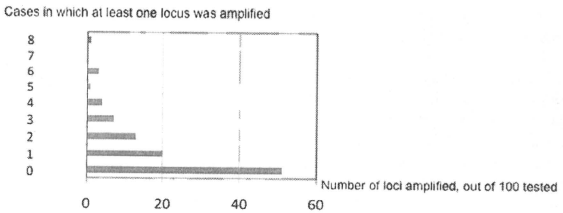


**Figure 4** Distribution of the numbers of the loci amplified in any of the 60 cases (20 gastric cancer cases, 20 lung cancer cases, and 20 colon cancer cases) in a discovery set. More than half (51) of the 100 loci tested were not amplified in any of the 60 cases tested. Five or more loci were amplified in 5 (8%) of the 60 cases tested.

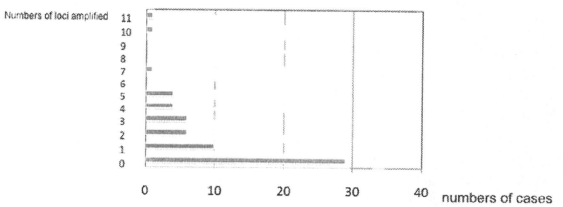


**Figure 5** Distribution of cases according to numbers of loci amplified (vertical axis). From 0 to 11 of the 70 or more (as many as 100) loci successfully tested were amplified. None of the 100 loci were amplified in 29 of the cases. Seven or more loci were amplified in 3 cases.

## WILL FISH BECOME A POPULAR AND ACCEPTED DIAGNOSTIC TOOL IN PATHOLOGY PRACTICE, ESPECIALLY IN GUIDING INDIVIDUAL CANCER THERAPY?

Only a few FISH kits have been authorized for clinical use, but many are available for use in research. Translocation detection kits are often used to confirm diagnoses.[63,64] Mori *et al.* recently used tens of BAC probes to make the differential diagnosis between adrenal tumors.[65] However, the clinical significance of copy number alterations warrants further accumulations of retrospective and prospective data. The rationale for the efficacy of molecularly targeted drugs varies with the mutation, overexpression, and genomic amplification of the target molecules, such as HER2 and EGFR. Fu *et al.* investigated copy number changes and expression of GATA-6 in pancreatic cancer and reported finding consistency between the results for overexpression and amplification of the genomic area of the GATA-6 locus,[66] and they also validated their findings observation by FISH. Amplification itself, however, does not always imply activation of the molecules or pathways of the genes on that genomic locus. Actually the EGFR immunohistological findings in lung cancer cells are not always consistent with the FISH data,[67] and borderline grades of immunostaining of HER2(2+) require FISH analysis to determine whether the HER2 gene has been amplified. Another receptor kinase gene, *MET*, has been evaluated as a potential target of tailor made therapy in the same manner as the *EGFR* gene and *HER2* gene have, and in some studies *MET* amplification has been found to predict shorter patient survival after surgical resection of non-small cell lung cancer.[68] Amplifications of PIK3CA is found in a considerable percentage of non-small cell lung cancers, and it and PIK3CA mutation are mutually exclusive.[69] The list of the amplified segments continues to increase, although validation of their clinical significance awaits further study. The list of tumors in which amplification of certain gene product(s)can be indentified has been growing, meaning that the list of the promising targets of therapy is also growing. Comprehensive copy number analysis by large-scale sequence technology has revealed that a copy number gain of an unexpectedly high proportion of genes that encode kinases in cancers.[11] We tested 100 BAC probes containing different kinase loci in a gastric, colorectal, and lung cancer detection sets (20 cases for each organ) by TMA-FISH technology, and found amplification of at least one kinase gene in a considerable number of cases, or, expressed another way, found that unexpected kinase loci were amplified in a significant proportion of human common solid tumors (Figs 3–5). The discovery blocks we used consisted of tumor tissues in both early and advanced stages, and various histological types. The observation above has also provided us with the following perspectives. Combinatory chemistry has already generated many drugs targeted to kinase genes or their products, thus amplifications of specific sites on certain kinase genes are amenable to pharmacological intervention which that will lead to the establishment of the target specific therapy. When observations like ours are validated and refined for clinical evaluation, the FISH diagnostic system with particular kinase probes may serve as another basis of tailor-made cancer therapy.

Major issues, however, remain to be resolved for before authorization of FISH-based diagnostic tools even if scientifically validated. Cost-benefit analysis of so-called targeted therapy is just starting in the tight-fisted health insurance environment, and there are gloom and doom forecasts that a bonanza of new authorized diagnostic kits is unlikely to arrive anytime soon. The time-line of the last few decades, however, in which many antibodies eventually became essential in pathology labs, evokes us a very different picture.

## CONCLUSIONS

The basic knowledge required to perform the combination of TMA and FISH with many BAC probes is familiar to diagnostic pathologists, but that is different from actually running it (TMA-FISH with BACs) in real pathology practice. Obtaining an ample numbers of BAC probes, labeling, and expensive fluorescence microscopes may be hurdles for modestly equipped community hospitals. In the previous issue of *Pathology International*, Kato *et al.*[70] have reported their experience with using of a commercialized product that applies chromogenic *in situ* hybridization, a friendlier method that allows the use of ordinary microscope.

Many DNA probes labeled 'research use only' are actually used in sarcoma diagnosis,[20] and standardization and quality control of only a few FISH diagnostic systems have been achieved. Most of DNA probes are expensive, and there are few 'generic' diagnostic kits.

Over the coming decades, DNA probes will become a familiar diagnostic tool to the pathologists in community hospitals, and the information obtained by using them will suggest therapeutic guidance as well as the diagnosis. At the same time, the accumulation of the data generated by TMA-FISH approach will complement numerous *OMICS* data that have been accumulating in other disciplines of medicine. In other words, the TMA-FISH approach may be one of the smartest harvest (exit) strategies among *OMICS* projects related to human cancer, and many investments have been made in it over the last two decades.

## ACKNOWLEDGMENTS

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# Detection of Lipid Peroxidation-Induced DNA Adducts Caused by 4-Oxo-2(*E*)-nonenal and 4-Oxo-2(*E*)-hexenal in Human Autopsy Tissues

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DNA adducts are produced both exogenously and endogenously via exposure to various DNA-damaging agents. Two lipid peroxidation (LPO) products, 4-oxo-2(*E*)-nonenal (4-ONE) and 4-oxo-2(*E*)-hexenal (4-OHE), induce substituted etheno-DNA adducts in cells and chemically treated animals, but the adduct levels in humans have never been reported. It is important to investigate the occurrence of 4-ONE- and 4-OHE-derived DNA adducts in humans to further understand their potential impact on human health. In this study, we conducted DNA adductome analysis of several human specimens of pulmonary DNA as well as various LPO-induced DNA adducts in 68 human autopsy tissues, including colon, heart, kidney, liver, lung, pancreas, small intestine, and spleen, by liquid chromatography tandem mass spectrometry. In the adductome analysis, DNA adducts derived from 4-ONE and 4-OHE, namely, heptanone-etheno-2′-deoxycytidine (HεdC), heptanone-etheno-2′-deoxyadenosine (HεdA), and butanone-etheno-2′-deoxycytidine (BεdC), were identified as major adducts in one human pulmonary DNA. Quantitative analysis revealed 4-ONE-derived HεdC, HεdA, and heptanone-etheno-2′-deoxyguanosine (HεdG) to be ubiquitous in various human tissues at median values of 10, 15, and 8.6 adducts per $10^8$ bases, respectively. More importantly, an extremely high level (more than 100 per $10^8$ bases) of these DNA adducts was observed in several cases. The level of 4-OHE-derived BεdC was highly correlated with that of HεdC ($R^2 = 0.94$), although BεdC was present at about a 7-fold lower concentration than HεdC. These results suggest that 4-ONE- and 4-OHE-derived DNA adducts are likely to be significant DNA adducts in human tissues, with potential for deleterious effects on human health.

## Introduction

Lipid peroxidation (LPO[1]) is a major source of DNA-damaging agents. Decomposition products generated from the LPO of polyunsaturated fatty acids (PUFAs) are highly DNA-reactive, including acrolein, crotonaldehyde, malondialdehyde, and other $\alpha,\beta$-unsaturated aldehydes (*1–3*). These electrophilic aldehydes may modify nucleic acid bases to form DNA adducts implicated in mutagenesis, carcinogenesis, accelerated aging, or neurological deterioration (*4–6*). Thus, investigation into the levels and tissue distributions of LPO-derived DNA adducts in humans is important to further understand their possible impact on human health.

