

Increased p53 mutation load in nontumorous human liver of Wilson disease and hemochromatosis: Oxyradical overload diseases

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Hemochromatosis and Wilson disease (WD), characterized by the excess hepatic deposition of iron and copper, respectively, produce oxidative stress and increase the risk of liver cancer. Because the frequency of p53 mutated alleles in nontumorous human tissue may be a biomarker of oxyradical damage and identify individuals at increased cancer risk, we have determined the frequency of p53 mutated alleles in nontumorous liver tissue from WD and hemochromatosis patients. When compared with the liver samples from normal controls, higher frequencies of G:C to T:A transversions at codon 249 ($P < 0.001$) and C:G to A:T transversions and C:G to T:A transitions at codon 250 ($P < 0.001$ and $P < 0.005$) were found in liver tissue from WD cases, and a higher frequency of G:C to T:A transversions at codon 249 ($P < 0.05$) also was found in liver tissue from hemochromatosis cases. Sixty percent of the WD and 28% of hemochromatosis cases also showed a higher expression of inducible nitric oxide synthase in the liver, which suggests nitric oxide as a source of increased oxidative stress. A high level of etheno-DNA adducts, formed from oxyradical-induced lipid peroxidation, in liver from WD and hemochromatosis patients has been reported previously. Therefore, we exposed a wild-type p53 TK-6 lymphoblastoid cell line to 4-hydroxynonenal, an unsaturated aldehyde involved in lipid peroxidation, and observed an increase in G to T transversions at p53 codon 249 (AGG to AGT). These results are consistent with the hypothesis that the generation of oxygen/nitrogen species and unsaturated aldehydes from iron and copper overload in hemochromatosis and WD causes mutations in the p53 tumor suppressor gene.

Wilson disease | hemochromatosis | p53 | iron | copper | liver carcinogenesis

Recent studies have linked oxidative stress and chronic inflammation with an increased risk of cancer (1). A number of oxyradical overload diseases, e.g., ulcerative colitis, viral hepatitis, pancreatitis, hemochromatosis, and Wilson disease (WD), are associated with an increased risk of cancer. Hemochromatosis and WD are genetic disorders characterized by excess hepatic deposition of iron and copper, respectively. In addition to cirrhosis and liver failure, the risk of hepatocellular carcinoma (HCC) is increased by 200-fold in hemochromatosis patients (2). A lower incidence of HCC has been reported in WD (3), possibly due to a reduced lifespan caused by hepatic failure and cardiac complications. Germ-line mutations in the genes responsible for both hemochromatosis, HFE (a major histocompatibility class I-like gene), and WD, ATP7B (a copper-transporting ATPase), have been described (4, 5). Whereas specific mutations in the HFE gene alter the regulation of iron uptake, a wide variety of mutations in the ATP7B gene seem to be responsible for copper accumulation in WD. A large propor-

tion of hemochromatosis patients (60–100%) are homozygous for the same missense mutation (C282Y), whereas a few are compound heterozygotes of C282Y and a second mutation H63D (3–7%) (6). However, evidence is accumulating that hemochromatosis can occur without the development of a homozygous C282Y mutation in the HFE gene (7). Animal models exist for both hemochromatosis and WD (8, 9). Long-Evans Cinnamon rats, an animal model for WD, accumulate excess copper in the liver and develop chronic hepatitis and liver cancer (10). Mice in which the HFE gene has been knocked out serve as a model for hemochromatosis (8, 11); however, although they develop an excessive accumulation of iron, there is no report on the pathology associated with the increase.

Evidence of oxidative stress and the subsequent generation of reactive aldehydes have been reported in hemochromatosis and WD patients from the iron or copper overload (12). Oxidative stress and the generation of reactive species can cause mutations in cancer-related genes (13). To study genetic events that can participate in the development of HCC in hemochromatosis and WD and to test the hypothesis that reactive species produced by oxidative stress in these diseases mutate the p53 tumor suppressor gene, we have measured the frequency of specific p53 mutated alleles in nontumorous liver samples from patients with either hemochromatosis or WD using a highly sensitive genotypic mutation assay (14). These data may identify endogenous carcinogen exposure and aid in the identification of individuals at increased cancer risk.

