Mitochondrial DNA Mutations and 8-hydroxy-2'-deoxyguanosine Content in Japanese Patients with Urinary Bladder and Renal Cancers

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Abstract. *Background: Several recent studies have demonstrated the presence of mitochondrial DNA (mtDNA) mutations in various human cancers. The origin of these mutations may be attributable to oxidative damage from reactive oxygen species (ROS). In order to investigate the relationship between mtDNA mutations and ROS in human cancers, urinary bladder and renal cancers were examined for mutations in the displacement-loop (D-loop) region of mtDNA and for 8-hydroxy-2'-deoxyguanosine (8-OHdG) content. Materials and Methods: The D-loop region of mtDNA of Japanese patients with urinary bladder or renal cancers was examined by direct sequencing. The level of 8-OHdG was measured in the patients who had undergone radical cystectomy or nephrectomy from excised specimens. Results: Somatic mutations in the D-loop region were detected in 7 (23%) out of 31 patients with bladder cancer and 3 (14%) out of 21 patients with renal cancer. The most frequent mutations were in the poly(C) mononucleotide repeat located at positions 303 to 309. The levels of 8-OHdG in cancer tissues were significantly higher than in the neighboring non-cancerous tissues, but many of the cancers with an elevated 8-OHdG level did not display D-loop mutations. Conclusion: These results suggest that the D-loop region of mtDNA might have a genetic instability in cancer tissues independently from the 8-OHdG level.*

The human mitochondrial genome is a small (16.5 kb), double-stranded, closed circular DNA molecule; it encodes 2 ribosomal RNAs, 22 transfer RNAs and 13 proteins, including some of the mitochondrial structural proteins and some of the respiratory complex enzymes involved in the production of adenosine triphosphate. The other mitochondrial structural proteins and respiratory complex enzymes, and all enzymes involved in replication and repair of mitochondrial DNA (mtDNA), are encoded by nuclear DNA.

Tumor development is often associated with mtDNA mutations, and mtDNA instability has been observed in many human cancers (1). Since most of the mitochondrial proteins are encoded by the nuclear DNA (nDNA), the role of mtDNA mutations in cancer development remains unclear. Alterations in expression of mtDNA-encoded polypeptides required for oxidative phosphorylation and cellular ATP generation may be a general characteristic of cancer cells. In some cases, these mutations may lead to abnormal metabolic and apoptotic processes in cancer cells (2). Findings of mtDNA mutations in tumor cells are consistent with reports showing that tumor cells are subjected to constitutive oxidative stress (3). Compared to nDNA, mtDNA is more susceptible to oxidative damage, and is, in general, more mutable. The higher mutation rate can be explained by three factors. First, mtDNA is located in the mitochondrial matrix in close proximity to the electron transport system where reactive oxygen species (ROS) are continuously generated. ROS function in both initiation and promotion of carcinogenesis, and they also decrease mitochondrial ATP production (4). Second, mtDNA is not protected by histones and chromatin structures, and third, mitochondria possess only limited DNA repair mechanisms. Moreover, since mtDNA lacks introns, it has been suggested that most mutations will occur in coding sequences and subsequent accumulation of mutations may lead to tumor formation. The region encoding NADH dehydrogenase subunits 3, 4 and 5 from respiratory complex I was found to be a mutational hot spot in some cancers (5). However, another mutation hot spot is the hypervariable regions of the non-coding displacement-loop (D-loop), which functions as a regulatory site for both replication and transcription (6).

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Mutations in the hypervariable regions are especially interesting since they are considered to cause no growth disadvantage for cells, thus, avoiding negative selective pressure. The mutations may alter the rate of DNA replication by modifying the binding affinity of important trans-acting factors (6). In the present study, urinary bladder and renal cancers were examined for genetic alterations in the D-loop region of mtDNA. Moreover, to gain further insight into the relationship between mtDNA mutations and oxidative damage, we measured the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is one of the major biomarkers of oxidative DNA damage induced by ROS, in cancer tissues and the neighboring normal tissues.