LPO-related DNA adducts identified in human tissues are mainly exocyclic etheno and propano adducts such as 1,$N^6$-etheno-2′-deoxyadenosine (εdA); 3,$N^4$-etheno-2′-deoxycytidine (εdC); 1, $N^2$-propano-2′-deoxyguanosines generated from acrolein, crotonaldehyde, and 4-hydroxy-2(*E*)-nonenal (4-HNE); and malondialdehyde-derived 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimido[1.2-α]purin-10(3*H*)-one (*7–9*). The long-chain aldehyde 4-HNE is an ω-6 PUFA-peroxidation product that reacts with DNA and protein (*10–12*); furthermore, 4-HNE-related DNA adducts have been reported to be associated with carcinogenesis and Alzheimer's disease (*13–15*). 4-Oxo-2(*E*)-nonenal (4-ONE), another decomposition product of ω-6 PUFAs, has also been shown to induce the formation of etheno DNA adducts carrying aliphatic side chains both in cells and in mouse models, including heptanone-etheno-2′-deoxycytidine (HεdC), heptanone-etheno-2′-deoxyguanosine (HεdG), and heptanone-etheno-2′-deoxyadenosine (HεdA) (*16–18*). 4-Oxo-2(*E*)-hexenal (4-OHE), an ω-3 PUFA-peroxidation product having a chemical structure similar to that of 4-ONE, was recently reported to be able to produce etheno DNA adducts as well.

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[1] Abbreviations: PUFA, polyunsaturated fatty acid; LPO, lipid peroxidation; 4-HNE, 4-hydroxy-2(*E*)-nonenal; 4-ONE, 4-oxo-2(*E*)-nonenal; 4-OHE, 4-oxo-2(*E*)-hexenal; HεdC, heptanone-etheno-2′-deoxycytidine; HεdG, heptanone-etheno-2′-deoxyguanosine; HεdA, heptanone-etheno-2′-deoxyadenosine; BεdC, butanone-etheno-2′-deoxycytidine; BεmedC, butanone-etheno-2′-deoxy-5-methylcytidine; BεdG, butanone-etheno-2′-deoxyguanosine; BεdA, butanone-etheno-2′-deoxyadenosine; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; εdA, 1,$N^6$-etheno-2′-deoxyadenosine; 8-OHAdG, 8-hydroxy-1,$N^2$-propanodeoxyguanosine; $CdG_2$, α-*R*-methyl-γ-hydroxy-1,$N^2$-propano-2′-deoxyguanosine;COX-2, cyclooxygenase-2; HPNE, 4-hydroperoxy-2(*E*)-nonenal; EDE, 4,5-epoxy-2(*E*)-decenal; 5-LO, 5-lipoxygenase.

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such as butanone-etheno-2′-deoxycytidine (BεdC), butanone-etheno-2′-deoxy-5-methyl-cytidine (BεmedC), butanone-etheno-2′-deoxyguanosine (BεdG) (*19–21*), and butanone-etheno-2′-deoxyadenosine (BεdA) (*22*). The levels of 4-ONE- and 4-OHE-related DNA adducts in humans are currently unknown because such adducts were discovered only very recently.

In addition to LPO-derived DNA adducts, various other types of DNA lesions are frequently formed in humans as a consequence of exposure to environmental carcinogens or endogenous DNA-reactive agents. Because a variety of DNA adducts are present in human tissues, comprehensive investigation of these base modifications is necessary to identify the ones most critical to mutagenesis and carcinogenesis in humans. Recently, we developed a novel technique to detect multiple known or unknown DNA adducts simultaneously by using LC-MS/MS (*23, 24*). This approach, named the DNA adductome approach, monitors the neutral loss of 2′-deoxyribose from positively ionized 2′-deoxynucleoside adducts over a certain range of transitions. A variety of DNA adducts detected in DNA samples can be presented and compared by creating an adductome map showing LC retention time, mass-to-charge ratio (*m/z*), and relative peak intensity of each potential DNA adduct. In this study, we applied the DNA adductome approach to several human pulmonary DNA specimens and identified major DNA adducts on the adductome maps. Interestingly, 4-ONE- and 4-OHE-related DNA adducts were found to be major adducts in at least one pulmonary DNA sample, and they were also detected in other DNA samples. We also analyzed the levels of 4-ONE- and 4-OHE-related DNA adducts in various organs of different individuals by using LC-MS/MS. The lesions were found to be widely distributed, with some being present in significant amounts, suggesting that they could be important causative factors in human disease.

## Experimental Procedures

**Human Autopsy Tissues.** Human autopsy tissue samples were collected at Hamamatsu University School of Medicine, Japan, and the study design was approved by the Institutional Review Board of Hamamatsu University School of Medicine (18−4). Sixty-eight samples were obtained from organs of 26 deceased persons, including the colon ($n = 6$), liver ($n = 19$), lung ($n = 12$), pancreas ($n = 9$), spleen ($n = 9$), kidney ($n = 9$), heart ($n = 2$), and small intestine ($n = 2$). The samples were taken within 24 h after death and frozen at −80 °C until DNA extraction. The ages of the subjects (17 males and 9 females) ranged from 26 to 90. Seventeen of them had malignancies as backgrounds, and final remarkable circulatory failures (shock, massive hemorrhage, and sepsis) were validated both clinically and pathologically in 6 cases. Detailed properties of the patients are listed in Supporting Information, Table S-1.

**DNA Adduct Standards and Stable Isotope Standards.** 4-ONE- and 4-OHE-related DNA adducts (HεdC, HεdA, HεdG, BεdC, BεmedC, BεdA, and BεdG) were synthesized according to previously published methods (*16–20*). The stereoisomers α-*S*- and α-*R*-methyl-γ-hydroxy-1,$N^2$-propano-2′-deoxyguanosine ($CdG_1$, $CdG_2$), 8-hydroxy-1,$N^2$-propanodeoxyguanosine (8-OH-AdG), and the two stereoisomers of 6-hydroxy-1,$N^2$-propanodeoxyguanosine ($6\text{-OH-AdG}_1$ and $6\text{-OH-AdG}_2$) were prepared as previously described (*24*). 8-OxodG and εdA were obtained from Sigma Aldrich Japan (Japan). [U-$^{15}N_5$]-8-oxodG was kindly provided by Dr. Shinya Shibutani, State University of New York, Stony Brook, NY, [$^{15}N_5$, $^{13}C_{10}$]-2-(2′-deoxyguanosine-8-yl)-3-aminobenzanthrone ([$^{15}N_5$, $^{13}C_{10}$]-C8-C2-ABA) was kindly provided by Dr. Takeji Takamura, Kanagawa Institute of Technology, Japan, and other DNA adduct stable isotope standards were synthesized according to previously described methods using [U-$^{15}N_5$]- or [U-$^{15}N_3$]-deoxynucleosides purchased from Cambridge Isotope Laboratories (Andover, MA).

**DNA Purification and Hydrolysis.** Genomic DNA was isolated and purified from human autopsy samples by using a Gentra Puregene Tissue Kit (QIAGEN, Valencia, CA). DNA extraction was undertaken according to the protocol provided by the manufacturer, with the addition of desferroxamine to all solutions to a final concentration of 0.1 mM.

For DNA adductome analysis, isolated DNA was enzymatically digested as follows: each DNA sample (100 μg) was mixed with 54 μL of digestion buffer (17 mM sodium succinate and 8 mM calcium chloride, pH 6.0) containing 67.5 units of micrococcal nuclease (Worthington, Lakewood, NJ) and 0.255 units of spleen phosphodiesterase (Worthington, Lakewood, NJ). After 3 h of incubation at 37 °C, 3 units of alkaline phosphatase (Sigma-Aldrich, St. Louis, MO), 30 μL of 0.5 M Tris-HCl (pH 8.5), 15 μL of 20 mM zinc sulfate, and 101 μL of Milli-Q water were added, and the mixture were incubated for another 3 h at 37 °C. After this incubation, the mixture was concentrated to 10−20 μL by a Speed-Vac concentrator, and 100 μL of methanol was added to precipitate the protein. After centrifugation, the methanol fraction (supernatant) was transferred to a new Eppendorf tube. The precipitate was extracted with 100 μL of methanol, and the methanol fractions were combined and evaporated to dryness.

For adduct quantification analysis, the DNA sample (50 μg) was mixed with 18 μL of digestion buffer (17 mM sodium succinate and 8 mM calcium chloride, pH 6.0) containing 22.5 units of micrococcal nuclease (Worthington, Lakewood, NJ) and 0.075 units of spleen phosphodiesterase (Worthington, Lakewood, NJ) and 10 units of stable isotope-labeled DNA adduct internal standards mix, including 27.8 nM [U-$^{15}N_5$]-8-oxodG, and 1.1 nM [U-$^{15}N_5$]-εdA, [U-$^{15}N_5$]-$CdG_1$, [U-$^{15}N_5$]-$CdG_2$, [U-$^{15}N_5$]-8-OH-AdG, [U-$^{15}N_3$]-HεdC, [U-$^{15}N_5$]-HεdA, [U-$^{15}N_5$]-HεdG, [U-$^{15}N_3$]-BεdC, and [U-$^{15}N_5$]-BεdA. After 3 h of incubation at 37 °C, 3 units of alkaline phosphatase (Sigma-Aldrich, St. Louis, MO), 10 μL of 0.5 M Tris-HCl (pH 8.5), 5 μL of 20 mM zinc sulfate, and 67 μL of Milli-Q water were added, and the mixture were incubated for another 3 h at 37 °C. After this incubation, the mixture was concentrated to 10−20 μL by a Speed-Vac concentrator, and 100 μL of methanol was added to precipitate the protein. After centrifugation, the methanol fraction (supernatant) was transferred to a new Eppendorf tube. The precipitate was extracted with 100 μL of methanol, and the methanol fractions were combined and evaporated to dryness.

