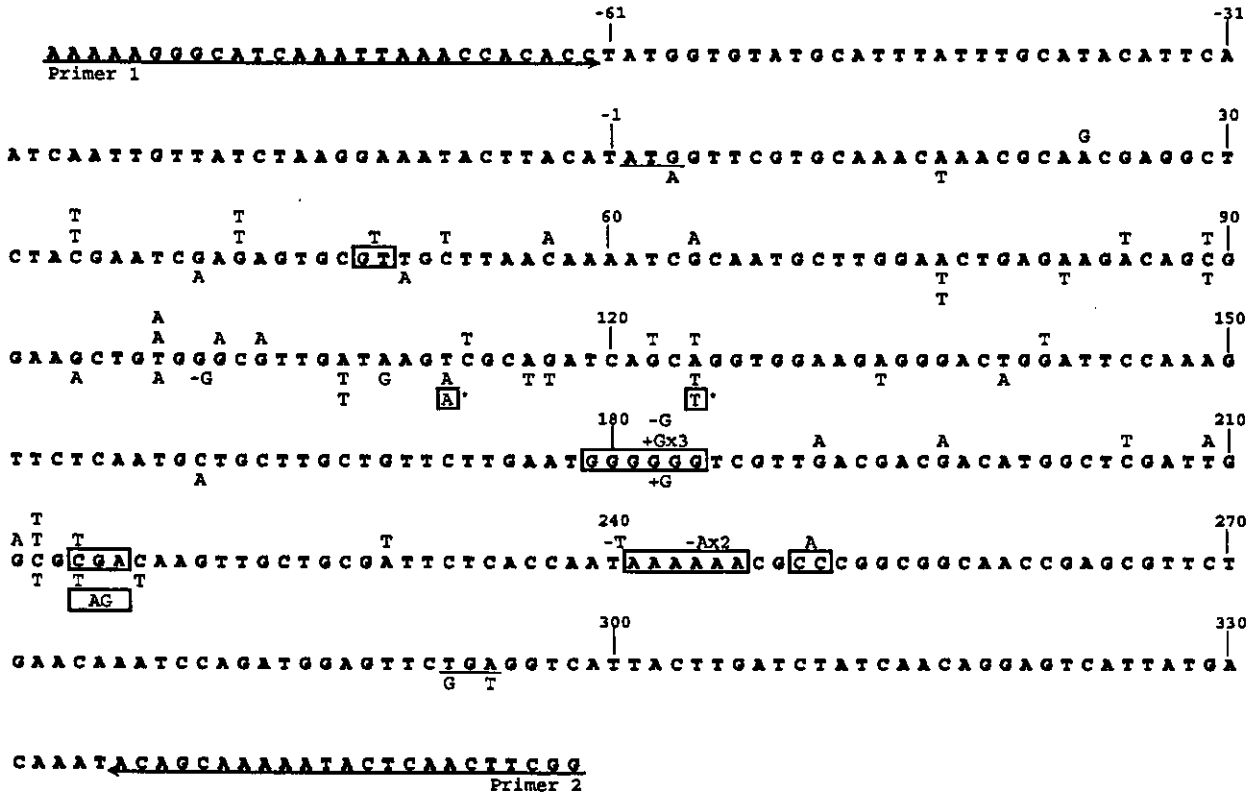

Fig. 3. Mutations in the kidney of *cII* gene obtained from control and AA-treated Muta™Mouse. The sequence from top to bottom represents the amplified lambda *cII* region. Mutations shown above the strands were detected in control mice, whereas those below the strands were detected in AA-treated mice. The symbol (•) denotes double mutation.

4. Discussion

It was reported that, in the bone marrow micronucleus study [26], the male NMRI mice treated with 6–60 mg/kg and the females treated with 20–60 mg/kg, by intravenous injection, showed significant increases in micronucleated polychromatic erythrocytes. In the present study, however, no statistical difference in MNRET frequency was observed in peripheral blood of Muta™Mouse after intragastric AA treatment at 15 mg/kg (1/4 of LD_{50} in mice). Different doses and routes of administration may account for the different results. But, it is important that the gene mutation in the target organs for carcinogenesis (kidney, bladder, and forestomach) were clearly detected after AA treatments although the simultaneous assessment of micronucleus induction gave a negative result. The lack of micronucleus induction agrees with a marginal increase of MF in the bone marrow that is not target organ for carcinogenesis.

