Molecular Pathogenesis of Genetic and Inherited Diseases

Contrasting Genome-Wide Distribution of 8-Hydroxyguanine and Acrolein-Modified Adenine during Oxidative Stress-Induced Renal Carcinogenesis

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Oxidative stress is a persistent threat to the genome and is associated with major causes of human mortality, including cancer, atherosclerosis, and aging. Here we established a method to generate libraries of genomic DNA fragments containing oxidatively modified bases by using specific monoclonal antibodies to immunoprecipitate enzyme-digested genome DNA. We applied this technique to two different base modifications, 8-hydroxyguanine and 1,N⁶-propanoadenine (acrolein-Ade), in a ferric nitrilotriacetate-induced murine renal carcinogenesis model. Renal cortical genomic DNA derived from 10- to 12-week-old

male C57BL/6 mice, of untreated control or 6 hours after intraperitoneal injection of 3 mg iron/kg ferric nitrilotriacetate, was enzyme digested, immunoprecipitated, cloned, and mapped to each chromosome. The results revealed that distribution of the two modified bases was not random but differed in terms of chromosomes, gene size, and expression, which could be partially explained by chromosomal territory. In the wild-type mice, low GC content areas were more likely to harbor the two modified bases. Knockout of *OGG1*, a repair enzyme for genomic 8-hydroxyguanine, increased the amounts of acrolein-Ade as determined by quantitative polymerase chain reaction analyses. This versatile technique would introduce a novel research area as a high-throughput screening method for critical genomic loci under oxidative stress. (Am J Pathol 2006, 169:1328–1342; DOI: 10.2353/ajpath.2006.051280)

Oxygen is essential for efficient energy production in aerobes. However, reactive oxygen species generated during this energy production represent a persistent threat to the integrity of the genome. Indeed, oxidative stress is associated with major causes of human mortality including cancer and atherosclerosis.¹ Reactive oxygen species react with the genomic DNA to cause strand scission, cross-links, or base modifications,² with base modifications thought to be the most frequent.³ Guanine base exposed either to hydroxyl radical, singlet oxygen, or photodynamic action is hydroxylated at C-8, leading to the formation of 8-hydroxyguanine (8-OHGua),⁴ which is

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present at a frequency of ~ 1 in 10^{5-6} guanine bases in the genomic DNA of various tissues under control conditions.⁵ Other modified guanine bases such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) may be produced via hydroxyl radicals.⁶ 8-OHGua in the genome may cause G to T transversion-type mutations during DNA replication.⁷ Thus far, three distinct repair enzymes (OGG1, MUTYH, and MTH1) have been identified to function at different levels to repair or prevent 8-OHGua in the genome. Germ-line mutations in *MUTYH* have been associated with recessive inheritance of multiple colorectal adenomas.^{8,9} Knockout of these repair enzymes in mice have been shown to produce a phenotype of higher cancer incidence.¹⁰⁻¹²

We have been studying the molecular mechanisms of how reactive oxygen species induce cancer using an iron-mediated rodent renal carcinogenesis model. Our recent genetic analyses identified *p16^{INK4A}* tumor suppressor gene as a major target gene, and we proposed a hypothesis that fragile sites exist in the genome against oxidative stress.^{13,14} Thus far, to the best of our knowledge, genome-wide screening for the detection of sites vulnerable to oxidative stress has not been undertaken.

This study aimed to establish a method to generate libraries of genomic DNA fragments containing oxidatively modified bases and to use the method to reveal common rules therein. We applied and optimized an immunoprecipitation technique for this purpose. In addition to 8-OHGua, we selected an aldehyde-modified adenine, 1,*N*⁶-propanoadenine, which is produced by the reaction of acrolein and adenine.¹⁵ Acrolein, a major lipid peroxidation product, is chemically reactive and mutagenic and may be one of the second messengers of oxidative stress.¹⁶ Previously, we produced and characterized monoclonal antibodies recognizing either 8-hydroxy-2'-deoxyguanosine (8-OHdG)¹⁷ or 1,*N*⁶-propano-2'-deoxyadenosine (acrolein-dA).¹⁵ Here we examined whether it was possible to specifically immunoprecipitate DNA fragments (DnaIP) containing these two different modified bases and whether there is any principle underlying the distribution of these modified bases *in vivo* in the genome of renal cortical cells in an oxidative stress (ferric nitrilotriacetate)-induced carcinogenesis model of rodents.¹⁸⁻²²

Materials and Methods

Animal Experiments

Male C57BL/6 mice (10 to 12 weeks old; Charles River Japan, Tokyo, Japan) were maintained in a specific pathogen-free environment. Twenty-four animals were divided into three groups of 18, three, and three animals, respectively, consisting of a time course group, untreated control group, and ferric nitrilotriacetate (Fe-NTA) group. Animals of the time course group were used for the selection of timing appropriate for the immunoprecipitation analyses, ie, not too much cellular necrosis but high genomic content of 8-OHdG and acrolein-dA as evalu-

ated by high-performance liquid chromatography and/or immunohistochemistry. The animals received an intraperitoneal injection of 3 mg of iron/kg of Fe-NTA prepared immediately before use²³ and were sacrificed at the indicated time after injection ($n = 3$, untreated, 3, 6, 9, 12, and 24 hours after injection; 6 hours was used for immunoprecipitation). Male *OGG1* knockout mice (C57BL/6 background)¹¹ of the same age were used ($n = 3$ for each time course group, untreated control, and Fe-NTA groups). The institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

Monoclonal Antibodies

Clone N45.1, which specifically recognizes 8-OHdG,¹⁷ and clone mAb21, which specifically recognizes acrolein-dA,¹⁵ were used.

Immunoprecipitation of Oligomeric DNA

A double-stranded 22-bp oligonucleotide containing one 8-OHdG paired with deoxycytidine on the complementary strand was prepared and labeled with fluorescein isothiocyanate (FITC) at the 5'-end of the (+) strand (FITC-5'-GGTGGCCTGACG*CATTCCCCAA-3'; *, 8-OHdG).²⁴ A double-stranded 22-bp oligonucleotide with the same sequence except without 8-OHdG worked as a control. One hundred fmol of the double-stranded 22-bp oligonucleotide was incubated at 4°C overnight with 0.1 or 100 μ g of N45.1 monoclonal antibody in 10 mmol/L phosphate buffer (pH 7.4) in a 50- μ l volume, followed by mixing with 50 μ l of protein A Sepharose CL-4B (Amersham Pharmacia Biotech, Tokyo, Japan) and incubation on ice for 1 hour. After washing with 100 mmol/L HEPES buffer (pH 8.0), the Sepharose beads were separated by centrifugation, lyophilized, and dissolved in 20 μ l of loading buffer [80% formamide, 10 mmol/L NaOH, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA)]. The solution was then denatured by heating at 95°C for 5 minutes. The sample solution was applied to a 20% denaturing polyacrylamide gel containing 8 mol/L urea in 1 $\times$ Tris-borate EDTA buffer and electrophoresed at 10 W for 30 minutes at room temperature. After electrophoresis, the fluorescence intensity of each band was evaluated using FMBio-100 (TakaraBio, Shiga, Japan).