**DNA Adductome Analysis.** Digested DNA used for adductome analysis was redissolved in 120 μL of 30% dimethyl sulfoxide containing 23 nM [$^{15}N_5$, $^{13}C_{10}$]-C8-C2-ABA as the internal standard and then subjected to DNA adductome analysis similar to that described by Kanaly et al. (*24*). Briefly, adductome analysis was carried out using a Quattro Ultima Pt triple stage quadrupole mass spectrometer (Waters-Micromass, Milford, MA) equipped with a Shimadzu LC system (Shimadzu, Japan). An aliquot of digested DNA sample (10 μL) was injected and separated by a Shim-pack XR-ODS column (3.0 mm × 75 mm, Shimadzu, Japan). The column was eluted in a linear gradient of 5% to 80% methanol in water from 0 to 40 min and kept in 80% methanol in water from 40 to 45 min at a flow rate of 0.2 mL/min. Multi-reaction monitoring (MRM) was performed in positive ion mode using nitrogen as the nebulizing gas. Experimental conditions were set as follows: ion source temperature, 130 °C; desolvation temperature, 380 °C; cone voltage, 35 V; collision energy, 15 eV; desolvation gas flow rate, 700 L/h; cone gas flow rate, 35 L/h; collision gas, argon. The strategy was designed to detect the neutral loss of 2′-deoxyribose from positively ionized 2′-deoxynucleoside adducts by monitoring the samples transmitting their $[M + H]^+ \rightarrow [M + H - 116]^+$ transitions. For each DNA sample, 241 MRM transitions were monitored over the *m/z* range from transition *m/z* 250 → 134 to transition 492 → 376. For each sample injection, a total of 31 channels were monitored simultaneously with one channel for each injection reserved to monitor the internal standard [$^{15}N_5$, $^{13}C_{10}$]-C8-C2-ABA at transition *m/z* 526 → 405. Each sample was injected 8 times to complete the monitoring of 241 MRM transitions. Transitions of normal deoxynucleosides, including 252 → 136 ([dA

+ H]$^+$) and 268 → 152 ([dG + H]$^+$), were not monitored in the adductome analysis.

Relative peak intensity of each potential DNA adduct was calculated as follows: (the peak area of the potential DNA adduct)/(the peak area of the internal standard)/(the amount of 2′-deoxyguanosine (dG)). The amount of dG in each DNA sample was estimated by monitoring the dG peak area at 254 nm using the Shimazu SPD-10Avp UV−visible detector connected in series with the LC/MS/MS. The relative peak intensity was plotted as a bubble chart in which the horizontal axis was retention time and the vertical axis is *m/z*. Sodium and potassium adducts of normal deoxynucleosides or other corresponding peaks, such as those detected in the retention times of 9.3−9.5 min (dC), 10.2−10.4 min (dG), 11.2−11.4 min (dT), and 14.0−14.2 min (dA), were not included in the plot.

**DNA Adduct Quantification.** The digested DNA sample used for quantification was resuspended in 50 μL of 30% dimethyl sulfoxide before LC-MS/MS analysis. An aliquot (20 μL) was injected and separated by the Shim-pack XR-ODS column, eluted in a linear gradient of 5% to 30% methanol in water from 0 to 27 min, 30% to 80% from 27 to 35 min, then kept in 80% from 35 to 40 min at a flow rate of 0.2 mL/min. For the quantification of 4-ONE-derived DNA adducts, HεdC, HεdG, and HεdA, another HPLC-gradient condition was employed because of the high hydrophobicity of these compounds. A remaining aliquot (20 μL) was injected and separated by the same column, eluted in a linear gradient of 45% to 90% methanol in water from 0 to 20 min at a flow rate of 0.2 mL/min. Experimental conditions were identical to those set for adductome analysis except that the cone voltage and collision energy were different for different DNA adducts. The collision energies and characteristic reactions monitored for the different DNA adducts are as follows (cone voltage (V), collision energy (eV), base ionS → product ion): [U-$^{15}N_5$]-8-oxodG (40, 12, 288.8 → 172.8), [U-$^{15}N_5$]-εdA (35, 14, 280.9 → 164.9), [U-$^{15}N_5$]-$CdG_1$ and [U-$^{15}N_5$]-$CdG_2$ (35, 10, 343.0 → 227.0), [U-$^{15}N_5$]-8-OH-AdG (35, 10, 329.3 → 213.3), [U-$^{15}N_3$]-HεdC (35, 10, 367.0 → 251.0), [U-$^{15}N_5$]-HεdA (35, 10, 393.0 → 277.0), [U-$^{15}N_5$]-HεdG (35, 10, 409.0 → 293.0), [U-$^{15}N_3$]-BεdC (35, 10, 324.8 → 208.6), and [U-$^{15}N_5$]-BεdA (35, 10, 351.0 → 234.8), 8-oxodG (40, 12, 283.8 → 167.8), εdA (35, 14, 275.9 → 159.9), $CdG_1$ and $CdG_2$ (35, 10, 338.0 → 222.0), 8-OH-AdG, 6-OH-$AdG_1$, and 6-OH-$AdG_2$ (35, 10, 324.3 → 208.3), HεdC (35, 10, 364.0 → 248.0), HεdA (35, 10, 388.0 → 272.0), HεdG (35, 10, 404.0 → 288.0), BεdC (35, 10, 321.8 → 205.6), BεmedC (35, 20, 335.9 → 220.0), and BεdA (35, 10, 351.0 → 234.8) and BεdG (35, 20, 362.0 → 245.9).

The amount of each DNA adduct was quantified by calculating the peak area ratio of the target DNA adduct and its specific internal standard ([U-$^{15}N_3$]-BεdC was used for BεdC and BεmedC, and [U-$^{15}N_5$]-BεdA was used for BεdA and BεdG). Calibration curves were obtained by using authentic standards spiked with isotope internal standards. The concentration of dG in each DNA sample was also monitored as described in the DNA Adductome Analysis section. The number of DNA adducts per $10^8$ bases was calculated by the following equation: number of DNA adducts per $10^8$ bases = adduct level (fmol/μmol dG) × 0.218(μmol dG/μmol dN) × $10^{-1}$, as described previously (25).

## Results

**Adductome Analysis of DNA Extracted from Human Lung Autopsy Tissues.** We applied adductome analysis to DNA extracted from four human lung autopsy samples to simultaneously detect a variety of known and unknown DNA adducts in human pulmonary DNA. Although adductome analysis is semiquantitative, this analysis would help to grasp a complete picture of the DNA adducts in human samples. Several peaks were identified as corresponding to known DNA adducts by showing identical *m/z* and LC retention times to DNA adduct standards. Figure 1 shows the adductome maps of two human pulmonary DNA samples having different patterns of DNA adduct composition. Numerous DNA adducts can be seen in Figure 1A, and LPO-induced DNA adducts were detected as major peaks, including HεdC, HεdA, BεdC, BεdA, BεmedC, BεdG, εdA, and 1,$N^2$-glyoxal-dG. Although fewer DNA adducts were found in the sample represented in Figure 1B, LPO-induced DNA adducts derived from 4-ONE and 4-OHE (i.e., HεdC, HεdA, and BεdC) were nonetheless detected. Adductome maps of two other human pulmonary DNA samples have patterns similar to that shown in Figure 1B (data not shown),

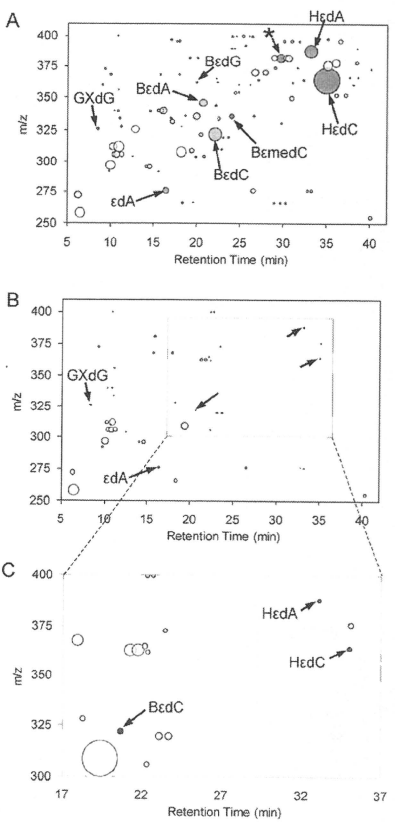


**Figure 1.** A and B show the DNA adductome maps of two human pulmonary DNA samples from different individuals, and C is a close-up of a selected area in B. Each circle represents one DNA adduct candidate detected by adductome analysis using LC-MS/MS. HPLC retention time, mass to charge ratio (*m/z*), and relative intensity (shown by the size of each circle, which is proportional to the peak area of each DNA adduct candidate divided by the peak area of the internal standard and the amount of 2′-deoxyguanosine) of each DNA adduct candidate can be found on the maps. Blue circles represent corresponding peaks of 4-ONE-related DNA adducts, while orange circles represent 4-OHE-related DNA adducts. Green circles are the other lipid-peroxidation derived DNA adducts, and yellow circles represent unidentified peaks. GXdG: 1,$N^2$-glyoxyal-dG. *: heptanone-ethano-2′-deoxycytidine.