Materials and Methods

Fresh-frozen surgical liver samples were collected from 12 WD and 8 hemochromatosis noncancerous cases undergoing liver transplant. The normal controls were obtained from the livers of organ donors and were without morphological evidence of pathology.

Determination of Iron and Copper Levels. Iron levels were measured employing inductively coupled plasma optical emission spectro-

Abbreviations: MS, mutant standard; WD, Wilson disease; NOS2, inducible nitric oxide synthase; 4-HNE, 4-hydroxynonenal; RFLP, restriction fragment length polymorphism.

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photometry. The spectrometer was a Perkin–Elmer Optima 3000 equipped with a computer module for instrument control, data acquisition, and handling. Copper levels were determined by electrothermal atomic absorption spectrophotometry with Zeeman background correction (Varian Spect AA-400Z). Before elemental analysis, tissue samples were digested with a microwave digester system (CEM Corp., Indian Trail, NC) at controlled temperature and pressure. Liver specimens were dried and brought to a constant dry weight before digestion. Tissue samples were digested using a mixture of 1 ml of 70% HNO₃ (Ultrex, J.T. Baker), 1 ml of 30% H₂O₂ (J.T. Baker), and 1 ml of distilled deionized water. Experimental measurements and analyte recoveries were compared using certified control samples from the National Institute of Standards and Technology (Gaithersburg, MD). Iron and copper levels were calculated as microgram (μg) of element per gram of dry tissue.

Analysis of C282Y and H63D Mutations in the HFE Gene. C282Y and H63D mutations were analyzed using PCR amplification and specific restriction digestion as described previously (15).

Exposure of TK-6 Cell Lines to 4-Hydroxynonenal (4-HNE). The TK-6 lymphoblastoid cell line was used to assess the mutability of p53 codons 248, 249, and 250 to 4-HNE (Calbiochem–Novabiochem Corp.). Cells were grown in CHAT medium (cytidine, hypoxanthine, aminopterin, and thymidine) for 2 days and then washed and resuspended at a density of 4×10^6 cells in 12 ml of RPMI-1640 medium. Cells were then exposed to doses of 12.5, 25, and 50 μM 4-HNE for 1 h. Cultures were centrifuged and resuspended in 100 ml of fresh medium. A dose-dependent decrease in survival was observed at the above concentrations of 4-HNE. Cells were allowed to grow for 6 days, and genomic DNA was isolated using Qiagen genomic DNA kit for p53 mutation analysis as described below.

Analysis of Codons 249 and 250 for Missense Mutations. Previously, we have described in detail a highly sensitive mutation assay to measure the frequency of p53 mutations at codons 249 and 250 (13, 14). These codons constitute the *Hae*III restriction site and, thus, are accessible to the restriction fragment length polymorphism (RFLP)/PCR approach used in this assay. Briefly, $3.5\text{--}4 \times 10^7$ copies of genomic DNA were digested exhaustively with *Hae*III restriction enzymes. To this DNA digest, 30 copies of an internal control or mutant standard (MS) were added. The samples were enriched in sequences with mutated *Hae*III site (harboring codons 249 and 250) by gel isolation of a 100- to 200-bp fragment population containing a mutated 159-bp p53 segment, which extends from flanking 5' *Hae*III site (residue 13981) to the flanking 3' *Hae*III site (residue 14139). The 117-bp MS also is co-isolated with the bona fide mutated sequences. This enriched DNA preparation was used as a template for the amplification of a final 101-bp exon VII fragment (residues 13999 to 14099) in two rounds of amplification using *Pyrococcus furiosus* (Stratagene) and *Taq*-DNA polymerase (Perkin–Elmer).