Genomic DNA Extraction and Production of 8-OHGua in the Genomic DNA

Nuclear genomic DNA was extracted from mouse renal cortical samples by the Nal method (Wako, Osaka, Japan).²⁵ Each solution was saturated with argon gas and supplemented with desferal (final concentration, 0.1 mmol/L) where applicable to prevent further DNA oxidation. To increase the 8-OHdG level without inducing strand breaks, genomic DNA (100 μ g/ml; 10 mmol/L Tris-HCl buffer, pH 8.0) in the presence of 5 to

Table 1. Detailed Information about Target Genome Areas in Quantitative PCR Analysis

Target area	Category	Map	Sau96I fragment examined*
Gpx3	Highly expressed in the kidney	11qB1.3	54,543,484 to 54,543,937 (454 bp) 54,544,840 to 54,545,400 (561 bp)
Actb	Housekeeping	5qG2	140,384,767 to 140,385,328 (562 bp) 140,389,282 to 140,389,785 (504 bp)
Gsta4	Response to oxidative stress	9qE1	78,563,608 to 78,564,135 (528 bp) 78,567,352 to 78,567,905 (554 bp)
Trio	Highly expressed at early developmental stage	15qB1	27,892,748 to 27,893,287 (540 bp)
Trp53	Tumor suppressor (gatekeeper)	11qB3	69,199,039 to 69,199,547 (509 bp) 69,201,537 to 69,202,006 (470 bp)
Hmox1	Response to oxidative stress	8qC1	74,162,238 to 74,162,760 (523 bp) 74,166,814 to 74,167,311 (498 bp)
Al480653	Unknown function	16qB2	30,842,039 to 30,842,562 (524 bp)
Pdzk3	Secreted protein	15qA1	12,394,886 to 12,395,429 (545 bp)
Pax7	Developmental stage-specific (silenced in the kidney)	4qD3	138,218,354 to 138,218,866 (513 bp) 138,279,020 to 138,279,573 (554 bp)
Cdkn2a	Tumor suppressor (gatekeeper)	4qC4	87,591,234 to 87,591,826 (593 bp) 87,597,610 to 87,598,071 (462 bp)
Pax5	Developmental stage-specific (silenced in the kidney)	4qB1	43,933,461 to 43,933,976 (516 bp) 43,936,346 to 43,936,888 (543 bp)
Gabra5	Tissue-specific (silenced in the kidney)	7qB4	45,084,988 to 45,085,544 (557 bp) 45,106,519 to 45,107,045 (527 bp)
Chr10_65	Intergenic area	10qB5.1	65,380,121 to 65,380,628 (508 bp) 65,381,573 to 65,382,083 (511 bp)
Chr15_21	Intergenic area	15qA2	21,813,091 to 21,813,675 (585 bp)
Chr15_37	Intergenic area	15qB3.1	37,211,512 to 37,212,101 (590 bp)
Chr15_58	Intergenic area	15qD1	58,952,316 to 58,952,844 (529 bp)
Chr16_33	Mouse mRNAs from GenBank	16qB3	34,035,996 to 34,036,524 (529 bp)
Chr16_47	Intergenic area	16qB5	47,283,849 to 47,284,395 (547 bp) 47,294,616 to 47,295,114 (499 bp)
Chr16_58	Intergenic area	16qC1.2	58,921,006 to 58,921,534 (529 bp)
Chr16_90	Mouse mRNAs from GenBank	16qC3.3	91,205,361 to 91,205,886 (526 bp)

*Alignment on NCBI Mouse Build 33 (and its length).

(table continues)

50 mmol/L methylene blue and 0.1 mmol/L desferal was incubated under a 60 W electric bulb (12-cm distance) for 30 minutes as described.²⁶ This procedure increased the amounts of 8-OHdG up to ~1000-fold.

8-OHdG Determination

The amount of 8-OHdG in DNA was estimated after nuclease P1 and alkaline phosphatase treatment by high-performance liquid chromatography with an electrochemical detector as described¹⁷ with the following minor modification. Desferal (final concentration, 0.1 mmol/L) was added before nuclease P1 digestion.

Differential Separation Analysis

A pGL3-catalase promoter vector²⁷ was digested with *Bam*HI and *Hind*III to produce 1.7-, 1.9-, and 4.4-kb fragments (vector-1). Another vector containing a cloned genomic DNA fragment (pCR4BluntTOPO-0078) was digested with *Eco*RI and *Kpn*I to produce 0.4-, 1.1-, 2.0-, and 3.9-kb fragments (vector-2). DNA fragments from the former vector were treated with methylene blue and light to increase the level of 8-OHdG by ~500-fold. Equal amounts of both of the DNA fragment preparations were mixed and subjected to DnaIP. Recovered DNA fragments were cloned and identified either by size or by

sequencing with an ABI Prism 377 sequencer (Tokyo, Japan).

Immunoprecipitation of Genomic DNA

Genomic DNA was digested either with *Hae*III (GG/CC) for library construction or with *Sau*96I (G/GNCC) for amplification in quantitative polymerase chain reaction (PCR) experiments. An aliquot of genomic DNA fragments (20 μ g for chromosome mapping; 30 μ g for amplification in quantitative PCR experiments) was incubated with each antibody (10 μ g of N45.1 or 2 μ g of mAb 21) in 10 mmol/L phosphate-buffered saline containing 0.1% bovine serum albumin for 3 hours at 4°C in a 900- μ L volume, mixed with 100 μ L of Dynabeads M-280 sheep anti-mouse IgG (DynaL, Oslo, Norway) and incubated for another 3 hours. The beads were then washed sequentially with four different buffers (buffer 1: 0.1% sodium deoxycholate, 1% Triton X-100, 1 mmol/L EDTA, 50 mmol/L HEPES-KOH, 140 mmol/L NaCl, pH 7.5; buffer 2: 0.1% sodium deoxycholate, 1% Triton X-100, 1 mmol/L EDTA, 50 mmol/L HEPES-KOH, 500 mmol/L NaCl, pH 7.5; buffer 3: 0.1% sodium deoxycholate, 0.5% Nonidet P-40, 1 mmol/L EDTA, 250 mmol/L LiCl, and 10 mmol/L Tris-HCl, pH 8.0; buffer 4: 1× TE). The beads were incubated with 80 μ L of elution buffer (10 mmol/L EDTA, 1% sodium dodecyl sulfate, and 50 mmol/L Tris-HCl, pH 8.0) at 65°C for 10 min-