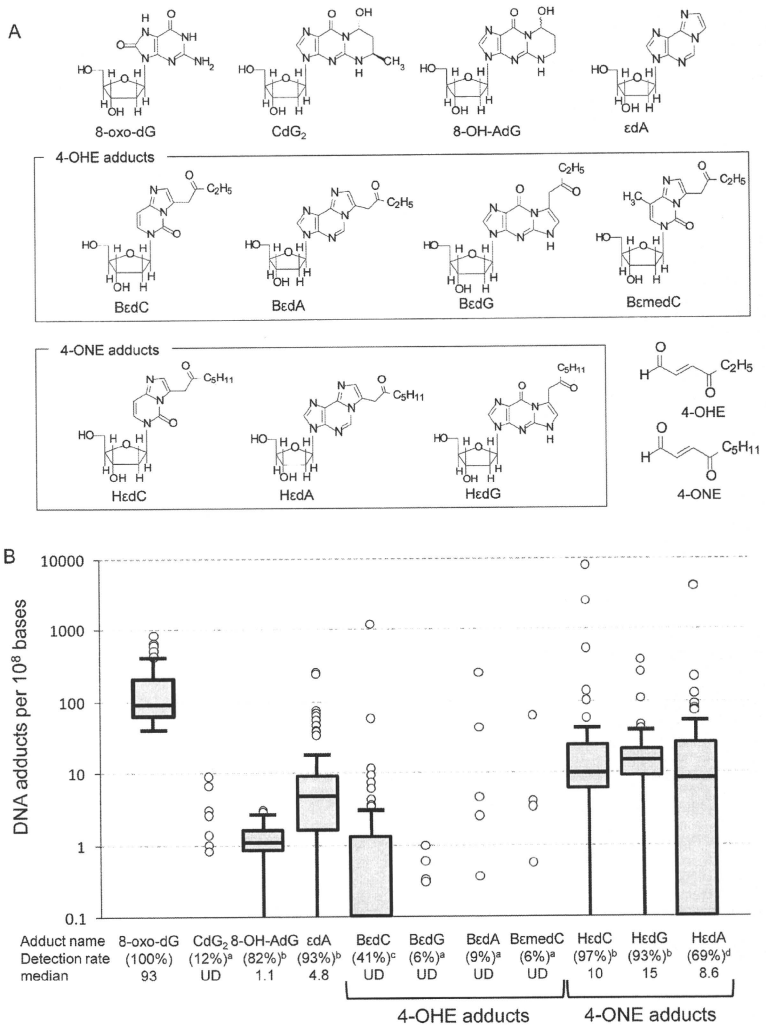


**Figure 2.** Level of LPO-induced DNA adducts in human tissues. (A) Chemical structure of DNA adducts detected in human tissues and the chemical structure of 4-OHE and 4-ONE. (B) Box-whisker plot of the levels of DNA adducts detected in human autopsy tissues, including the colon, liver, lung, pancreas, spleen, kidney, heart, and small intestine ($n = 68$). The boxes indicate the 75th percentile, the median, and the 25th percentile. The ends of the whiskers indicate the minimum and maximum data values unless outliers are present, in which case the whiskers extend to a maximum of 1.5 times the interquartile range. Circles above the whisker indicate outliers. Although crotonaldehyde-induced $CdG_1$ and acrolein-induced 6-OH-$AdG_1$ and 6-OH-$AdG_2$ were also monitored, we could not detect those adducts. Detected rate and median are shown under each DNA adduct. UD: under the detection limit. a, 75th percentile was UD; b, minimum was UD; c, median was UD; d, 25th percentile was UD.

indicating that DNA adducts induced by 4-ONE and 4-OHE are often formed in human lungs.

**Detection of 4-ONE- and 4-OHE-Induced DNA Adducts in Human Autopsy Tissues.** To elucidate whether the levels of

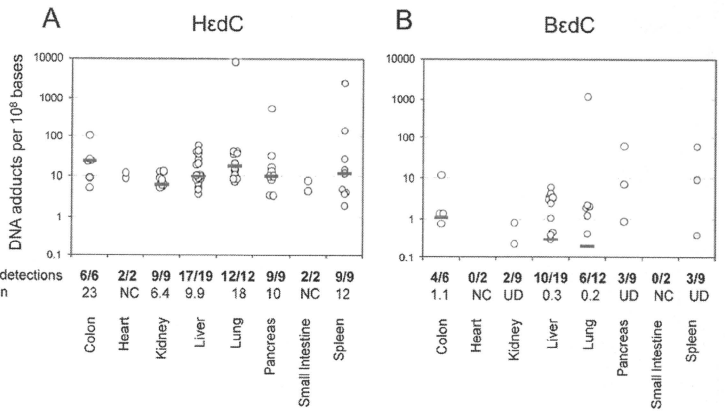


**Figure 3.** DNA adduct levels of HεdC and BεdC detected in various human autopsy tissues. Data from the DNA of 6 colons, 2 hearts, 9 kidneys, 19 livers, 12 lungs, 9 pancreases, 2 small intestines, and 9 spleens were plotted as circles, and the blue bars indicate the median values. NC: not calculated because the sample number was only 2. UD: median was under the detection limit.

4-ONE- and 4-OHE-related DNA adducts are comparable to those of other DNA adducts frequently found in human tissues, we measured the levels of various DNA adducts by using LC-MS/MS in 68 human autopsy specimens obtained from 26 persons, including samples of colon ($n = 6$), liver ($n = 19$), lung ($n = 12$), pancreas ($n = 9$), spleen ($n = 9$), kidney ($n = 9$), heart ($n = 2$), and small intestine ($n = 2$). The approximate detection limit of the DNA adducts (in the case that 50 $\mu$g of DNA was digested and 40% of the portion was injected to the LC/MS/MS) were as follows: 8-oxodG (1.65 adduct per $10^8$ bases), εdA (0.17), $CdG_1$ and $CdG_2$ (0.17), 8-OH-AdG (0.05), 6-OH-$AdG_1$ and 6-OH-$AdG_2$ (0.08), HεdC (0.33), HεdA (1.65), HεdG (1.65), BεdC (0.17), BεmedC (0.17), and BεdA (0.83) and BεdG (0.83) (Supporting Information Figures S-2 and S-3, Table S-8), and the calibration curves of each DNA adducts are shown in Supporting Information, Figure S-4. We could detect the target DNA adducts in several human tissue samples (the representative chromatographs are shown in Supporting Information, Figures S-5, S-6, and S-7). The results revealed that the levels of target DNA adducts varied considerably among individuals or organs (Figure 2 and Supporting Information, Table S-8). Figure 2 shows the DNA adduct levels of the oxidative lesion 8-oxodG as well as the LPO-related lesions $CdG_2$, 8-OH-AdG, εdA, BεdC, BεdG, BεdA, BεmedC, HεdC, HεdG, and HεdA. 8-OxodG was detected in all autopsy tissues, and high detection rates were also found for εdA (93%) and 8-OH-AdG (82%). 4-ONE-related DNA adducts were also frequently detected in various tissue samples: total detection rates for HεdC, HεdG, and HεdA were 97%, 93%, and 63%, respectively. 4-OHE-related BεdC, having a total detection rate of 41%, was commonly found in the colon, liver, and lung, with detection rates higher than 50%. However, the other 4-OHE-related adducts, BεdG, BεdA, and BεmedC, showed lower detection rates of 6%, 9%, and 6%, respectively. The detection rate of the crotonaldehyde-derived DNA adduct $CdG_2$ was 12%. Although crotonaldehyde-induced $CdG_1$ and acrolein-induced 6-OH-$AdG_1$ and 6-OH-$AdG_2$ were also monitored, we could not detect those adducts in any sample. The level of each DNA adduct per $10^8$ bases ranged as follows: 8-oxo-dG, 41.6−837 (median 93.2); $CdG_2$, not detected (ND) to 8.98 (median was under the detection limit); 8-OH-AdG, ND to 3.04 (median 1.14); εdA, ND to 259 (median 4.83); BεdC, ND to 1186 (median was under the detection limit); BεdG, ND to 0.99 (median was under the detection limit; BεdA, ND to 254 (median was under the detection limit); BεmedC, ND to 63.8 (median was under the detection limit); HεdC, ND-8204 (median 10.3); HεdG, ND to 377 (median 15.0); and HεdA, ND to 4186 (median 8.63).