The RFLP/PCR products were cloned into $\lambda\text{gt}10$, and the phages were plated onto *Escherichia coli* C600 Hfl. For each sample, a total of 1,500–1,700 plaques were grown on 8–10 different L–B agar plates, each containing 150–200 plaques. The plaques were lifted on plaque screens and hybridized separately with a total of 14 different ³²P-labeled, 19-mer oligonucleotide probes that were each specific for a single base-pair mutation at the *Hae*III site (containing codons 249 and 250), wild-type sequence, and MS. After hybridization with each probe, positive plaques representing a specific mutation were counted on autoradiographs, and the percentage of positive mutant plaques was determined for each of the 8–10 membranes (each representing the replica of 1 plate containing a total of 150–200 total

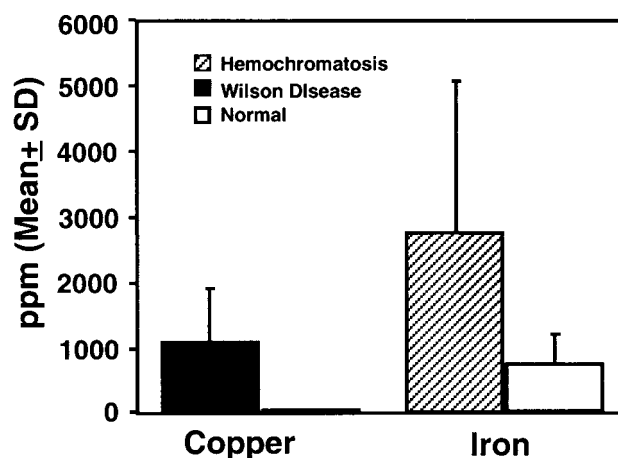


Fig. 1. Levels of iron and copper in liver samples of hemochromatosis and WD cases, respectively, compared with liver samples from normal individuals.

plaques). Then, the mean \pm standard deviation of the percentage of mutant plaques was calculated for the 8–10 membranes representing different plates. Likewise, the percentage of mutant plaques was determined after hybridization with each of the 14 probes. The mean of the percentage of positive plaques from a total of 1,500–1,700 plaques for each of the 12 possible mutations was calculated. Then, the mean value was used to calculate the mutation frequency by comparison with the mean of the percentage of MS (internal control) plaques arising from the known copy number of MS copies added into the genomic DNA.

Inducible Nitric Oxide Synthase (NOS2) Immunohistochemistry and Staining of Metals. The immunohistochemical staining procedure was performed as reported (16), using primary anti-NOS2 mAb (Transduction Laboratories, Lexington, KY) at a dilution of 1:125. Positive controls were represented by lung cancers known to express NOS2. Negative controls were produced by substituting the primary antibody with non-immune rabbit serum. Sample sections of WD and hemochromatosis cases also were stained with rhodamine and Perl's (Prussian blue reaction) stains, for copper and iron, respectively (17).

Results

We determined the frequency of p53 codons 249 and 250 mutated alleles in nontumorous liver tissue from WD and hemochromatosis patients as well as from normal controls. Histological analysis showed regenerative nodule and fibrosis with inflammation indicating the presence of cirrhosis in all of the hemochromatosis and WD cases. Eight of 12 WD and 2 of 8 hemochromatosis cases showed chronic active hepatitis; the remainder showed minimal change. All of the liver samples from normal donors were without pathological abnormalities.