Table 1. *Continued*

Primer sequence		Product size
5'-CTGTGCCTTTAGTGTGGGAAC-3'	and 5'-TCACCATCTCTCCTTCATTCT-3'	113 bp
5'-TGTAACCAAGCTTGAGACCTGA-3'	and 5'-ATTCACCTATTGGGGGAGGAT-3'	119 bp
5'-TGACGTTGACATCCGTAAAGAC-3'	and 5'-GCAGTAATCTCCTTCTGCATCC-3'	102 bp
5'-TCCAACAACCAAGAGAAATCC-3'	and 5'-CGACCTCTGAAACAATCTGGT-3'	108 bp
5'-CATTGTTGACTGCCTAGCAAAG-3'	and 5'-GGTAAGAAAAGCTCCTGTGTGG-3'	141 bp
5'-TCTAGCCTCTGGCTCATGGTAT-3'	and 5'-CACTGTCTGATGGAATGCTTGT-3'	101 bp
5'-TATATTCGTGGGGGTCGATTTC-3'	and 5'-AACAGCCTGTAAGGTGGGAGAG-3'	130 bp
5'-TAAGCCATAGGGGTTTGTGT-3'	and 5'-AGGCTGATATCCGACTGTGACT-3'	122 bp
5'-ACTTCACCTGGATCCTGTGTCT-3'	and 5'-ACCTGGAGTCTTCCAGTGTGAT-3'	142 bp
5'-AATCATTCACAGACATCGCTTG-3'	and 5'-CTTCATGAACCTCAGCATCTCTCG-3'	137 bp
5'-GGGAGATACCTGACACAGTTCC-3'	and 5'-GCTCAGAACAGCTGCTTTTACA-3'	126 bp
5'-ATGCTGTCTCCTCCCACTTTC-3'	and 5'-CTTGCTGGTCAGTTTGAAGTGG-3'	126 bp
5'-AGGAATGGAGGCTTTGTGAGTC-3'	and 5'-AGAGGGCATATGTTGCCTCATT-3'	130 bp
5'-CCTTTATCAAACGGGGATTTCAC-3'	and 5'-CTCCATCTTCCGGAAACAAAC-3'	128 bp
5'-AAGTTGAGGCTTCGGTTAGTC-3'	and 5'-AGCTAGAGCCCTCTTCTCAAGC-3'	138 bp
5'-ATGACTTCACCCGTCACCTTTT-3'	and 5'-GGTACCACGCCAGTATGAAAT-3'	121 bp
5'-GAAGGAATGGTATCCAGCAGAG-3'	and 5'-TTTACTCTTTCCACCATGACC-3'	114 bp
5'-TGTCACCACAGAAGCTGCTTTT-3'	and 5'-GACAGTGAGACTTGCCTTGGTG-3'	114 bp
5'-AGAGCTGGCTCTTCTCCTTTCA-3'	and 5'-ATCCTGGCTCAGGAACTCAAG-3'	130 bp
5'-TAAAGCTGTCTCGGTTGGGTTT-3'	and 5'-GTGATGGATTGGTACACGGA-3'	104 bp
5'-GTGTTTCTGACATGTGCTTTTC-3'	and 5'-CTGAAATATTTGCCACACGTTTC-3'	101 bp
5'-GCTAGAGCAAGAGCAAAACCAT-3'	and 5'-GAAGCCATGCTTCTAAATGAGG-3'	128 bp
5'-AAGTTGTCAAGGGAAGGAAGC-3'	and 5'-CCACTATTGCATCTTCGGGTA-3'	102 bp
5'-TGTGGACTCACAGAATGCCTTA-3'	and 5'-GACCAGTGGATTCCATGATTGT-3'	125 bp
5'-TCTCTGTCTTCTGCAGCATCC-3'	and 5'-GAACTGCTGGAGTATGGCTCCT-3'	141 bp
5'-TTTTTCAGCACGGGTACTTGAG-3'	and 5'-GGCAGCAGTATCAAGCAGAGAC-3'	147 bp
5'-GTACTGAGGATGCTGTGTTGC-3'	and 5'-CATGAGCCAGACAGGTAACAG-3'	115 bp
5'-AGGCAGAGGTTTGTACTTGGAG-3'	and 5'-TCGACAACTGTAATGCCAACC-3'	130 bp
5'-CCAATTGGAGCTAACAGAAACC-3'	and 5'-AGCTGGTCAACTGCCTACTCTC-3'	113 bp
5'-TGTTTTCCGGAGTTTCAGTTCA-3'	and 5'-ACCCTTGTTTCTCCCATGTTGT-3'	133 bp
5'-CTAGCATGCCACTTCCATGTTTC-3'	and 5'-TGCGGTCTTTCACTTGTGTT-3'	107 bp

utes. This procedure was performed twice. For cloning only, the eluent was treated with calf intestinal alkaline phosphatase (TakaraBio). Then, the recovered DNA was digested with proteinase K at 37°C for 1 hour, subjected to phenol-chloroform extraction, and precipitated with ethanol. The amounts of recovered DNA were quantified by the Saran Wrap method using ethidium bromide.²⁸

Cloning and Chromosome Mapping

Cloning was done with a Zero Blunt TOPO PCR cloning kit for sequencing (Invitrogen, Tokyo, Japan) as suggested by the manufacturer, and the cloned fragments were sequenced with an ABI Prism 377 sequencer. The locations of the cloned fragments on chromosomes were assigned according to the May 2004 assembly of the mouse genome (NCBI m33 build) at UCSC (<http://www.genome.ucsc.edu/>). RefSeq database (<http://www.ncbi.nlm.nih.gov/RefSeq/>) was also used as a reference.

Gene Expression Analysis

Mouse Genome 430, 2.0 Arrays (Affymetrix Inc., Santa Clara, CA) were used. Total RNA was isolated using a

RNeasy mini kit (Qiagen, Tokyo, Japan). Pooled RNA from three animals of each group was analyzed. The degree of gene expression was then evaluated with Affymetrix GeneChip operating software (GCOS).

Fragment Amplification and Quantitative PCR Analysis

Immunoprecipitated DNA fragments were amplified by PCR after ligation with an adaptor (*Sau*96I, 5'-GNCT-GCGGTGA-3' and 5'-AGCACTCTCCAGCCTCTCAC-CGCA-3'; underline, complementary sequence; Ligation pack, Nippon Gene, Toyama, Japan) and then subjected to exonuclease I treatment and phenol-chloroform extraction as described.²⁹ The amplified DNA fragments were subjected to quantitation using an ABI 7300 real-time PCR system with the specific primer pairs shown in Table 1. One or two primer pairs were prepared for each gene or intergenic area, and the mean value was used as a result when two primer pairs were selected. The corresponding genomic DNA after *Sau*96I digestion and amplification was used as a control. The final results were adjusted by the amounts of immunoprecipitated DNA fragments.

Analysis of Chromosomal Territory

A touch preparation of renal cortex was obtained as previously described. More than 90% were nuclei of proximal tubular cells.³⁰ The specimens were subjected to fluorescent *in situ* hybridization analysis with chromosome painting probes according to the manufacturer's instructions (dual-color biotin/Texas Red-FITC; Cambio, Cambridge, UK) and were observed with a confocal laser microscope (Fluoview; Olympus, Osaka, Japan). The center of gravity of the nucleus and that of the chromosome were measured to assign the relative radial location.

Histology and Immunohistochemistry

Histological and immunohistochemical analyses were performed as previously described.^{15,17} Neutral formalin-fixed paraffin-embedded sections were used with the avidin-biotin complex method (primary antibody concentration: N45.1, 10 μ g/ml; mAb 21, 1.3 μ g/ml). Three registered pathologists (T.S., T.T., and S.T.) conducted all of the pathological analyses.

Statistical Analysis

Statistical analyses were performed with an unpaired *t*-test, which was modified for unequal variances when necessary, χ^2 test, Kolmogorov-Smirnov test,³¹ or one-way analysis of variance. *P* < 0.05 was considered to be statistically significant.

Results

Specificity of DnaIP for Modified DNA Bases

To test our strategy (Figure 1A), double-stranded 22-mer oligo-DNA fragments containing a single 8-OHdG were synthesized and subjected to DnaIP. Although oligo-DNA fragments were bound to the beads in the absence of antibody (Figure 1B, large arrowhead), the inclusion of a small amount of antibody was enough to dramatically decrease this nonspecific binding (Figure 1B, small arrowhead). In the presence of specific antibody against 8-OHdG (clone N45.1), only DNA fragments containing

8-OHdG, but not those of the same sequence without 8-OHdG, were immunoprecipitated (Figure 1B). Then, genomic DNA extracted from mouse cortical kidney was used after digestion with *Hae*III (GG/CC). The average size of the DNA fragments was $\sim$ 1 kbp. N45.1 but not control antibody of the same isotype (IgG₁) immunoprecipitated the DNA fragments. On the contrary, an excess of antibody decreased the final recovery of DNA fragments (Figure 1, C and D) probably because of the limited binding capacity of the beads for IgG. Similar results to those in Figure 1, C and D, were obtained for acrolein-dA and its specific monoclonal antibody (data not shown). The amounts of immunoprecipitated DNA was basically in proportion with the amounts of loaded DNA (Figure 1E) or the fraction of 8-OHdG (Figure 1F). When equal amounts of enzyme-digested double-stranded DNA vector-1 and -2 (only vector-1 was incubated with methylene blue under light to increase the 8-OHdG level by $\sim$ 500-fold) were mixed and subjected to DnaIP, 20 fragments of 21 (95.2%) obtained were from vector-1.