**Adduct Levels of HεdC and BεdC in Different Organs.** As shown in Figure 3, DNA adduct levels of HεdC and BεdC range broadly in different organs. HεdC was detected in all tissue samples except for two liver specimens, whereas BεdC was detected in the colon, kidney, liver, lung, spleen, and pancreas. The median level of HεdC in different organs ranged from 6.4 (kidney) to 23 (colon) adducts per $10^8$ bases, whereas the median of BεdC was 1 or 2 orders of magnitude lower. However, an extremely high level of HεdC (more than 100 adducts per $10^8$ bases) was found in one colon, one lung, one pancreas and two spleen DNA samples, all from different individuals. Also, an extremely high level of BεdC was observed in one lung DNA sample, the same one that showed a high HεdC level as described above. The results suggest that 4-ONE- and 4-OHE-related DNA adducts are widely distributed in various tissues.

Figure 4 shows the correlations of BεdC, εdA, and 8-oxodG with HεdC in human tissue autopsy samples. The DNA adduct level of HεdC was strongly correlated to LPO-induced BεdC ($R^2 = 0.94$) and εdA ($R^2 = 0.70$), but no correlation could be seen between HεdC and the oxidative damage-related lesion 8-oxodG ($R^2 = 0.02$).

## Discussion

In this study, we clearly demonstrated that DNA adducts derived from 4-ONE and 4-OHE occur commonly in human tissues. The levels of the 4-ONE-related DNA adducts HεdC, HεdA, and HεdG in human tissue samples were similar to each other (Supporting Information, Figure S-9), and their median values were 2- to 3-fold higher than that of εdA. However, the 4-OHE-related adducts BεdC, BεmedC, BεdA, and BεdG were detected at lower levels and frequencies; in most samples, their

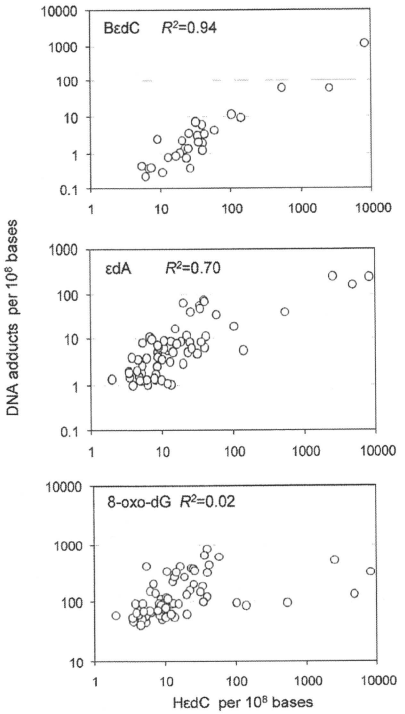


**Figure 4.** Correlations among DNA adduct levels of HεdC vs BεdC, εdA, and 8-oxodG. $R^2$: coefficient of determination. For the $R^2$ calculation, not detected data was treated as 0.

levels were similar to that of crotonaldehyde-derived $CdG_3$ or acrolein-derived 8-OH-AdG. Importantly, in some cases, the levels of these 4-ONE- and 4-OHE-derived DNA adducts were comparable to or even higher than that of the most abundant DNA adduct, 8-oxo-dG. Thus, these recently recognized DNA adducts may be an important source of somatic mutations and could significantly contribute to cancer formation in humans.

The tissues adjacent to those taken for adductome analysis were microscopically examined for the absence of tumor cells. The histological findings varied in terms of inflammation, not otherwise specified. Details of histological characteristics and their relationship to the DNA adducts level are under investigation.

Mutagenic properties of HεdC have been demonstrated in mammalian cell lines and *Escherichia coli* (*26, 27*). Pollack et al. (*26*) reported that in human cell lines HεdC blocked DNA synthesis and also miscoded markedly during the replication of a shuttle vector site-specifically modified with HεdC. The miscoding frequency was higher than 90%, and dT and dA were preferentially inserted opposite the lesion in human cells. HεdC was also shown to be genotoxic in a similar host-vector system consisting of mouse fibroblasts and a replicating plasmid bearing a site-specific HεdC (*25*). Moreover, the results indicated that the Y family DNA polymerases $\eta$, $\kappa$, and $\iota$ preferentially catalyzed the insertion of dT opposite HεdC, whereas an unidentified DNA polymerase was suggested to catalyze the insertion of dA opposite HεdC (*27*). Information about the potential mutagenic properties of the other 4-ONE- and 4-OHE-derived DNA adducts found in human autopsy tissues is still unavailable; thus, further studies concerning the mutagenicity and DNA repair pathways of these newly identified DNA adducts are necessary.

Human tissues could be exposed to 4-ONE and 4-OHE endogenously and exogenously. The endogenous formation of 4-ONE and 4-OHE is via the oxidation of $\omega$6- and $\omega$3-PUFAs in tissues. Because all bodily tissues contain both $\omega$6- and $\omega$3-PUFAs, 4-ONE and 4-OHE could be produced simultaneously under oxidative stress conditions. The near-perfect correlation between the levels of HεdC and BεdC ($R^2$ = 0.94) shown in Figure 4 strongly suggests that there is endogenous and simultaneous formation of 4-ONE- and 4-OHE-derived DNA adducts. According to the slope of the regression curve, the level of HεdC was about 7 times greater than that of BεdC. This also supports the endogenous-formation hypothesis because in all tissues except the brain, the total concentration of $\omega$6- PUFAs is several times higher than that of $\omega$3-PUFAs (*28, 29*).

However, no correlation was observed between the level of HεdC and the level of the oxidative DNA lesion 8-oxo-dG (Figure 4). This discrepancy may be explained by the contribution of enzymatic formation pathways to 4-ONE. For example, Blair's group demonstrated that overexpression of cyclooxygenase-2 (COX-2) increased the level of 4-ONE-derived DNA adducts in both rat intestinal epithelial cells (*30*) and the small intestine of C57BL/6J $APC^{min}$ mice (*31*). COX-2 is an enzyme that is responsible for the formation of the important biological mediator prostaglandin H2. COX-2 can also convert arachidonic acid into 15(*S*)-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-HPETE), which undergoes homolytic decomposition to the DNA-reactive bifunctional electrophiles 4-hydroperoxy-2(*E*)-nonenal (HPNE), 4,5-epoxy-2(*E*)-decenal (EDE), 4-HNE, and 4-ONE (*31*). 4-ONE is also produced enzymatically from arachidonic acid by the 5-lipoxygenase (5-LO)-related pathway (*32*). 5-LO is an enzyme that is responsible for the formation of leukotriene $A_4$. The precursor of leukotriene $A_4$, 5(*S*)-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5(*S*)-HPETE), generated from arachidonic acid by 5-LO, decomposes to form 4-ONE and HPNE (*32*). The considerably good correlation between the DNA adduct levels of HεdC and εdA, as described in Figure 4 ($R^2$ = 0.70), also suggests the involvement of this metabolic pathway, because εdA is known to be produced by HPNE and EDE (*31*). If 4-OHE is also produced enzymatically from abundant $\omega$3-PUFAs such as docosahexaenoic acid, this would help to explain why the level of BεdC nearly perfectly correlates with the level of HεdC but the level of 8-oxo-dG does not. Further study is needed to elucidate this point.

The exogenous sources of 4-ONE and 4-OHE are foods and cooking vapor. Kasai and Kawai reported that several types of cooked fishes and cooking oils contain 4-OHE in the range of a few to tens of micrograms per gram (*21*). They further reported that the cooking vapor emitted during fish broiling also contains 4-OHE (*21*). In an animal experiment, orally administered 4-OHE resulted in the formation of BεdC, BεdG, and BεmedC in cells of the gastrointestinal tract, but no increase in the level of DNA adducts was observed in the liver and kidney (*19*), indicating that, except for the gastrointestinal tract, the oral route is probably not a significant source of 4-OHE. However, the

impact of cooking vapor in terms of the formation of DNA adducts in pulmonary tissues remains to be resolved.

In conclusion, DNA adducts caused by 4-ONE and 4-OHE are ubiquitous in various human tissues, and even predominant in some cases. It is very likely that these DNA adducts cause somatic mutations and cancers, contribute to aging, and have other adverse effects related to DNA damage. Further studies of their exposure routes and biological properties should be carried out to elucidate the impact of these DNA lesions on human health.

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**Supporting Information Available:** Properties of the patients; sensitivity of LC/MS/MS analysis for each DNA adduct (1 and 2); calibration curves of each DNA adduct; representative chromatographs of DNA adducts, 4-OHE-derived DNA adducts, and 4-ONE-derived DNA adducts in human spleen DNA; DNA adducts level in human tissues; and correlations among the 4-ONE-derived DNA adduct level of HedC vs HedA (A) and HedG (B). This material is available free of charge via the Internet at http://pubs.acs.org.