Materials and Methods

Patients. This study was approved by the Ehime University School of Medicine Committee on Human Genomic or Genetic Research. After written informed consent was obtained from each patient, primary tumor tissues and corresponding normal tissues were collected at surgery in the Ehime University School of Medicine between May 2002 and April 2004 from 31 urinary bladder cancer and 21 renal cancer patients who had been diagnosed histologically. Fresh tissues were collected, snap-frozen in liquid nitrogen, and stored at –80˚C until analysis of DNA and 8-OHdG. For histological examination, specimens were fixed in 10% buffered formalin and embedded in paraffin. Grading and staging of each tumor were done according to TNM classifications (UICC; 1997). For functional histochemistry, specimens were embedded in TISSUE-TEK (Miles Inc., Fulkhart, IN, USA) and stored at –80˚C until staining.

Histochemistry of mitochondrial respiratory chain enzymes. Functional histochemical analysis of mitochondrial respiratory chain enzymes was carried out on frozen sections of each patient. Six-micrometer-thick cryostat sections were stained for both cytochrome C oxidase (COX) and succinate dehydrogenase (SDH) in a double-staining procedure, as described elsewhere (7). In brief, SDH reaction solution (pH 7.6) was prepared as follows: 5 mM phosphate buffer (pH 7.4), 5 mM EDTA, 1 mM potassium cyanide, 0.2 mM phenazine methosulfate, 50 mM succinate, and 1.5 mM nitroblue. COX reaction solution (pH 7.4) was prepared as follows: 5 mM phosphate buffer (pH 7.4), 0.1% 3, 3'-diaminobenzidine (DAB), 0.1% cytochrome C, 0.02% catalase. Frozen sections were incubated with COX reaction solution for 30 min at 37˚C, followed by incubation with SDH reaction solution for 15 min. Sections were washed with distilled water and mounted in gelatin. SDH yields a blue reaction product, whereas COX gives a brown reaction product. Normal cells are stained blue-brown, whereas cells with mtDNA dysfunction are observed in blue because of the loss of COX activity.

PCR amplification and sequence analysis for D-loop region of mtDNA. Total cellular DNA from each tissue was extracted by QIAamp DNA mini kit (Qiagen, Tokyo, Japan) using the manufacturer's protocol. The two pairs of primers used in the study were as follows: forward 5'-TTAACTCCACCATTAGCACCC-3' (position: 15971-15991 in MITOMAP) and reverse 5'-ACCAAATGCATGGAG AGCTC-3' (position 36-55); forward 5'-CACCCTATTAACCACTC ACG-3' (position 15-34) and reverse 5'-GTGATGTGAGCCCGTCT AAACA-3' (position 616-637). PCR was performed in a final volume of 50 μl with 0.2 mM dNTPs, 1 mM $MgSO_4$, 0.3 μM of each primer, 1.0 unit KOD-plus-DNA polymerase (Toyobo, Osaka, Japan) and various amounts of total cellular DNA as template. The PCR consisted of an initial incubation at 94˚C for 2 min, followed by 35 cycles of denaturation at 94˚C for 15 sec, annealing at 60˚C for 30 sec and extension at 68˚C for 30 sec, and further extension at 72˚C for 5 min. PCR products were purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and sequenced with an ABI PRISM 310 (Applied Biosystems, Tokyo, Japan).

Measurement of 8-hydroxy-2'-deoxyguanosine (8-OHdG). Total cellular DNA was extracted as described above. Fifteen microliters of 30 mM acetate buffer (pH 5.3) and 15 μg nuclease P1 (Roche Diagnostics, Tokyo, Japan) were added to 135 μl DNA samples. The mixtures were incubated at 37˚C for 1 h to digest the DNA to nucleotides. Then, 20 μl of 500 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 2 units of *Escherichia coli* alkaline phosphatase (Toyobo) were added to the samples. The mixtures were incubated at 37˚C for 1 h to hydrolyze the nucleotides to nucleosides. The nucleoside samples were used for the measurement of 8-OHdG by competitive enzyme-linked immunosorbent assay kit (8-OHdG Check Highly Sensitive; Japan Institute for the Control of Aging, Shizuoka, Japan).