Animal Experiments

An oxidative stress-induced mouse model of renal carcinogenesis was used.¹⁹ In this model, the Fenton reaction occurs in the renal proximal tubules after an intraperitoneal injection of ferric nitrilotriacetate (Fe-NTA).²⁰ Our quantitation by high-performance liquid chromatography with electrochemical detector showed significantly higher levels of 8-OHdG 6 to 9 hours after an injection of Fe-NTA (Figure 1G). Increased levels of 8-OHdG and acrolein-dA in the nuclei of renal proximal tubular cells were observed with immunohistochemistry. Most of the cells (>95%) with intense nuclear immunostaining were proximal tubular cells (Figure 1, I–N). We selected 6 hours after Fe-NTA injection for DnaIP after these results. Genomic DNA was extracted from the nuclear fraction of the cortical kidney of control and Fe-NTA-treated mice. DNA fragments after enzyme digestion were subjected to DnaIP with N45.1 or a monoclonal antibody recognizing acrolein-dA (clone mAb 21). The amounts of immunoprecipitated DNA were significantly different between the control samples and those after Fe-NTA treatment ei-

Figure 1. Validation of immunoprecipitation of DNA fragments (DnaIP) containing oxidatively modified bases and its application to the mouse cortical kidney under oxidative stress induced by intraperitoneal administration of ferric nitrilotriacetate (Fe-NTA). **A:** Principle of DnaIP. **B:** Immunoprecipitation of FITC-labeled 22-mer oligo-DNA fragments containing single or no 8-hydroxy-2'-deoxyguanosine (8-OHdG), as shown by 20% denaturing polyacrylamide electrophoresis. DNA attachment to beads (**large arrowhead**) was significantly reduced by including a small amount of antibody (**small arrowhead**, trace band). Oligo-DNA with 8-OHdG, but not that without 8-OHdG, was immunoprecipitated. Refer to the Materials and Methods for details. **C:** Specificity of N45.1 antibody for genomic DNA containing 8-OHdG ($\sim 0.5/10^5$ dG) as determined by the Saran Wrap method using ethidium bromide (Dynabeads of 100 μ l and 30 μ g of *Hae*III-digested genomic DNA were used in the system). **D:** Quantitation of results in **C**. **E:** Linear correlation with the amount of input DNA. Each point denotes the mean of duplicate experiments (Dynabeads of 100 μ l and 10 μ g of N45.1 monoclonal antibody were used in the system). **F:** Linear correlation with the fraction of 8-OHdG in the input DNA. Each point denotes the mean of duplicate experiments (Dynabeads of 100 μ l, 20 μ g of *Hae*III-digested genomic DNA and 20 μ g of N45.1 monoclonal antibody were used in the system). Refer to Results and Discussion for details. **G:** Time course of 8-OHdG levels in male wild-type C57BL/6 mice (*n* = 3 to 4, mean $\pm$ SEM; **P* < 0.05, ***P* < 0.01 versus untreated control). **H:** Amounts of immunoprecipitated DNA fragments from kidney containing either 8-OHdG or acrolein-dA under control conditions or oxidative stress (6 hours after Fe-NTA administration; *n* = 3, mean $\pm$ SEM; **P* < 0.05, ***P* < 0.01 versus control; Dynabeads of 100 μ l, 20 μ g of *Sau*96I-digested genomic DNA and 10 μ g of N45.1/2 μ g of mAb21 monoclonal antibody were used in the system). **I:** H&E staining of control kidney. **J:** 8-OHdG immunostaining (N45.1) of control kidney. **K:** Acrolein-dA immunostaining (mAb21) of control kidney. **L:** H&E staining of the kidney under oxidative stress (6 hours after an intraperitoneal injection of Fe-NTA; **arrows**, degeneration of proximal tubular cells). **M:** 8-OHdG immunohistochemical analysis of the kidney under oxidative stress shows nuclear staining. **N:** Acrolein-dA immunohistochemical analysis of the kidney under oxidative stress shows nuclear staining. Refer to the Materials and Methods for details. GL, glomerulus. Original magnifications, $\times 200$.

Table 2. χ^2 Test for Even Distribution of 8-OHdG Among the Chromosomes

Chromosome	Length (Mbp)	Probability (if H_0 true)	Untreated ($P = 0.107^*$)				Fe-NTA treated ($P = 0.327^*$)			
			Observed frequency	Expected frequency	O-E/E	P value [†]	Observed frequency	Expected frequency	O-E/E	P value [†]
1	195.2 × 2	0.078	24	24.1	-0.6%	0.975	24	28.6	-16.0%	0.372
2	181.7 × 2	0.073	19	22.5	-15.5%	0.447	25	26.6	-6.0%	0.746
3	160.6 × 2	0.064	16	19.9	-19.5%	0.370	30	23.5	27.6%	0.167
4	154.1 × 2	0.062	17	19.1	-10.8%	0.625	26	22.6	15.2%	0.457
5	149.2 × 2	0.060	20	18.5	8.3%	0.712	24	21.9	9.8%	0.636
6	149.7 × 2	0.060	19	18.5	2.6%	0.909	13	21.9	-40.7%	0.049
7	133.1 × 2	0.053	15	16.5	-8.9%	0.712	21	19.5	7.8%	0.724
8	128.7 × 2	0.051	16	15.9	0.5%	0.983	15	18.8	-20.4%	0.363
9	124.2 × 2	0.050	14	15.4	-8.9%	0.722	26	18.2	43.0%	0.060
10	130.6 × 2	0.052	11	16.2	-31.9%	0.187	12	19.1	-37.3%	0.094
11	121.6 × 2	0.049	12	15.0	-20.3%	0.420	21	17.8	17.9%	0.439
12	115.1 × 2	0.046	12	14.2	-15.7%	0.544	18	16.9	6.8%	0.775
13	116.5 × 2	0.046	13	14.4	-9.8%	0.704	20	17.1	17.3%	0.465
14	117.1 × 2	0.047	15	14.5	3.6%	0.889	11	17.1	-35.8%	0.128
15	104.1 × 2	0.042	11	12.9	-14.6%	0.592	19	15.3	24.6%	0.327
16	98.8 × 2	0.039	26	12.2	112.7%	0.00006	20	14.5	38.2%	0.138
17	93.6 × 2	0.037	15	11.6	29.6%	0.305	12	13.7	-12.4%	0.639
18	91.1 × 2	0.036	9	11.3	-20.1%	0.491	11	13.3	-17.5%	0.514
19	60.7 × 2	0.024	13	7.5	73.2%	0.042	9	8.9	1.3%	0.970
X	160.6 × 1	0.032	13	9.9	30.8%	0.323	10	11.8	-15.0%	0.601
Total	5011.9	1.000	310	310.0			367	367.0		

χ^2 test for goodness of fit under the null hypothesis (H_0) that 8-OHdGs occur equally in proportion to length of each chromosome.