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ORIGINAL ARTICLE

# Association between neuropeptide Y receptor 2 polymorphism and the smoking behavior of elderly Japanese

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**Molecular heterogeneity of neuropeptide Y (NPY) and its three receptors (1, 2 and 5) has recently been discovered. *NPY2R* polymorphisms have been shown to be related to cocaine and alcohol dependence in European Americans. To test our hypothesis that these polymorphisms influence the smoking behavior of Japanese population, we investigated the prevalence of the rs4425326 and rs6857715 polymorphisms, which have been suggested to be related to alcohol dependence in European Americans, in 2517 Japanese elderly subjects for whom information on smoking behaviors was available. The prevalence of current smokers was greater among Japanese men having the rs4425326 C allele than ex-smokers. Among the ever-smokers, the Fagerström Test for Nicotine Dependence scores were higher in men having the rs4425326 homozygous T allelotype, and the numbers of cigarettes smoked per day were also significantly higher in the male smokers having the TT genotype. No correlations between the Tobacco Dependence Screener scores and any genotypes were detected. These results suggest that rs4425326 polymorphisms may be related to smoking behavior in the Japanese elderly population. This study for the first time suggests NPY2R genotype as a possible genetic factor in nicotine dependence.**
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**Keywords:** addiction; Fagerström Test for Nicotine Dependence (FTND); neuropeptide Y (NPY); *NPY2R*; single nucleotide polymorphism; smoking behavior; Tobacco Dependence Screener (TDS); nicotine dependence

## INTRODUCTION

Neuropeptide Y (NPY) is a neuromodulator in the leptin–melanocortin axis. There are three types of receptors of NPY receptors (NPYRs) in humans: NPY receptor type 1 (NPY1R), NPY receptor type 2 (NPY2R) and NPY receptor type 5 (NPY5R). They are all G-protein-coupled receptors having a 7-transmembrane domain,[1] and the genes that encode them are located on chromosome 4.

The physiological and pathological functions of NPY and NPYRs have been widely investigated in relation to the pathogenesis of obesity (food-seeking behavior),[2] hypertension[3] and other neurovascular disorders.[4] Some psychological conditions have recently been claimed to be related to the NPY–NPYR system,[5] and the claim has generated controversy.[6] Abuse of several substances, including methamphetamine, phencyclidine, cocaine, marijuana and alcohol, is thought to be associated with the NPY–NPYR axis.[7] Polymorphisms in the *NPY* gene locus (chromosome 7) and *NPY* receptor loci are known, and they are currently being investigated for possible associations with individual differences in addictive behaviors. The *NPY* locus polymorphism has been extensively studied in regard to various aspects of many physiological and psychological disorders. For example, the Leu7Pro (rs16139) single nucleotide polymorphism (SNP) has been reported to be associated with alcohol consumption and the alcohol withdrawal syndrome in a European-American population.[8–10] However, it has a different prevalence in several other populations besides a European-American population,[11] and the association was not reproducible in Swedes and Finns.[12] Other polymorphisms in the *NPY* gene have been also reported to be related to alcohol dependence/preference.[13–15]

The roles of variants of the NPYRs, however, have not been thoroughly investigated. Wetherill *et al.*[16] carried out an extensive investigation of polymorphisms of *NPY* and *NPY* receptors: 7 SNPs in *NPY*, 15 SNPs in *NPY2R* including the 5′ end, and 17 SNPs in *NPY1R* and *NPY5R* in alcohol-dependent population; in populations with various degrees of the alcohol withdrawal syndrome and in populations with cocaine dependence. They found that several polymorphisms, including rs4425326 and rs6857715 located in the upstream of

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*NPY2R*, were associated with these pathological conditions in non-Hispanic, European-Caucasian families.

In this study, we investigated the relationships between the polymorphisms rs4425326 (C/T) and rs6857715 (T/C), both of which were claimed to be associated with alcohol and cocaine dependence in the report by Wetherill *et al.*,[16] and nicotine dependence in an elderly Japanese population.

## MATERIALS AND METHODS

### Questionnaire

Blood was collected from 2517 subjects in the clinical laboratory of Iwata City Hospital during the 5-year period from 2003 to 2008. The criteria for recruitment as subjects of this study were being ambulant, able to communicate orally and 60 years of age or older. All subjects provided written informed consent regarding participation in this study. A leaflet containing a questionnaire about life style, including alcohol consumption, smoking, diet and cancer history, was handed to each subject, and professional interviewers assisted them in filling them out and confirmed their answers. Some of the questions on smoking behavior were the same as those in the revised Fagerström Tolerance Questionnaire,[17] that is, the Fagerström Test for Nicotine Dependence (FTND),[18] which contains six of the original eight questions in the Fagerström Tolerance Questionnaire, and the Tobacco Dependence Screener (TDS) (a screening questionnaire for tobacco/nicotine dependence according to the *International Statistical Classification of Diseases and Related Health Problems* (ICD)-10, *Diagnostic and Statistical Manual of Mental Disorders* (DSM)-III-R and DSM-IV),[19] which consists of 10 questions. The questionnaire also included questions about the numbers of cigarettes smoked per day (CPD), age when the subject started smoking, how many times current smokers had tried to quit and how many times ex-smokers had tried to quit smoking before they succeeded. FTND scores were available for 1296 subjects (1220 men, 76 women (90.4 and 91.6%, respectively, of the ever-smokers)), and TDS scores were available for 1252 subjects (1183 men, 69 women (87.6 and 83.1%, respectively, of the ever smokers)).

The study design was approved by the institutional review board of Hamamatsu University School of Medicine (19-87 and 21-8).

### Genotype analysis

DNA was extracted from whole blood by using a QIAamp DNA Blood Maxi kit according to the manufacturer's instructions (Qiagen, Hamburg, Germany). A 50 ng sample of each subject's DNA was used for PCR amplification with the primer sets for NPY2R polymorphisms rs4425326 and rs6857715 by using the Start One (Applied BioSystems, Carlsbad, CA, USA), and assayed by using the Custom TaqMan SNP Genotyping Assay C__26159385_10 and C__29013142_10, respectively. The locations of the *NPY2R* polymorphisms and the exons are shown in Figure 1. The distribution of both genotypes distributions differs among populations. The minor alleles of rs4425326 and rs6857715 are Ts in the European populations, but C in rs4425326 and T in rs6857715 are listed as the ancestral alleles in the SNP database. The allele frequency of the C allele at the rs4425326 in Japanese is 0.341 and at the rs6857715 is 0.557. The background information regarding rs4425326 and rs6857715 is available at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=4425326 and at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=6857715, respectively.

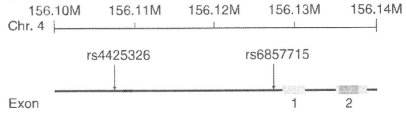


Figure 1

### Statistical analysis

Genotype distributions were tested for Hardy–Weinberg equilibrium by using SPSS statistics 17.0. software (SPSS Japan, Tokyo, Japan). $\chi^2$-Tests of each genotype or dominant model were performed for smoking status. The odds ratios were estimated by using a logistic model. The CPD values, FTND scores and TDS scores were evaluated according to smoking status and a dominant model of the polymorphisms by the Kruskal–Wallis test or Mann–Whitney *U*-test (SPSS Japan).

## RESULTS

The age, sex and smoking status of the subjects are shown in Table 1. The subjects ranged in age from 60 to 94 years, and they consisted of generations born between 1910 and 1948. There were 1350 ever-smokers (current smokers and ex-smokers) (83.6%) among the 1615 male subjects and 83 (9.2%) among the 902 female subjects, and 21.5% of the men and 3.4% of the women were current smokers. These values are slightly lower than in a recent report[20] stating that the prevalence of male and female Japanese current smokers 60 years of age or more is 27.8 and 6.2%, respectively. The mean CPD values, FTND scores and TDS scores in this study were lower than those previously reported in Japanese[19,21] or American smokers (Table 1).[22] The CPD values of male ex-smokers were higher than those of male current smokers, and TDS scores of the current smokers of both sexes were higher than the TDS scores of ex-smokers of both sexes.

The drinking status and lung cancer history of the subjects are also shown in Table 1. Current drinkers were overrepresented among current smokers, and never-smokers had tendencies to be never-drinkers in both sexes (Supplementary Table 1).

Figure 2, a (male) and b (female), shows the distributions of the FTND scores, and Figure 3, a (male) and b (female), shows the distributions of the TDS scores. The FTND scores of the male smokers ranged from 0 to 10, and the mode value and the mean value were 2 and 3.59, respectively. The FTND scores of the female smokers ranged from 0 to 7, and the mode value and mean value were 1 and 2.41, respectively. The TDS scores of the male smokers ranged from 0 to 10, and the mode value and mean value were 2 and 3.06, respectively. The TDS scores of the female smokers ranged from 0 to 9, and the mode value and mean value were 0 and 2.87, respectively (Figures 2 and 3).

Spearman's rank correlation coefficient for the correlation between the FTND scores and the TDS scores of the male ever-smokers and the female ever-smokers was 0.307 and 0.347, respectively. The *P*-values of both rank correlation coefficients indicated that they were significant at the 1% level. The frequency distribution of both rs4425326 (men: $\chi^2$=1.155, *P*=0.566; women: $\chi^2$=1.595, *P*=0.451) and rs6857715 (men: $\chi^2$=0.020, *P*=0.991; women: $\chi^2$=1.035, *P*=0.593) obeyed the Hardy–Weinberg law in both sexes.