Previous studies have demonstrated the carcinogenicity of AA in rats [12,27]. In those studies, AA was orally administrated over a period of 3–6 months in doses ranging from 0.1 to 10.0 mg/kg. The rats developed metastasizing squamous cell carcinomas of the forestomach as well as benign and malignant tumors of the kidney and the urinary tract. In mice on the other hand, during the year following 3 weeks of 5.0 mg/kg AA papillomatous changes occurred in forestomach, and later, the authors observed squamous cell carcinomas in the forestomach in all animals, one adenocarcinoma of the glandular stomach, malignant lymphomas and adenomas of the kidneys, carcinomas of the lungs, and hemangiomas of the uteri [23]. In our study, AA increased the kidney, bladder, and forestomach MFs in both *lacZ* and *cII* genes, which

correlated with the carcinogenic data in mice, but the lung MF was not so increased. One of the mutagenic and carcinogenic properties of AA was the very high selectivity of target organs. It is interesting that the MF of glandular stomach increased only slightly, whereas forestomach showed a very high MF. The reason for the difference is not clear. The MFs of the *cII* gene were, in general, slightly lower than those of the *lacZ* gene (Table 1). This similarity suggests that the *cII* gene has a higher sensitivity than the *lacZ* gene because the length of the *cII* gene (294 bp) is about 1/10 of the *lacZ* gene (3 kb). It is possible that some *lacZ* mutations do not affect the activity of its product, but subtle conformational changes in *cII* protein diminish transcriptional activation of the downstream genes responsible for lambda lysogeny.

The characteristic spontaneous mutations were G:C to A:T transitions, and most of those occurred at CpG sites. The sequence analysis suggested that AA was activated and reacted with deoxyadenine, causing A to T transversions in the target organs. G to A transitions at non-CpG site increased in the target organs probably caused by the dG-AA adduct [28,29], although its contribution is less than the adenine adducts. Induction of A:T to T:A transversions can be explained by an intrinsic property of the polymerase that inserts dA residues opposite the lesions that block DNA replication. The mechanism for G:C to A:T transitions is not known. The mutation spectrum of AA differed from the spectra of other nitro- or amino-polycyclic compounds, such as dinitropyrene (unpublished data), heterocyclic amines [30–33], and *o*-aminoazotoluene [34] because those compounds induce mainly G:C to T:A transversions. Also, those compounds are activated through the formation of hydroxylamine but formation of cyclic nitrenium cation is unique for AA, which may account for the different reactivity.

It was demonstrated by a zinc-catalyzed reduction system in vitro that AAII modifies DNA more extensively than AAI, and AAI strongly favors guanine adduct formation while AAII reacts mainly with dA residues [19]. AAI, however, is metabolized more efficiently than AAII in vitro (by rat liver homogenate) [35], and in vivo [36]. 7-(Deoxyadenosin-N^6-yl)-aristololactam I (dA-AAI) persists much longer than 7-(deoxyguanosin-N^2-yl)-aristololactam I (dG-AAI) or 7-(deoxyadenosin-N^6-yl)-aristololactam II (dA-AAII) in rat forestomach DNA [37]. That may be because the imino structure of the N^6-adenosine adduct is more rigid than the amino structure of the N^2-deoxyguanosine adduct (Fig. 2). Moreover, the aristolactam moiety of guanine adducts is located in the minor groove of the B-DNA strand, whereas the aristolactam moiety of adenine adducts is situated in the major groove [38]. Therefore, it seems likely that the structural characteristic of these bulky DNA adducts account for differences in the response by polymerase. Thus, the AA-induced A:T to T:A mutation observed in this study might have been caused by the dA-AAI adduct, but evaluation of each component is necessary to elucidate their contribution to AA-mutagenesis. Furthermore, nearest neighbor binding analysis at the arrest site of DNA polymerase indicates that flanking pyrimidines had the greatest effect on polymerase arrest and therefore on DNA binding by AA [19], but such preference was not observed for the site of *cII* mutation, which showed no obvious hot spot.

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