*All the frequencies for 20 chromosomes were tested together (d.f. = 19).

†The frequency for one chromosome was tested against the combined frequency for the other 19 chromosomes. (d.f. = 1).

ther for 8-OHdG and acrolein-dA as shown in Figure 1H.

Mapping of Cloned DNA Fragments Containing Oxidatively Modified DNA Bases

Fragments ranging 86 to 159 from each animal ($n = 3$ for each combination of treatment and antibody) were cloned, sequenced, and mapped to chromosomes based on the May 2004 assembly of the mouse genome (NCBI m33 build) at UCSC (<http://www.genome.ucsc.edu/>) (Figure 2, A and B). First, Kolmogorov-Smirnov analysis was applied to the distribution of oxidatively modified DNA bases on the assumption that the genome is a unit of continuous information from chromosome 1 to X disregarding the presence of chromosomal restriction. This analysis revealed no significant difference between any combination of the two conditions in the distribution of 8-OHdG and acrolein-dA (data not shown). However, the χ^2 test showed a significant deviation for chromosome-specified analysis of the acrolein-dA distribution in Fe-NTA-treated mice ($P = 0.034$). No significant deviation was observed for the other three conditions (Tables 2 and 3). Then, the χ^2 test was applied to combinations of each chromosome and the other chromosomes. We

found that one to three chromosomes in each condition showed significant deviation, as shown in Tables 2 and 3.

Analysis of Chromosome Territory

We selected chromosomes 15 and 16 based on the similarity of physical size and statistical significance of our results (Tables 2 and 3), and performed fluorescence *in situ* hybridization analysis to localize the chromosome territory during interphase in renal proximal tubular cells (Figure 2C). Chromosome 16 was located toward the nuclear center and chromosome 15 close to the nuclear border. The results were significant according to Kolmogorov-Smirnov analysis ($D = 0.333$, $P = 9.7 \times 10^{-8}$) (Figure 2D) and could be even underestimated because of the nature of two-dimensional analyses.

Genome-Wide Analysis in Association with Genes and Their Expression

When we aligned the RefSeq genes ($\sim 18,000$) along the chromosomes, 27.94% of the mouse genomic areas were covered with their exons and introns. We used this value as a reference. The fraction of DNA

Figure 2. Chromosome mapping of nuclear genomic DNA fragments containing modified DNA bases from mouse renal cortical cells under control and oxidatively stressed conditions and analysis of chromosome territory of mouse renal proximal tubular cells after touch preparation. **A:** Distribution of 8-OHdG. **B:** Distribution of acrolein-dA (green, control kidney; red, kidney 6 hours after an intraperitoneal injection of Fe-NTA). Extracted genomic DNA of the kidney was digested with *Hae*III, followed by immunoprecipitation, cloning, sequencing, and identification in the UCSC genome database. The numbers of clones mapped were as follows: 8-OHdG, control, $n = 310$; 8-OHdG, oxidative stress, $n = 367$; acrolein-dA, control, $n = 302$; acrolein-dA, oxidative stress, $n = 295$. Long ticks mean two independent clones at the identical loci. **C:** Representative results of chromosome 15 and 16 territories by fluorescent *in situ* hybridization (red, chromosome 15; green, chromosome 16 counterstained with DAPI). **D:** Distribution of the position of chromosome territory from the nuclear center of gravity. One hundred cells were analyzed (chromosome 15, mean = 0.598; chromosome 16, mean = 0.426; Kolmogorov-Smirnov test, $D = 0.333$, $P = 9.7 \times 10^{-8}$).

Table 3. χ^2 Test for Even Distribution of Acrolein-dA Among the Chromosomes

Chromosome	Length (Mbp)	Probability (if H_0 true)	Untreated ($P = 0.068^*$)				Fe-NTA treated ($P = 0.034^*$)			
			Observed frequency	Expected frequency	O-E/E	P value [†]	Observed frequency	Expected frequency	O-E/E	P value [†]
1	195.2 × 2	0.078	31	23.5	31.80%	0.108	21	23.0	-8.60%	0.667
2	181.7 × 2	0.073	19	21.9	-13.20%	0.521	22	21.4	2.90%	0.891
3	160.6 × 2	0.064	26	19.4	34.40%	0.118	25	18.9	32.30%	0.147
4	154.1 × 2	0.062	17	18.6	-8.50%	0.706	20	18.1	10.20%	0.653
5	149.2 × 2	0.060	28	18.0	55.70%	0.015	18	17.6	2.50%	0.915
6	149.7 × 2	0.060	13	18.0	-28.00%	0.221	12	17.6	-31.90%	0.167
7	133.1 × 2	0.053	9	16.0	-43.90%	0.071	13	15.7	-17.00%	0.489
8	128.7 × 2	0.051	12	15.5	-22.60%	0.360	15	15.1	-1.00%	0.969
9	124.2 × 2	0.050	11	15.0	-26.50%	0.293	15	14.6	2.60%	0.918
10	130.6 × 2	0.052	13	15.7	-17.40%	0.478	21	15.4	36.60%	0.141
11	121.6 × 2	0.049	11	14.7	-25.00%	0.327	5	14.3	-65.10%	0.012
12	115.1 × 2	0.046	10	13.9	-27.90%	0.288	8	13.5	-40.90%	0.123
13	116.5 × 2	0.046	18	14.0	28.30%	0.278	15	13.7	9.40%	0.721
14	117.1 × 2	0.047	20	14.1	41.70%	0.108	18	13.8	30.60%	0.245
15	104.1 × 2	0.042	16	12.6	27.50%	0.320	20	12.3	63.10%	0.024
16	98.8 × 2	0.039	11	11.9	-7.60%	0.789	7	11.6	-39.80%	0.166
17	93.6 × 2	0.037	10	11.3	-11.30%	0.699	6	11.0	-45.50%	0.124
18	91.1 × 2	0.036	10	11.0	-8.90%	0.764	11	10.7	2.60%	0.931
19	60.7 × 2	0.024	3	7.3	-59.00%	0.106	6	7.1	-16.00%	0.665
X	160.6 × 1	0.032	14	9.7	44.60%	0.158	17	9.5	79.80%	0.013
Total	5011.9	1.000	302	302.0			295	295.0		

χ^2 test for goodness of fit under the null hypothesis (H_0) that acrolein-dAs occur equally in proportion to length of each chromosome.

*All the frequencies for 20 chromosomes were tested together (d.f. = 19).

†The frequency for one chromosome was tested against the combined frequency for the other 19 chromosomes. (d.f. = 1).

fragments containing oxidatively modified bases that landed on RefSeq genes was significantly smaller than the reference probability in the samples of acrolein-dA under oxidative stress by Fe-NTA administration (χ^2 test, $P = 0.033$) (Figure 3A). We also performed microarray analysis to examine the correlation of our results with gene expression. Figure 3B illustrates the status of expression of the cloned genes included in both the GeneChip probes and RefSeq under the control conditions or oxidative stress. Fe-NTA-mediated oxidative stress increased the genome fraction of expressed RefSeq genes by 1.45%. The fraction of the expressed genes was slightly lower than the results obtained by microarray analysis in all of the conditions except for 8-OHdG under oxidative stress, whereas that of 8-OHdG under oxidative stress was similar to the expressed fraction of genes (Figure 3B).