The distribution of rs4425326 of the smokers revealed that the subjects having the C allele (genotypes CC and CT) were over-represented among male current smokers (Table 2), or expressed another way, men having the C allele in this SNP tended to continue to smoke (current smokers). This relation between the rs4425326 C allele and smoking status category was not found in female smokers. There were no differences in the prevalence of the rs6857715 polymorphism in any smoking category in either sex.

Male, but not female ever-smokers who had the rs4425326 TT genotype had significantly higher FTND scores and greater CPD than those with other genotypes (Table 3). In addition, male ever-smokers having the rs6857715 TT genotype had greater CPD than those having the C allele, but not significantly. Any other relations between the

**Table 1 Subject profile**

| *Variables* | *Male* | *P-value* | *Female* | *P-value* |
|---|---|---|---|---|
| Number of subjects | 1615 | | 902 | |
| Mean age, years (± s.d.) | 73.1 (± 6.2) | | 73.0 (± 6.4) | |
| *Age distribution, n (%)* | | | | |
| 60–64 | 81 (5.0) | | 51 (5.7) | |
| 65–69 | 426 (26.4) | | 251 (27.8) | |
| 70–74 | 455 (28.2) | | 240 (26.6) | |
| 75–79 | 418 (25.9) | | 197 (21.8) | |
| 80–84 | 170 (10.5) | | 134 (14.9) | |
| 85–89 | 51 (3.2) | | 25 (2.8) | |
| 90– | 14 (0.9) | | 4 (0.4) | |
| *Smoking status, n (%)* | | | | |
| Current smokers | 348 (21.5) | | 31 (3.4) | |
| Ex-smokers | 1002 (62.1) | | 52 (5.8) | |
| Never-smokers | 265 (16.4) | | 819 (90.8) | |
| *Mean age according to smoking status, years (± s.d.)* | | | | |
| Current smokers | 72.1 (± 6.0) | 0.002[a] | 71.1 (± 5.2) | 0.078[a] |
| Ex-smokers | 73.4 (± 6.0) | | 71.7 (± 6.4) | |
| Never-smokers | 73.4 (± 7.0) | | 73.2 (± 6.4) | |
| *Mean age at start of smoking, years (± s.d.)* | | | | |
| Ever smokers | 19.7 (± 3.7) | | 34.5 (± 13.3) | |
| Current smokers | 19.9 (± 4.3) | 0.282[b] | 37.5 (± 15.0) | 0.136[b] |
| Ex-smokers | 19.6 (± 3.5) | | 32.7 (± 11.9) | |
| *Mean numbers of CPD (± s.d.)* | | | | |
| Ever smokers | 21.1 (± 13.0) | | 13.3 (± 8.1) | |
| Current smokers | 16.6 (± 9.1) | <0.001[b] | 12.4 (± 6.1) | 0.776[b] |
| Ex-smokers | 22.7 (± 13.7) | | 13.8 (± 9.1) | |
| *Mean FTND score (± s.d.)* | | | | |
| Ever smokers | 3.59 (± 2.21) | | 2.41 (± 2.00) | |
| Current smokers | 3.63 (± 2.10) | 0.495[b] | 2.26 (± 1.81) | 0.777[b] |
| Ex-smokers | 3.57 (± 2.25) | | 2.51 (± 2.13) | |
| *Mean TDS score (± s.d.)* | | | | |
| Ever smokers | 3.06 (± 2.49) | | 2.87 (± 2.47) | |
| Current smokers | 3.69 (± 2.41) | <0.001[b] | 3.78 (± 2.50) | 0.018[b] |
| Ex-smokers | 2.84 (± 2.48) | | 2.41 (± 2.35) | |
| *Drinking status, n (%)* | | | | |
| Current drinkers | 854 (52.9) | | 177 (19.6) | |
| Ex-drinkers | 319 (19.8) | | 50 (5.5) | |
| Never-drinkers | 442 (27.4) | | 675 (74.8) | |
| *Lung cancer history, n (%)* | | | | |
| Yes | 47 (2.9) | | 12 (1.3) | |
| No | 1568 (97.1) | | 890 (98.7) | |

Abbreviations: CPD, cigarettes smoked per day; FTND, the Fagerström Test for Nicotine Dependence; s.d., standard deviation; TDS, the Tobacco Dependence Screener.
Ever smokers: current smokers and ex-smokers.
[a]Kruskal–Wallis test comparing three statuses.
[b]Mann–Whitney *U*-test comparing current smokers and ex-smokers.

rs6857715 polymorphism and the FTND scores or CPD value were not found in either sex (Table 3). Neither the rs4425326 nor the rs6857715 polymorphism was associated with the TDS scores in either sex (Table 3).

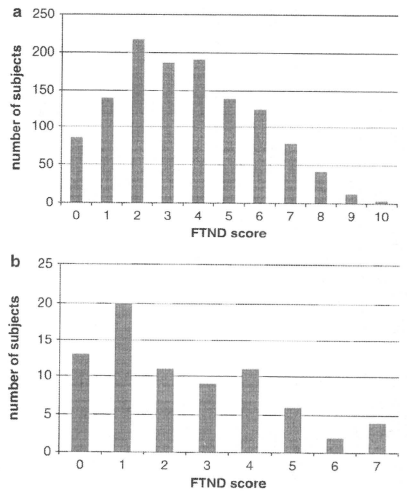


**Figure 2** (**a**) Distribution of the Fagerström Test for Nicotine Dependence (male). (**b**) Distribution of the Fagerström Test for Nicotine Dependence (female).

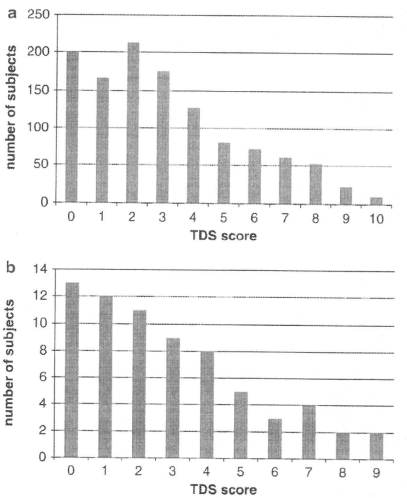


**Figure 3** (**a**) Distribution of the Tobacco Dependence Screener (male). (**b**) Distribution of the Tobacco Dependence Screener (female).

**Table 2 Comparison of subjects distribution of current smokers and ex-smokers according to two polymorphisms of *NPY2R***

| | Smoking status | | | | Dominant model (CC+CT vs TT) | | Dominant model (CC+CT vs TT) | |
|---|---|---|---|---|---|---|---|---|
| | Total n (%) | Current smokers n (%) | Ex-smokers n (%) | P-value[a] | OR[b] (95% CI) | P-value | OR[c] (95% CI) | P-value |
| *Males* | | | | | | | | |
| *rs4425326* | | | | | | | | |
| TT | 680 (50.4) | 158 (45.4) | 522 (52.1) | | 1 | | 1 | |
| CT | 566 (41.9) | 156 (44.8) | 410 (40.9) | 0.055 | 1.308 (1.024–1.670) | 0.034 | 1.300 (1.017–1.662) | 0.036 |
| CC | 104 (7.7) | 34 (9.8) | 70 (7.0) | | | | | |
| *rs6857715* | | | | | | | | |
| TT | 319 (23.6) | 82 (23.6) | 237 (23.7) | | 1 | | 1 | |
| CT | 679 (50.3) | 164 (47.1) | 515 (51.4) | 0.244 | 1.005 (0.754–1.339) | 1.000 | 1.013 (0.759–1.351) | 0.931 |
| CC | 352 (26.1) | 102 (29.3) | 250 (25.0) | | | | | |
| *Females* | | | | | | | | |
| *rs4425326* | | | | | | | | |
| TT | 37 (44.6) | 17 (54.8) | 20 (38.5) | | 1 | | 1 | |
| CT | 44 (53.0) | 14 (45.2) | 30 (57.7) | 0.281 | 0.515 (0.209–1.268) | 0.175 | 0.512 (0.208–1.264) | 0.147 |
| CC | 2 (2.4) | 0 (0) | 2 (3.8) | | | | | |
| *rs6857715* | | | | | | | | |
| TT | 19 (22.9) | 10 (32.3) | 9 (17.3) | | 1 | | 1 | |
| CT | 47 (56.6) | 14 (45.2) | 33 (63.5) | 0.205 | 0.440 (0.155–1.245) | 0.176 | 0.446 (0.157–1.268) | 0.130 |
| CC | 17 (20.5) | 7 (22.6) | 10 (19.2) | | | | | |

Abbreviations: CI, confidence interval; OR, odds ratio.
Allele frequency of C in rs4425326: 0.278. Allele frequency of T in rs6857715: 0.489. The risk alleles of these two polymorphisms are designated as C.
[a]The $\chi^2$-tests were performed based on 3×2 tables.
[b]The odds ratios were calculated for the genotypes concerned in the current smoking status.
[c]The odds ratios were adjusted for age and calculated for the genotypes concerned in the current smoking status.