Quantitation and Localization of Iron and Copper. We measured the amounts of iron and copper in hemochromatosis and WD samples, respectively, as well as in normal samples as described in *Materials and Methods*. The mean level of copper in WD samples was $1125 \pm 836 \mu\text{g/g}$ dry tissue weight, compared with $21 \pm 7 \mu\text{g/g}$ in the normal samples, whereas the mean iron level in hemochromatosis samples was $2,777 \pm 2,331 \mu\text{g/g}$ compared with $762 \pm 484 \mu\text{g/g}$ in the normal samples (Fig. 1). These values are within the range described for iron and copper accumulation in hemochromatosis and WD, respectively (6, 18, 19). We also stained the sections of WD and hemochromatosis samples with special rhodamine and Perl's stains specific for copper and iron,

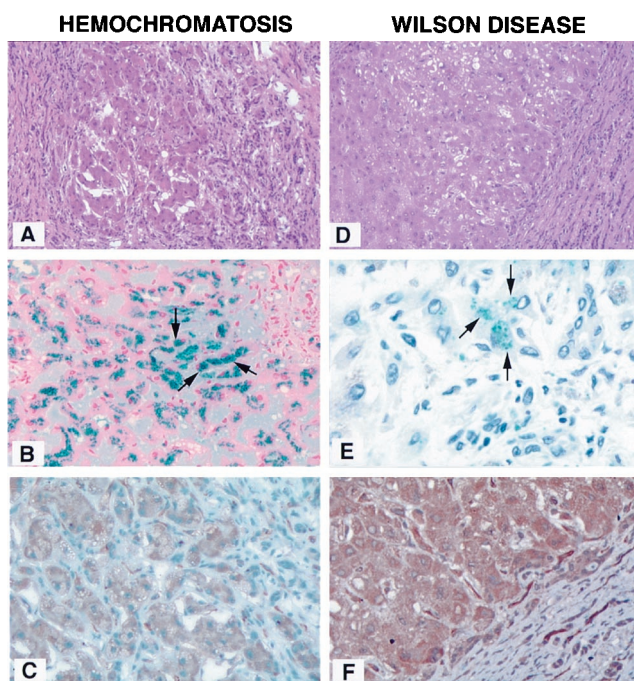


Fig. 2. Sections from patients with hemochromatosis (A–C) or WD (D–F). (A) Hematoxylin/eosin stain, which shows hyperplastic nodule. Surrounding the nodule, regenerative fibrous tissues and mononuclear inflammatory cells are present, indicating the presence of cirrhosis ($\times 100$). (B) The iron stain (Prussian blue), which displays iron granules in hepatocytes (arrowheads, $\times 400$). The NOS2 expression is seen predominantly in the cytoplasm of hepatocytes. (C) In addition, NOS2 also is overexpressed in endothelial cells within areas of bridging fibrosis. The H&E stain in WD (D) shows regenerative and hyperplastic nodule with fibrosis and inflammation, indicating the presence of cirrhosis ($100\times$). Confirmatory rhodamine stain shows the intracellular deposition of copper (E, $\times 400$). The immunohistochemical stain with anti-NOS2 is shown (F). NOS2 overexpression is seen within hepatocytes of hyperplastic nodule ($\times 200$).

respectively (Fig. 2B and E). Intracellular localization of these metals was observed in hepatocytes.

C282Y and H63D Mutations in HFE Gene. The presence of C282Y (cysteine to tyrosine) and H63D (histidine to aspartic acid) mutations in the HFE gene have been associated with hemochromatosis. Although homozygosity for C282Y mutations has been reported as ranging from 60–100% in hemochromatosis patients (reviewed in ref. 6), only four of the eight (50%) hemochromatosis samples showed C282Y homozygous mutations in the present sample set. No H63D mutations were found.

NOS2 Immunohistochemistry. We analyzed NOS2 expression by immunostaining liver tissue sections from hemochromatosis and WD cases, in addition to the normal controls. Six of 10 WD samples (60%) showed immunoreactivity at an intensity of 2 (>25 to $<50\%$ reactivity) or 3 ($>50\%$ to $<90\%$ reactivity), whereas only 2 of 7 (28%) of the hemochromatosis samples showed immunoreactivity of similar intensity (Fig. 2C and F). NOS2 expression was observed predominantly in the cytoplasm of hepatocytes, but some endothelial cells within areas of bridging fibrosis also showed NOS2 expression. The normal liver samples did not contain detectable anti-NOS2 immunoreactivity.