Then, we analyzed the distributions of two characteristic properties of the genes in which modifications occurred. Under oxidative stress, the distribution of the expression level of genes was significantly different between the groups for 8-OHdG and acrolein-dA (Figure 3C). The plots revealed that 8-OHdG preferred highly expressed genes (expression >1000), but acrolein-dA did not. Furthermore, the distribution of the size of genes was significantly different between the groups for 8-OHdG and acrolein-dA under oxidative stress (Figure 3, D and E). This significance was mainly contributed by the fraction of expressed genes. In the expressed genes especially with size less than 150 kb, 8-OHdG occurred more frequently than acrolein-dA (Figure 3, E and F).

Quantitative PCR after DnaIP in The Kidneys of Wild-Type and OGG1 Knockout Mice

Our analysis confirmed that the genomic DNA of knockout mice of a repair enzyme gene *OGG1*,³² which removes genomic 8-OHdGua, contained higher levels of 8-OHdG than that of wild-type mice. Furthermore, 8-OHdG was repaired in *OGG1* knockout mice as well, and even more rapidly than in wild-type mice (Figure 4A). This may be attributed to an accelerated response to large amounts of oxidative damage via yet undetermined repair or antioxidative mechanisms. Twenty representative genomic areas were selected after consideration of the characters of genes, namely their size, their chromosomal position, and their level and pattern of expression (Table 1 and Figure 4B). The amounts of 8-OHdG DNA fragments increased in every genomic area of wild-type mice after oxidative stress (Figure 4C). *OGG1* knockout mice in general (19 genomic areas of 20) showed larger amounts of immunoprecipitated 8-OHdG DNA fragments under control conditions (Figure 4C). However, no further increase in the amount of immunoprecipitated 8-OHdG DNA was obvious in the selected genomic areas after oxidative stress in *OGG1* knockout mice. The exceptions were chr15_58 and chr16_58 in which persistent significant increases over control conditions were observed (Figure 4C). The amounts of acrolein-dA DNA fragments increased in 8 of 20 genomic areas of wild-type mice after oxidative stress. In every locus of *OGG1* knockout mice examined, increased amounts of acrolein-dA DNA fragments over wild-type mice were observed (Figure 4D). The average PCR product ratio (DnaIP/genome) of

Figure 3. Analysis of the DNA fragments containing oxidatively modified bases in association with genes and their properties. **A:** Analysis of gene and nongene areas according to the RefSeq database. Numerals show actual numbers of clones studied in this experiment. The dashed line shows the calculated fraction of RefSeq gene areas in the mouse genome (* $P = 0.033$ with χ^2 test). **B:** Analysis of expressed and nonexpressed genes based on microarray experiments for quantitating their expression level. Numerals show actual numbers of clones in this experiment. The dashed line shows the calculated ratio of expressed versus unexpressed RefSeq gene areas. Refer to the Materials and Methods for details. **C:** Kolmogorov-Smirnov test for the expression level of genes among the four conditions (* $D = 0.300$, $P = 0.048$). **D:** Kolmogorov-Smirnov test for the gene size among the four conditions (all of the genes; * $D = 0.226$, $P = 0.038$). **E:** Kolmogorov-Smirnov test for the gene size among the four conditions (only expressed genes; * $D = 0.355$, $P = 0.011$). **F:** Kolmogorov-Smirnov test for the gene size among the four conditions (only unexpressed genes).

Figure 4. Analysis of the amounts of 8-OHdG and acrolein-dA in selected genomic areas by quantitative PCR. **A:** Time course of 8-OHdG levels in wild-type and *OGG1* knockout mice ($n = 3$ to 4, mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$ versus untreated control; * $P < 0.05$, ** $P < 0.01$ versus wild type at the same time point). **B:** Map position, size, and expression level of genes analyzed. Red letters indicate no significant expression with Affymetrix GeneChip Operating Software (GCOS). **C:** Analysis of 8-OHdG amounts. **D:** Analysis of acrolein-dA amounts. Refer to the Materials and Methods and Results sections for details ($n = 3$, mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$ versus untreated control with the same genotype; * $P < 0.05$, ** $P < 0.01$ versus wild type with the same treatment; † $P < 0.05$, †† $P < 0.01$ versus chr10_65 as a standard locus).

the untreated wild-type mice was much lower for acrolein-dA than for 8-OHdG. Furthermore, one-way analysis of variance for comparison between multiple genomic areas revealed statistical significance in seven of eight conditions (8-OHdG: wild-type, untreated control, $P = 0.0046$; wild-type, Fe-NTA 6 hours, $P = 0.00029$; OGG1 knockout, untreated control, $P = 0.00019$; OGG1 knockout, Fe-NTA 6 hours, $P = 1.6 \times 10^{-10}$; acrolein-dA: wild-type, untreated control, $P = 0.0030$; wild-type, Fe-NTA 6 hours, $P = 0.11$; OGG1 knockout, untreated control, $P = 3.3 \times 10^{-5}$; OGG1 knockout, Fe-NTA 6 hours, $P = 1.3 \times 10^{-11}$), demonstrating the existence of fragile sites against oxidative stress in the genome.

We observed a trend ($0.05 < P < 0.1$), only in the case of wild-type mice, that genomic areas low in GC content were more likely to harbor 8-OHdG or acrolein-dA (Figure 5, A–D). In fact, combined analyses of the control and Fe-NTA treated groups of wild-type mice presented a statistically significant correlation ($P = 0.036$ for 8-OHdG; $P = 0.024$ for acrolein-dA).

Discussion

The present study shows, for the first time, the existence of fragile sites against oxidative stress in the genome. As far as we know, no attempts have been made to detect fragile sites with respect to oxidative stress in the whole genome, although there have been a few studies on the relative vulnerability of certain DNA bases within a single gene.^{33,34} We previously hypothesized the presence of fragile sites against oxidative stress in the genome,¹⁴ and to test this hypothesis we developed a novel method to collect DNA fragments containing oxidatively modified DNA bases.

The antibodies used successfully immunoprecipitated DNA fragments containing each modified base in a dose-dependent manner (Figure 1, E and F). The strong affinity of these antibodies probably depends on the fact that clone N45.1 recognizes not only the C-8 hydroxyl moiety of 8-OHdG but also the 2'-deoxy portion of dG¹⁷ and that acrolein-dA is a relatively bulky product.¹⁵ During the procedures, ample precautions, such as the use of the Nal method and argon gas for DNA extraction, were taken to avoid artifactual DNA oxidation. The fraction obtained by DnaIP was $\sim 0.1\%$ of the loaded DNA, which was close to the theoretical value. The obtained fraction reached a plateau in the presence of an excess of loading DNA or 8-OHdG content. However, this could be overcome by adjusting the system within the proportional range.

This analysis was made possible by the completion of the mouse genome project.³⁵ When DNA information is considered to be continuous starting from the centromere of chromosome 1 and continuing to the telomere of the chromosome X, there was no significant deviation in the distribution of 8-OHdG or acrolein-dA. However, when the chromosomal restrictions were considered, significant deviations were detected (Tables 2 and 3). The chromosomal units in the nuclei could at least partly explain the results. Therefore, we conclude that the dis-

tribution of 8-OHdG or acrolein-dA is not random in the genome.