Statistical analysis. All values are expressed as means±S.E. Statistical analysis was performed by paired *t*-test for the comparison of 8-OHdG contents and Mann-Whitney *U*-test for comparison between mtDNA mutations and clinicopathological data. A p value <0.05 was considered statistically significant.

Results

COX and SDH activity were first studied by functional histochemistry in normal and urinary bladder cancer tissue (Figure 1). In normal urothelial epithelium (Figure 1A), blue-brown staining reveals that COX enzymatic activity was retained. In contrast, cancerous tissue shows a mosaic pattern of brown-blue alternating with pure blue (Figure 1B), consistent with focal loss of COX enzymatic activity.

The D-loop region of mtDNA in the cancer tissues and in the neighboring normal tissues were sequenced. Clinicopathological data are summarized in Table Ia and Ib. Seven out of 31 (23%) urinary bladder cancers contained somatic mutations in the D-loop region, among which 3 had a point mutation and 4 had a 1 bp deletion (Table IIa). In two patients (Case B3a and b and Case B18a and b in Table IIa), the same mutations were detected in the initial cancer and in a second cancer that appeared in a different site several months later. Three out of 21 (14%) renal cancers contained somatic mutations in the D-loop region, one was a point mutation, 2 were 1 bp deletions and 1 was a 2 bp deletion at nucleotide position 303-309 of the poly(C) region (Table IIb). Mutations in the poly(C) mononucleotide repeat