Analysis of p53 Mutation Load in Nontumorous Liver from Hemochromatosis and WD Cases. Using a highly sensitive genotypic mutation assay, we assayed the frequency of p53 codons 249 and 250 mutated alleles in nontumorous liver from hemochromatosis and

WD cases as well as normal controls. We observed a higher frequency of G:C to T:A transversions at codon 249 ($P < 0.001$) and C:G, as well as to A:T transversions and C:G to T:A transitions at codon 250 ($P < 0.001$, and $P < 0.005$) in tissue from WD cases when compared with samples from normal controls (Fig. 3). We also found a higher frequency of G:C to T:A transversions at codon 249 ($P < 0.05$) in tissue from hemochromatosis cases when compared with normal controls (Fig. 3). However, the frequency of C:G to T:A transitions in hemochromatosis cases was not significantly higher than in normal controls. The frequency of other base-pair changes at codons 249 and 250 was not significantly different from that of controls in either hemochromatosis or WD samples.

Mutability of p53 Codons 248, 249, and 250 to 4-HNE. To evaluate the mutability of p53 codons 248, 249, and 250, genomic DNA from lymphoblastoid cells exposed to $50 \mu\text{M}$ 4-HNE was analyzed for p53 mutations. We found that 4-HNE preferentially induced G:C to T:A transversions at codon 249 (AGG to AGT). The frequency of other base-pair changes at codons 249 and 250 did not show any difference compared with controls (Fig. 4). Mutations were not observed at codon 248.

Discussion

p53 mutations represent the most prominent genetic alteration in the development of human cancer (20, 21). About 30–40% of all HCC contain a mutation in the p53 tumor suppressor gene (ref. 22, <http://www.iarc.fr/p53/homepage.html>), and a high frequency of a specific p53 mutant allele in noncancerous liver suggests that a p53 mutation can be an early event in the process of hepatocellular carcinogenesis (23). Although the available literature on the p53 mutational spectra in hemochromatosis and WD-associated HCC is limited, a high frequency of p53 mutations in hemochromatosis-associated HCC has been reported (ref. 24; C.C.H. and coworkers, unpublished data). In the present study, we tested the hypothesis that reactive species, e.g., oxygen and nitrogen species as well as reactive aldehydes, in WD and hemochromatosis patients may contribute to the mutation of the p53 tumor suppressor gene. We found a significantly high frequency of G:C to T:A transversions at codon 249 and C:G to A:T transversions and C:G to T:A transitions at codon 250 in nontumorous liver tissue from WD cases when compared with liver samples from normal controls. Nontumorous liver from hemochromatosis cases also showed a significantly higher frequency of G:C to T:A transversions at codon 249 when compared with normal controls. Therefore, an increased p53 mutation load may be one of the determinants that predispose an individual with either WD or hemochromatosis to the development of cancer.

Liver samples from hemochromatosis and WD cases showed characteristic high levels of iron and copper, respectively, when compared with the normal liver. This excessive accumulation of either iron or copper in hepatocytes causes hepatocellular injury, which leads to the development of fibrosis and cirrhosis (reviewed in ref. 12) and is mediated by the direct interaction of metals with the cellular targets or by the formation of reactive species (25, 26). Fenton or Haber–Weiss chemistry and lipid peroxidation are the major pathways through which iron and copper catalyze the formation of reactive hydroxyl and lipid radicals. In this inflammatory microenvironment, high levels of nitric oxide and reactive nitrogen oxide species also are generated. The metal ions and the reactive oxygen and nitrogen species can induce a number of alterations in cellular macromolecular targets, which include point mutations in genes (refs. 25 and 27 and reviewed in refs. 28 and 29). Cellular injury, like cirrhosis caused by oxidative damage, leads to increased cellular proliferation. Increased cell division contributes to the conversion of DNA lesions into point mutations. In the present study,