Our results further suggest the importance of a concept called chromosome territories, which posits specific localization of chromosomal areas during interphase.^{36,37} The increase of acrolein-dA in chromosome 15 after oxidative stress may be explained by the fact that chromosome 15 is located close to the nuclear border (Figure 2, C and D). Acrolein, a lipid peroxidation product and a possible second messenger after oxidative stress,¹⁶ may travel for a limited distance leading to modification of genomic DNA located near the nuclear membrane. In contrast, 8-OHdG generation occurs in the proximity of genomic DNA in the presence of catalytic transition metals and is regulated by H_2O_2 or other reducing agents. The reaction is extremely fast.¹ It is possible that the accumulation of 8-OHdG in the centrally located chromosome 16 under control condition is attributable to the relatively open chromatin structure and the inability of OGG1 repair enzyme to arrive frequently. Thus, the genomic distribution of the two oxidatively modified DNA bases is distinct and contrasting.

The fraction of DNA fragments that hit RefSeq gene areas was comparable with the ratio of RefSeq gene to RefSeq nongene areas in the genome, but this was significantly decreased only for acrolein-dA under oxidative stress. Furthermore, acrolein-dA under oxidative stress was observed in genes with larger size and lower expression, whereas 8-OHdG was observed in genes with smaller size and higher expression (Figure 3, C–F). These overall data again suggest a prominent difference in the generation and repair mechanisms between 8-OHdG and acrolein-dA.

In the selected genomic loci, an absence of OGG1 halted further increase in the 8-OHdG-level under oxidative stress (Figure 4C). This is consistent with the possible alteration of chromatin structure leading to a decrease in the expression of β -actin. We also need to consider the presence of a backup repair or antioxidative mechanism in the absence of OGG1. This mechanism appears to be switched on in the presence of high levels of 8-OHdG and accelerated by further oxidative stress, leading to rapid elimination of modified bases (Figure 4A). Thus, the genomic distribution of 8-OHdG in OGG1 knockout mice after Fe-NTA administration might reflect the bias in repair efficiency more strongly than in the control condition (Figure 4C). Notably an absence of OGG1 also influenced the amounts and distribution of acrolein-dA with no further increase 6 hours after Fe-NTA administration (Figure 4D). This may be associated with the protective role of OGG1 protein against modification of genomic DNA by acrolein in consideration of the fact that this enzyme is prone to oxidation.³⁸

There was also a difference in the levels of 8-OHdG and acrolein-dA in the kidney of wild-type mice among the 20 genomic loci studied although it may be too early to deduce certain principles out of these data. The differences in the immunoprecipitation/genome ratio between 8-OHdG and acrolein-dA fragments in the quantitative PCR strategy and the association with genomic GC content (a trend for AT-rich region both in 8-OHdG and

Figure 5. Association in the genomic DNA fragments between the levels of oxidatively modified DNA bases and their GC content. **A, C, E, and G:** Association between 8-OHdG levels and GC content in the genomic DNA fragments for each group (**A**, wild-type, untreated control; **C**, wild-type, Fe-NTA 6 hours; **E**, *OGG1* knockout, untreated control; **G**, *OGG1* knockout, Fe-NTA 6 hours). **B, D, F, and H:** Association between acrolein-dA levels and GC content in the genomic DNA fragments for each group (**B**, wild-type, untreated control; **D**, wild type, Fe-NTA 6 hours; **F**, *OGG1* knockout, untreated control; **H**, *OGG1* knockout, Fe-NTA 6 hours). *y* Axes indicate the results of the quantitative PCR analyses that in this figure are not adjusted by the amounts of immunoprecipitated DNA fragments. Refer to the Materials and Methods section for details.

acrolein-dA) are to be explained in future works. These results warrant further experiments analyzing local patterns of DNA base modifications and mapping analyses of *OGG1* knockout as well as wild-type mice, which would clarify the contribution of formation and repair of oxidatively modified DNA bases.

DnaIP is a versatile technique comparable with chromatin immunoprecipitation. It can be applied to any base modification or other kinds of DNA damage if specific antibodies appropriate for immunoprecipitation are available. Especially, this technique can be used not only for *in vitro* fine experiments but also for *in vivo* animal experiments regardless of the species used. Additional flexibility is obtained by combining this technique with quantitative PCR or genome chip analyses. There are a few drawbacks with this method: identification of the hot spot sequences with shorter size than those that can be amplified by PCR or hybridized to gene chips is not possible, and plus/minus strands are not differentiated, so after genome-wide screening with this method other techniques may be necessary to accomplish finer evaluation.

Although oxidative DNA base modifications have been studied for more than 2 decades, the results have been shown as mere fractional values, and genome-wide information has not been integrated with them. The levels of oxidatively modified DNA bases are the sum of their generation and repair in the genome. Theoretically, with the present method we can evaluate where in the genome certain base modifications have occurred or been repaired at a single-cell level. This research area in close association with the postgenome era has just begun.³⁹ The current study with the two probe modifications produced novel observations that probably reflect three-dimensional and functional aspects of the genome. We believe that this method would greatly contribute to the understanding of pathology associated with oxidative stress.