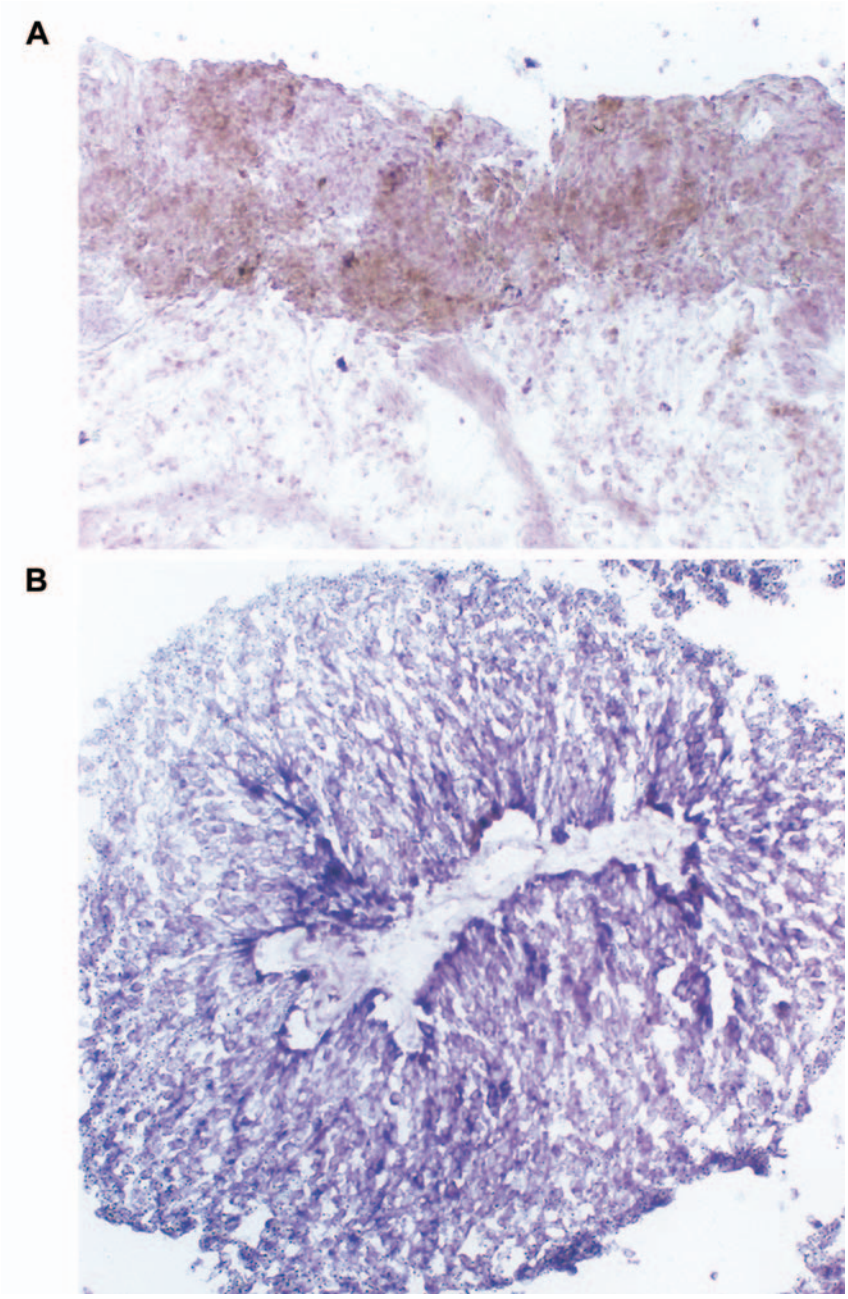

Figure 1. *Functional histochemistry for mitochondrial enzymes. Double staining reveals blue-brown staining in normal urothelial cells (A) and pure blue staining in cancer cells (B) (Original magnification x200).*

located from position 303 to 309 were the most frequent (7 out of 10 cases) (Table II). There were no significant correlations between mtDNA mutations and tumor grades or pathological stages.

Because of the limited volume of tissue available from transurethral punch biopsy, 8-OHdG measurements were carried out on only 3 urinary bladder cancer samples collected by means of total cystectomy and 13 renal cancer samples from radical nephrectomy. The 8-OHdG content of DNA in renal cancers was significantly higher than that of the neighboring normal tissues (66.5±10.4 *vs.* 32.8±4.1 ng/ml/mg DNA, $p=0.007$), whereas the difference in 8-OHdG content of DNA between urinary bladder cancers and the neighboring normal tissues (72.7±16.6 *vs.* 42.2±15.3 ng/ml/mg DNA, $p=0.072$) was not shown to be significant. Among the renal cancers, the 8-OHdG content of DNA in

Table Ia. *Clinicopathological characteristics of the patients with urinary bladder cancer.*

Case	Gender	Age	Grade	Stage	Histology	Operative procedure	mtDNA mutation	8-OH-dG (T:N)
B1	M	53	G3	pT3a	SCC	TC	-	N.E.
B2	M	74	G2	pTa	UC	TUR-Bt	-	N.E.
B3a	M	64	G1	pTa	UC	TUR-Bt	+	N.E.
B4	M	91	G1	pTa	UC	TUR-Bt	+	N.E.
B5	M	79	G2	pT2	UC	TUR-Bt	-	N.E.
B6	F	75	G2	pT1	UC	TUR-Bt	+	N.E.
B7	M	67	G3	pT1	UC	TUR-Bt	-	N.E.
B8	M	54	G1	pTa	UC	TUR-Bt	-	N.E.
B9	M	79	G2	pT1	UC	TUR-Bt	-	N.E.
B10	M	79	G2	pTa	UC	TUR-Bt	+	N.E.
B11	M	71	G2	pT1	UC	TUR-Bt	+	N.E.
B12	M	50	G2	pT2	UC	TUR-Bt	-	N.E.
B13	F	84	G3	pT2	UC	TUR-Bt	-	N.E.
B14	M	65	G2	pTa	UC	TUR-Bt	-	N.E.
B15	M	75	G1	pTa	UC	TUR-Bt	-	N.E.
B3b	M	65	G1	pTa	UC	TUR-Bt	+	N.E.
B16	F	78	G2	pTa	UC	TUR-Bt	-	N.E.
B17	M	78	G3	pT1	UC	TUR-Bt	-	N.E.
B18a	M	70	G1	pTa	UC	TUR-Bt	+	N.E.
B19	M	71	G2	pT2	UC	TUR-Bt	-	N.E.
B20	M	54	G1	pTa	UC	TUR-Bt	-	N.E.
B21	F	68	G2	pTa	UC	TUR-Bt	-	N.E.
B22	M	85	G2	pTa	UC	TUR-Bt	-	N.E.
B23	F	76	G3	pT1	SCC	TUR-Bt	-	N.E.
B24	M	71	G2	pTa	UC	TUR-Bt	-	N.E.
B25	M	68	G1	pTa	UC	TUR-Bt	-	N.E.
B26	M	80	G2	pTa	UC	TUR-Bt	-	N.E.
B27	F	66	G2	pT1	UC	TUR-Bt	-	N.E.
B28	M	69	G2	pTa	UC	TUR-Bt	-	N.E.
B29	F	70	G3	pT3b	SCC	TC	-	1.54
B18b	M	70	G2	pT1	UC	TC	+	1.29
B30	M	68	G3	pT4a	UC	TC	-	3.03
B31	F	78	G2	pTa	UC	TUR-Bt	+	N.E.