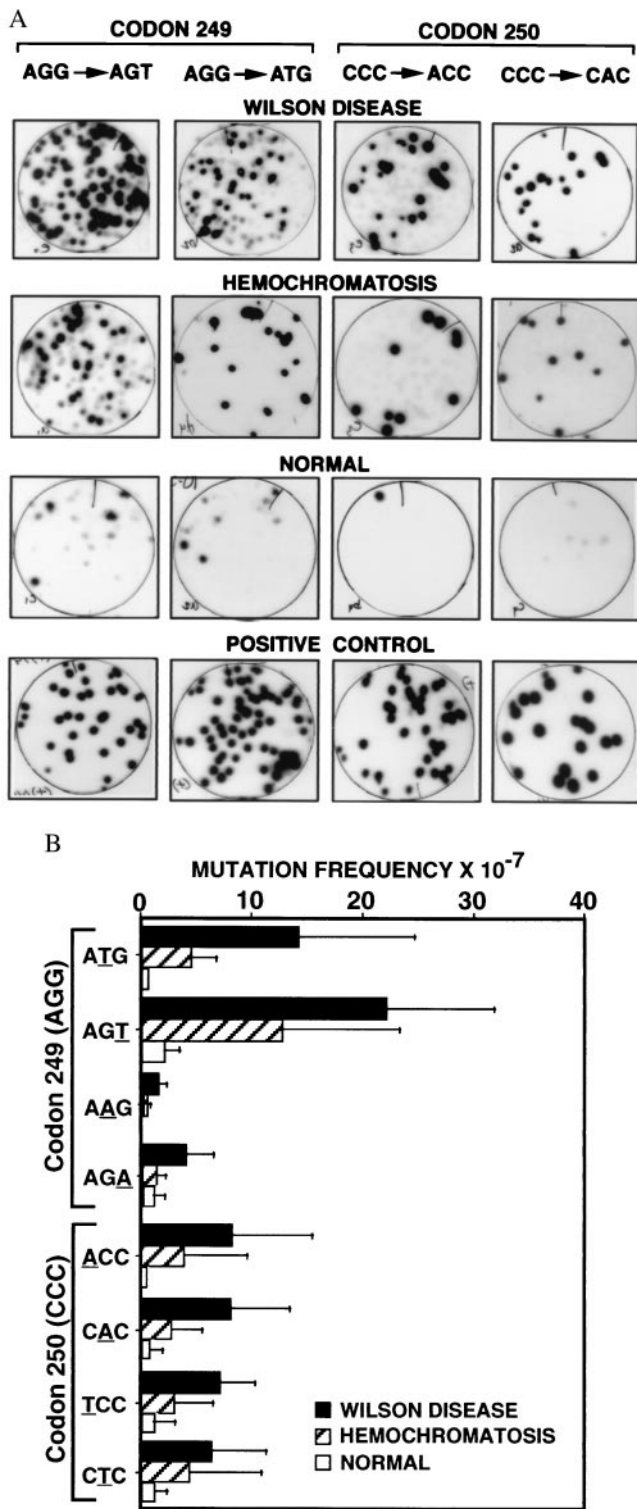


Fig. 3. (A) Identified mutant λ -plaques for G to T and C to A transversions in representative filters from each group of samples. Liver DNA samples from hemochromatosis, WD, and normal controls were analyzed for base-pair changes at *Hae*III site (codons 249 and 250), corresponding to p53 nucleotide residues 14072–14075 by *Hae*III RFLP/PCR. Thirty copies of the MS or internal control were added to $3.5\text{--}4 \times 10^7$ initial copies of the p53 allele. The RFLP/PCR products were cloned into λ gt10 and plated on *E. coli* C600 Hfl, and plaque-screens were analyzed by hybridization with 12 mutant-specific oligonucleotide probes as well as probes for the wild-type sequence and MS. With each hybridization and washing, positive control membranes containing the authentic mutants were added to serve as positive controls. Analyses