**Table 3 Comparison of the numbers of CPD, FTND scores and TDS scores of ever smokers according to the dominant model (CC+CT vs TT) of two polymorphisms of *NPY2R***

| | CPD | | | FTND | | | TDS | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Mean ± s.d. | P-value[a] | n | Mean ± s.d. | P-value[a] | n | Mean ± s.d. | P-value[a] |
| *Males (CPD, n=1348; FTND, n=1220; TDS, n=1183)* | | | | | | | | | |
| *rs4425326* | | | | | | | | | |
| TT | 680 | 22.3 ± 13.3 | <0.001 | 614 | 3.80 ± 2.31 | 0.003 | 598 | 2.95 ± 2.49 | 0.085 |
| CT+CC | 668 | 19.9 ± 12.5 | | 606 | 3.37 ± 2.08 | | 585 | 3.18 ± 2.51 | |
| *rs6857715* | | | | | | | | | |
| TT | 319 | 21.8 ± 12.6 | 0.054 | 291 | 3.76 ± 2.35 | 0.197 | 281 | 2.88 ± 2.32 | 0.314 |
| CT+CC | 1029 | 20.9 ± 13.1 | | 929 | 3.53 ± 2.16 | | 902 | 3.12 ± 2.55 | |
| *Females (CPD, n=83; FTND, n=76; TDS, n=69)* | | | | | | | | | |
| *rs4425326* | | | | | | | | | |
| TT | 37 | 14.0 ± 8.0 | 0.417 | 34 | 2.56 ± 1.99 | 0.480 | 29 | 3.31 ± 2.99 | 0.501 |
| CT+CC | 46 | 12.7 ± 8.2 | | 42 | 2.29 ± 2.02 | | 40 | 2.55 ± 2.00 | |
| *rs6857715* | | | | | | | | | |
| TT | 19 | 11.2 ± 6.1 | 0.218 | 17 | 1.88 ± 1.50 | 0.334 | 14 | 2.21 ± 2.29 | 0.264 |
| CT+CC | 64 | 13.9 ± 8.5 | | 59 | 2.56 ± 2.10 | | 55 | 3.04 ± 2.51 | |

Abbreviations: CPD, cigarettes smoked per day; FTND, the Fagerström Test for Nicotine Dependence; s.d., standard deviation; TDS, the Tobacco Dependence Screener.
[a]Mann–Whitney *U*-test.

## DISCUSSION

A long list of genes is considered candidates for a relation to smoking behavior or nicotine dependence. Li and Burmeister,[23] recently conducted an extensive reviewed of research on genes related to addictions, and in that seminal review, they listed genes that are considered candidates for an association with at least one drug addiction (62

genes) in two large tables and genes having one or more of whose variants have been associated with addiction to at least one substance (41 genes) in supplementary tables. *NPY*, but not *NPYR*, was included in the tables, and thus far there have been few studies on associations between nicotine dependence and the NPY–NPYR axis.[23]

We attempted to determine whether the rs4425326 and rs6857715 polymorphisms of the *NPY2R* gene are related to another addictive behavior in humans, nicotine dependence, because Wetherill *et al.*[16] have recently shown an association between these polymorphisms and both alcohol dependence and cocaine addiction in humans. Dependence on nicotine is one of the addictions that has been extensively studied, and the genetic aspects of smoking behavior are being extensively investigated now.[23]

Our study on smoking behavior is the first to report a correlation between *NPY2R* gene polymorphism and nicotine dependence.

We found a significant correlation between rs4425326 C-containing allelotypes, which have been hypothesized to be high-risk allelotypes for addiction and current smoking status in male smokers. This is the first report to show that the *NPY2R* polymorphism is related to human addictive behaviors in a non-Caucasian population. This is also the first time an association between *NPY2R* and nicotine dependence has been shown. However, no relation was found between TDS scores and any of the *NPY2R* allelotypes, perhaps because the traits detected by the scores on two questionnaires (FTND and TDS) are different, and how the genetic components control these traits in establishing individual nicotine dependence is not elucidated.

The mechanistic significance of the rs4425326 locus, 0.2 Mb upstream from the first exon of *NPY2R* (Figure 1), is unknown.

Interestingly, 'addiction'-allele rs4425326 C holders (according to Wetherill *et al.*[16]) were more prevalent in the current smokers in this study than among the ex-smokers in spite of the lower FTND scores of the allele C holders, meaning that the rs4425326 C allele holders have milder nicotine dependence based on FTND scores but they do not quit smoking (Supplementary Table 2). Like the FTND scores, the CPD values of the rs4425326 C allele holders were lower, too. The analysis of male current smokers also showed lower CPD values and FTND scores in the rs4425326 C holders ($P$=0.100 and 0.098, respectively), but not significantly (Supplementary Table 3). Thus, Japanese male rs4425326 C allele holders are modest but very persistent nicotine-seekers. This paradoxical result may reflect the complex decision-making process regarding smoking in the elderly Japanese. No genetic tendencies were detected in any of the nicotine dependence scores among the female smokers in our study. The failure to find any genetic tendencies among them was mainly because of the relatively small numbers of female smokers in our study.

Smoking is a complicated personal behavior that is influenced by multiple factors, including the social, cultural and sometimes even the political environment. The role of a single genetic polymorphism must not be overestimated in explaining individual smoking behavior. The interpretations of this study have several limitations. We recruited the elderly people in a rural city, and this population has demographical and occupational characteristics different from those in urban cities or agricultural villages. We expected our study subjects have established smoking behavior. This might imply the dependence scores collected here have some biases, causing the limitation of our study. In a different point of view, the information on the established smoking behavior as a life-long habit in individuals may make a unique contribution to understand the genetic effect on whole life of humans. Anyway, it will be necessary to validate our observations by replication studies in the future. However, we think our data provide a major clue to understanding human smoking behavior.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# Association between neurexin 1 (*NRXN1*) polymorphisms and the smoking behavior of elderly Japanese

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Neurexin 1 (*NRXN1*, MIM 600565) is a molecule that expressed in neurons and is a receptor for α-latrotoxin (Rowen *et al.*, 2002). Bierut *et al.* (2007) pinpointed the *NRXN1* gene locus as being responsible for nicotine dependence in a case–control analysis in which the cases were American and Australian smokers whose Fagerström test for nicotine dependence (FTND) score was 4 or more. The other group also documented a significant contribution of the polymorphism at the *NRXN1* locus to nicotine dependence in the European–American and African–American populations (Nussbaum *et al.*, 2008). They evaluated 21 *NRXN1* polymorphisms in European–American and African–American smokers for associations with FTND scores, number of cigarettes smoked per day (CPD), and the heaviness of smoking index, which is a combination of CPD and time before the first cigarette of the day. The results showed that two of 21 single nucleotide polymorphisms, rs2193225 and rs6721498, were associated with heaviness of smoking index and FTND scores in some American populations of European and African origin. In this letter, we report the evaluation of these polymorphisms in an elderly Japanese population (60–94 years old) according to smoking history. rs6721498 and rs2193225 were genotyped in 2516 Japanese with various smoking habits [1348 ever-smokers (current smokers and exsmokers; 83.6%) among the 1612 male participants and 82 (9.1%) among the 904 female participants, and current smokers were 21.6 and 3.4% of the male and female participants, respectively]. FTND and the tobacco dependence screener (TDS), which was developed so that the characteristics detected would be more closely correlated with those defined by the International Statistical Classification of Diseases and Related Health Problems, 10th version, *Diagnostic and Statistical Manual of Mental Disorders*, Revised Third Edition, and *Diagnostic and Statistical Manual of Mental Disorders*, Fourth Edition (Kawakami *et al.*, 1999), were applied to almost all of the ever-smokers among the participants.

Genotype frequencies of these polymorphisms were 0.168 (AA), 0.486 (AG), and 0.347 (GG) in the rs6721498 (A allele 0.411) and 0.659 (AA), 0.304 (AG), and 0.037 (GG) in the rs2193225 (G allele 0.189). Male ever-smokers with the rs2193225 GG type were more prevalent in the higher TDS score category ($P = 0.056$), but not in the higher FTND score category. The CPD was greater in the male ever-smokers with the rs2193225 GG genotype ($P = 0.06$ by analysis of variance; 0.032 and 0.025 by the Dunnett multiple test). In contrast, the rs2193225 GG type was overrepresented in the male never-smokers ($P = 0.006$). There were no differences in the distributions of the FTND scores or TDS scores according to the rs6721498 genotype. We concluded that *NRXN1* polymorphisms are associated with the smoking behavior of elderly Japanese. The contrasting associations between rs2193225 GG type and smoking initiation (ever vs. never) and smoking persistence (according to the TDS score) may imply a difference between the role of *NRXN1* in the initiation and persistence of smoking. This genotype may be more closely associated with the smoking behavior patterns detected by the TDS than that by the FTND.

Smoking is a highly complicated behavior, and no single genetic polymorphism has much influence on it. Further validation and replication in several populations are important.

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