M: male, F: female, UC: urotherial cancer, SCC: squamous cell carcinoma, TC: total cystectomy, TUR-Bt: transurethral resection of bladder cancer, T: tumor, N: normal, N.E.: not examined.
Case B3a/B3b and B18a/18b: same patients with recurrent tumors.

the cancer samples with mtDNA mutation was 38.7±9.0 ng/ml/mg DNA (N=3), whereas the content in the cancer samples without mtDNA mutation was 74.9±12.2 ng/ml/mg DNA (N=10). The difference between them was not shown to be significant.

Discussion

Much attention has been directed to genetic events in nDNA, such as activation of oncogenes, inactivation of tumor suppressor genes and defects of mismatch DNA repair genes. However, several aspects in the process of carcinogenesis are still unclear. In recent years several studies have reported mtDNA alterations in many human cancers. A recent report showed an extremely high incidence of somatic mutations in the D-loop region in prostatectomy cancer specimens (8). Horton *et al.* reported a case of renal cell carcinoma with a 264 base pair deletion in the first subunit (ND1) of NADH (9). Fliss *et al.* screened 14 urinary bladder cancers for somatic mutations in the D-loop region, and found mutations in 4 (29%) (6). In the present study frequencies of mtDNA mutations in Japanese patients with urinary bladder and renal cancers were found to be similar to those reported in Western countries. Deletions in the poly(C) tract at nucleotide positions 303-309 were frequently observed in this study. Recent reports identified this homopolymeric C tract as a mutational hot spot in several primary tumors (10, 11). The D-loop region of mtDNA is important for both replication and expression of the mitochondrial genome because it contains the

Table Ib. *Clinicopathological characteristics of the patients with renal cancer.*

Case	Gender	Age	Grade	Stage	Cell type	mtDNA mutation	8-OH-dG (T:N)
R1	F	59	G1	pT1a	C	-	N.E.
R2	M	69	G2	pT3a	C	+	1.37
R3	M	67	G1	pT3c	C	-	N.E.
R4	F	65	G2	pT2	C	-	N.E.
R5	M	87	G1	pT1a	C	-	N.E.
R6	M	67	G2	pT1a	C	-	1.25
R7	M	60	G2	pT1a	C	-	N.E.
R8	F	54	G1	pT1b	C	-	1.3
R9	M	47	G1	pT1a	C	-	N.E.
R10	F	66	G2	pT1b	G	-	N.E.
R11	M	76	G2	pT1b	C	-	1.46
R12	F	88	G2	pT3a	M	+	1.14
R13	F	74	G2	pT1b	C	-	N.E.
R14	M	73	G2	pT1a	C	-	1.61
R15	M	63	G2	pT3a	C	-	1.08
R16	M	68	G1	pT2	C	-	1.02
R17	M	68	G2	pT1b	C	-	10.9
R18	M	56	G3	pT3b	C	+	1.79
R19	F	29	G2	pT3a	G	-	3.13
R20	M	51	G1	pT1a	C	-	6.05
R21	F	60	G3	pT1b	C	-	2.78

M: male, F: female, N.E.: not examined, T: tumor, N: normal, C: clear cell carcinoma, G: granular cell carcinoma, M: mixed tumor.

leading-strand origin of replication and transcriptional promoters (12). It is possible that alterations in this region lead to increased numbers of respiratory chain polypeptides or altered electron transport, conferring a growth and/or survival advantage to tumor cells in hypoxic conditions (13).