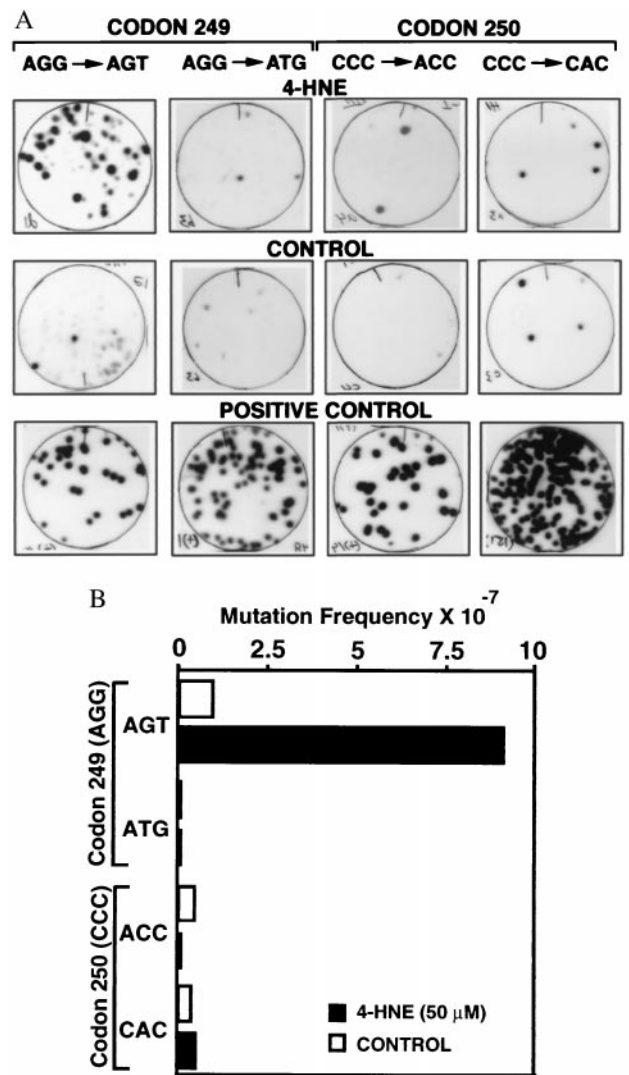


Fig. 4. (A) Identified mutant λ -plaques for G to T and C to A transversions at p53 codons 249 and 250 in representative filters from 4-HNE-treated and control TK-6 cells. Analyses were performed as described in the Fig. 3A legend. (B) Absolute p53 mutation frequencies of G to T and C to A transversions at p53 codons 249 and 250 in 4-HNE-treated and control TK-6 cells. No mutation was found at codon 248. Mutation frequencies were calculated as described in the Fig. 3B legend.

all of the WD and hemochromatosis cases showed liver cirrhosis that may enhance the generation of mutations following the initial DNA lesions caused by reactive oxygen and nitrogen species, and reactive aldehydes. In addition, a high level of non-heme iron in the cells can inhibit NO-induced apoptosis by converting NO from a pro-apoptotic molecule to an anti-apoptotic molecule (30). The development of a sustained oxidative and nitrosative stress, and the subsequent generation of lipid

included 1,500–1,700 plaques on 8–10 LB-Agar plates. (B) Absolute p53 mutation frequencies of G to T and C to A transversions and C to T and G to A transitions in hemochromatosis, WD, and normal controls. Mutation frequencies were calculated from the percentage of the identified λ -plaques (the mean of percentage was taken from 8–10 membranes, each with a total of 150–200 plaques, for each specific oligonucleotide probe) by calibration with the MS contents of the RFLP/PCR products, the initial number of p53 copies, and the number of copies of MS added at the outset of the experiment.

peroxidation intermediates can produce an environment suited for the development of human cancer including the mutation of tumor suppressor genes such as p53 (1, 31–33). Therefore, the increased p53 mutation load in hemochromatosis and WD cases can arise before the development of cancer through a number of different mutagenic pathways.