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References

- Halliwell B, Gutteridge JMC: Free Radicals in Biology and Medicine. Oxford, Clarendon Press, 1999
- Steenken S: Purine bases, nucleosides, and nucleotides: aqueous solution redox chemistry and transformation reactions of their radical reactions and e^- and $\cdot OH$ adducts. *Chem Rev* 1989, 89:503–520
- Bernstein C: Sex as a response to oxidative DNA damage. *DNA and Free Radicals*. Edited by B Halliwell, OI Aruoma. Chichester, Ellis Horwood, 1993, pp 193–210
- Kasai H, Nishimura S: Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res* 1984, 12:2137–2145
- Kasai H: Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res* 1997, 387:147–163
- Tudek B: Imidazole ring-opened DNA purines and their biological significance. *J Biochem Mol Biol (Korea)* 2003, 36:12–19
- Shibutani S, Takeshita M, Grollman AP: Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* 1991, 349:431–434
- Al-Tassan N, Chmiel N, Maynard J, Fleming N, Livingston A, Williams G, Hodges A, Davies D, David S, Sampson J, Cheadle J: Inherited variants of MYH associated with somatic G:C→T:A mutations in colorectal tumors. *Nat Genet* 2002, 30:227–232
- Sieber O, Lipton L, Crabtree M, Heinimann K, Fidalgo P, Phillips R, Bisgaard M, Orntoft T, Aaltonen L, Hodgson S, Thomas H, Tomlinson I: Multiple colorectal adenomas, classic adenomatous polyposis, and germ-line mutations in MYH. *N Engl J Med* 2003, 348:791–799
- Tsuzuki T, Egashira A, Igarashi H, Iwakuma T, Nakatsuru Y, Tominaga Y, Kawate H, Nakao K, Nakamura K, Ide F, Kura S, Nakabeppu Y, Katsuki M, Ishikawa T, Sekiguchi M: Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase. *Proc Natl Acad Sci USA* 2001, 98:11456–11461
- Sakumi K, Tominaga Y, Furuichi M, Xu P, Tsuzuki T, Sekiguchi M, Nakabeppu Y: Ogg1 knockout-associated lung tumorigenesis and its suppression by Mth1 gene disruption. *Cancer Res* 2003, 63:902–905
- Kunisada M, Sakumi K, Tominaga Y, Budiyo A, Ueda M, Ichihashi M, Nakabeppu Y, Nishigori C: 8-Oxoguanine formation induced by chronic UVB exposure makes Ogg1 knockout mice susceptible to skin carcinogenesis. *Cancer Res* 2005, 65:6006–6010
- Tanaka T, Iwasa Y, Kondo S, Hiai H, Toyokuni S: High incidence of allelic loss on chromosome 5 and inactivation of p15^{INK4B} and p16^{INK4A} tumor suppressor genes in oxystress-induced renal cell carcinoma of rats. *Oncogene* 1999, 18:3793–3797
- Hiroyasu M, Ozeki M, Kohda H, Echizenya M, Tanaka T, Hiai H, Toyokuni S: Specific allelic loss of p16^{INK4A} tumor suppressor gene after weeks of iron-mediated oxidative damage during rat renal carcinogenesis. *Am J Pathol* 2002, 160:419–424
- Kawai Y, Furuhashi A, Toyokuni S, Aratani Y, Uchida K: Formation of acrolein-derived 2'-deoxyadenosine adduct in an iron-induced carcinogenesis model. *J Biol Chem* 2003, 278:50346–50354
- Uchida K, Kanematsu M, Sakai K, Matsuda T, Hattori N, Mizuno Y, Suzuki D, Miyata T, Noguchi N, Niki E, Osawa T: Protein-bound acrolein: potential markers for oxidative stress. *Proc Natl Acad Sci USA* 1998, 95:4882–4887
- Toyokuni S, Tanaka T, Hattori Y, Nishiyama Y, Ochi H, Hiai H, Uchida K, Osawa T: Quantitative immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Lab Invest* 1997, 76:365–374
- Ebina Y, Okada S, Hamazaki S, Ogino F, Li JL, Midorikawa O: Nephrotoxicity and renal cell carcinoma after use of iron- and aluminum-nitrilotriacetate complexes in rats. *J Natl Cancer Inst* 1986, 76:107–113
- Li JL, Okada S, Hamazaki S, Ebina Y, Midorikawa O: Subacute nephrotoxicity and induction of renal cell carcinoma in mice treated with ferric nitrilotriacetate. *Cancer Res* 1987, 47:1867–1869
- Toyokuni S, Okada S, Hamazaki S, Minamiyama Y, Yamada Y, Liang P, Fukunaga Y, Midorikawa O: Combined histochemical and biochemical analysis of sex hormone dependence of ferric nitrilotriacetate-induced renal lipid peroxidation in ddY mice. *Cancer Res* 1990, 50:5574–5580
- Nishiyama Y, Suwa H, Okamoto K, Fukumoto M, Hiai H, Toyokuni S: Low incidence of point mutations in H-, K- and N-ras oncogenes and p53 tumor suppressor gene in renal cell carcinoma and peritoneal mesothelioma of Wistar rats induced by ferric nitrilotriacetate. *Jpn J Cancer Res* 1995, 86:1150–1158
- Okada S: Iron-induced tissue damage and cancer: the role of reactive oxygen free radicals. *Pathol Int* 1996, 46:311–332
- Toyokuni S, Uchida K, Okamoto K, Hattori-Nakakuki Y, Hiai H, Stadtman ER: Formation of 4-hydroxy-2-nonenal-modified proteins in the renal proximal tubules of rats treated with a renal carcinogen, ferric nitrilotriacetate. *Proc Natl Acad Sci USA* 1994, 91:2616–2620
- Kondo S, Toyokuni S, Tanaka T, Hiai H, Onodera H, Kasai H, Imamura

- M: Overexpression of the hOGG1 gene and high 8-hydroxy-2'-deoxyguanosine (8-OHdG) lyase activity in human colorectal carcinoma: regulation mechanism of the 8-OHdG level in DNA. *Clin Cancer Res* 2000, 6:1394-1400
25. Nakae D, Mizumoto Y, Kobayashi E, Noguchi O, Konishi Y: Improved genomic/nuclear DNA extraction for 8-hydroxydeoxyguanosine analysis of small amounts of rat liver tissue. *Cancer Lett* 1995, 97:233-239
26. Schneider JE, Price S, Maidt L, Gutteridge JM, Floyd RA: Methylene blue plus light mediates 8-hydroxy 2'-deoxyguanosine formation in DNA preferentially over strand breakage. *Nucleic Acids Res* 1990, 18:631-635
27. Toyokuni S, Tanaka T, Kawaguchi W, Fang N, Ozeki M, Akatsuka S, Hiai H, Aruoma O, Bahorun T: Effects of the phenolic contents of Mauritian endemic plant extracts on promoter activities of antioxidant enzymes. *Free Radic Res* 2003, 37:1215-1224
28. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*. New York, Cold Spring Harbor Laboratory Press, 2001
29. Lucito R, Nakimura M, West J, Han Y, Chin K, Jensen K, McCombie R, Gray J, Wigler M: Genetic analysis using genomic representations. *Proc Natl Acad Sci USA* 1998, 95:4487-4492
30. Toyokuni S, Iwasa Y, Kondo S, Tanaka T, Ochi H, Hiai H: Intranuclear distribution of 8-hydroxy-2'-deoxyguanosine. An immunocytochemical study. *J Histochem Cytochem* 1999, 47:833-836
31. Stephens MA: Use of the Kolmogorov-Smirnov, Cramer-Von Mises and related statistics without extensive tables. *J Roy Stat Soc B* 1970, 32:115-122
32. Nakabeppu Y, Tsuchimoto D, Furuichi M, Sakumi K: The defense mechanisms in mammalian cells against oxidative damage in nucleic acids and their involvement in the suppression of mutagenesis and cell death. *Free Radic Res* 2004, 38:423-429
33. Nomoto M, Yamaguchi R, Kawamura M, Kohno K, Kasai H: Analysis of 8-hydroxyguanine in rat kidney genomic DNA after administration of a renal carcinogen, ferric nitrilotriacetate. *Carcinogenesis* 1999, 20:837-841
34. Kawanishi S, Hiraku Y, Oikawa S: Mechanism of guanine-specific DNA damage by oxidative stress and its role in carcinogenesis and aging. *Mutat Res* 2001, 488:65-76
35. Gregory S, Sekhon M, Schein J, Zhao S, Osoegawa K, Scott C, Evans R, Burridge P, Cox T, Fox C, Hutton R, Mullenger I, Phillips K, Smith J, Stalker J, Threadgold G, Birney E, Wylie K, Chinwalla A, Wallis J, Hillier L, Carter J, Gaige T, Jaeger S, Kremitzki C, Layman D, Maas J, McGrane R, Mead K, Walker R, Jones S, Smith M, Asano J, Bosdet I, Chan S, Chittaranjan S, Chiu R, Fjell C, Fuhrmann D, Girn N, Gray C, Guin R, Hsiao L, Krzywinski M, Kutsche R, Lee S, Mathewson C, McLeavy C, Messervier S, Ness S, Pandoh P, Prabhu A, Saeedi P, Smailus D, Spence L, Stott J, Taylor S, Terpstra W, Tsai M, Vardy J, Wye N, Yang G, Shatsman S: A physical map of the mouse genome. *Nature* 2002, 418:743-750
36. Cremer T, Cremer C: Chromosome territories, nuclear architecture and gene regulation in mammalian cells. *Nat Rev Genet* 2001, 2:292-301
37. Parada L, McQueen P, Misteli T: Tissue-specific spatial organization of genomes. *Genome Biol* 2004, 5:R44.1-R44.9
38. Jaiswal M, La Russo N, Nishioka N, Nakabeppu Y, Gores G: Human Ogg1, a protein involved in the repair of 8-oxoguanine, is inhibited by nitric oxide. *Cancer Res* 2001, 61:6388-6393
39. Toyokuni S, Akatsuka S: What has been learned from the studies of oxidative stress-induced carcinogenesis: proposal of the concept of oxygenomics. *J Clin Biochem Nutr* 2006, 39:3-10