Interestingly, in 2 patients with urinary bladder cancer, identical mtDNA mutations were identified in the initial and second sites of cancer. There are three possible interpretations of this observation: that identical point mutations occurred independently in each tumor, that the cells already had mtDNA mutations before the development of definite cancer, or that the recurrent tumors developed by intraluminal seeding from the respective initial tumors. The third possibility is supported by the speculation that multifocal urothelial tumors are derived from a single progenitor cell (14, 15). Moreover, Parrela *et al.* showed that each patient had the same mutations in urine sediments and tissue samples (16). Each mitochondrion contains 2-10 copies of mtDNA, and each human cell contains more than 1,000 copies of mtDNA (17). At birth, the vast majority of these copies are identical in sequence (homoplasmic). Mutated and wild-type mtDNA molecules can coexist in the same cell, tissue, or organ (a state called heteroplasmy). However, in this study, the majority of somatic mutations were homoplasmic, suggesting that the mutant mtDNA became dominant in tumor cells. Supporting this notion, a recent cell fusion study found that mitochondria rapidly

Table IIa. *Sequence date of mtDNA mutation in urinary bladder cancer.*

Case	Stage	Nucleotide position	Mutation	State
B3a	pTa G1	16390	A to G	He
B4	pTa G1	182	C to T	Ho
B6	pT1 G2	16184	C to T	Ho
B10	pTa G2	303-309	deletion C	Ho
B11	pT1 G2	303-309	deletion C	Ho
B3b	pTa G1	16390	A to G	He
B18a	pTa G1	303-309	deletion C	Ho
B18b	pT1 G2	303-309	deletion C	Ho
B31	pTa G2	303-309	deletion C	Ho

Ho, homoplasmic; mitochondria containing only mutated DNA.
He, heteroplasmic; coexistence of mutated and wild-type mtDNA molecules.

Table IIb. *Sequence date of mtDNA mutation in renal cancer.*

Case	Stage	Nucleotide position	Mutation	State
R2	pT3a G2	16093	T to C	Ho
		303-309	deletion C	Ho
R12	pT3a G2	303-309	deletion C	Ho
R18	pT3b G3	303-309	deletion C	Ho

Ho, homoplasmic; mitochondria containing only mutated DNA.
He, heteroplasmic; coexistence of mutated and wild-type mtDNA molecules.

become homogeneous in colorectal cancer cells (5). Thus, there seems to be a selective mechanism that maintains a certain genotype of mtDNA in tumorigenesis. However, it is still unclear how the mtDNA molecules with point mutations provide a selective advantage that allows them to predominate in cancer cells.

Excessive exposure of mtDNA to ROS results in extensive oxidative damage that can induce T-to-C and G-to-A base transitions (18). In our study, only 1 out of 10 mutations identified was a T-to-C mutation, potentially indicating less exposure to ROS-derived mutagens in urinary bladder and renal cancers than in other cancers such as colorectal cancers. However, the wide variety of mutation types observed in tumor mtDNA would be compatible with an increase in oxidative DNA damage. Increased oxidative damage in tumors could be caused by subtle alterations of the mitochondrial respiratory chain function. In fact, functional histochemistry showed focal deficiencies of COX activity with preservation of SDH activity in cancer tissues, suggesting disorder of mitochondrial respiratory chain function. Moreover, significantly higher levels of 8-OHdG were found in cancer tissues than in the surrounding cancer-free tissues at various stages of the disease. Our results indicate that those cancer tissues constitutively produce more ROS than is produced by non-cancer tissues. However, the correlation between the increased rate of mtDNA mutation in tumor cells and increased free radical production in their mitochondria is highly speculative. mtDNA mutations in the D-loop were, indeed, not detected in some cancers that had a much higher level of 8-OHdG content compared to the neighboring normal tissue. It is possible that mtDNA mutations occur more frequently in regions other than the D-loop.

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