Nitric oxide (NO[•]) is produced by a family of enzymes known as nitric oxide synthases (NOS). NOS2 produces a sustained and high concentration of NO[•] compared with NOS1 and NOS3, and increased expression of NOS has been reported in a variety of human cancers as well as in cancer-prone, chronic inflammatory diseases (34–36). NO[•] may cause both deamination of bases and oxidative damage to DNA (37, 38). Treatment of TK6 human lymphoblastoid cells with NO[•] deaminated guanine and adenine, and induced mutations at *HPRT* and *TK* gene loci (39). Deamination of methylcytosine or cytosine and guanine can produce G:C to A:T transitions and G:C to T:A transversions, respectively (40, 41). We have reported previously a positive correlation between higher NOS2 activity and increased p53 G:C to A:T transitions in human colon cancer (42). In the inflammatory microenvironment, NO[•] can react with superoxide anion (O₂^{•-}) to form peroxynitrite (ONOO⁻). ONOO⁻ is highly reactive to guanine and produces 8-oxo-dG (43) and 8-nitroguanine (44) adducts. 8-oxo-dG can induce G:C to T:A transversions (45). In the present study, we found increased NOS2 expression in 60% of the WD cases and in 28% of the hemochromatosis cases, together with a significantly high frequency of C to T transitions in WD cases. These findings are consistent with a mutagenic mechanism of NO-induced deamination of cytosine and 5-methylcytosine.

The most prominent point mutations induced by oxidative damage are G:C to T:A transversions and G:C to A:T transitions. Excess iron or copper can participate in the Fenton reaction to generate highly reactive hydroxyl radicals (OH[•]). The administration of iron or copper induced the production of OH[•] radicals in the livers of experimental animals (46, 47). Treatment of normal human fibroblasts with H₂O₂ and FeCl₃ induced G:C to T:A and C:G to A:T transversions at p53 codons 249 and 250,

respectively (13). Iron and copper also bind directly to DNA and can induce site-specific DNA damage very effectively by producing Fenton oxidants (26, 48).

Evidence of lipid peroxidation has been reported in both WD and hemochromatosis patients (49–51). Reactive aldehydes such as 4-HNE, malondialdehyde, and crotonaldehyde are produced during lipid peroxidation and can generate exocyclic DNA adducts (reviewed in ref. 32). Whereas 4-HNE forms etheno-DNA adducts such as ethenodeoxyguanine, ethenodeoxycytosine, or ethenodeoxyadenine, the major DNA adduct produced by malondialdehyde is a pyrimidopurine or M₁G. A high level of etheno-DNA adducts have been reported in liver from hemochromatosis and WD patients (52). Both etheno-DNA adducts and M₁G can induce a number of different point mutations (reviewed in refs. 32, 53, and 54). Our results are consistent with these reports, in that, 4-HNE preferentially induced G:C to T:A transversions at p53 codon 249 (AGG to AGT) in TK6 lymphoblastoid cells.

In addition to inducing DNA alteration including point mutations in genes, oxidative stress can modulate cell growth and tumor promotion. Reactive oxygen species can activate signal transduction pathways, which result in the transcriptional induction of growth competence-related proto-oncogenes, e.g., *c-fos*, *c-jun*, and *c-myc* (55). Mechanistic studies have shown that protein phosphorylation and polyADP-ribosylation of chromosomal proteins are involved in the transcriptional induction of *c-fos* by oxidants (56), and that a pro-oxidant state can promote neoplastic growth (reviewed in ref. 31).

WD and hemochromatosis patients bear an excessive burden of oxidative stress. Our data are consistent with the hypothesis that the metal overload-generated reactive oxygen/nitrogen species and reactive aldehydes such as 4-HNE can induce mutations in the p53 tumor suppressor gene in WD and hemochromatosis patients. These results warrant further studies to determine the correlation between p53 mutation load and cancer risk in these oxyradical overload diseases.

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