Oxidative Stress and p53 Mutations in the Carcinogenesis of Iron Overload-Associated Hepatocellular Carcinoma


The incidence of hepatocellular carcinoma (HCC) in individuals with hereditary hemochromatosis is 200 times greater than in the general population (1). This increased risk of HCC may be the result of deregulation of oxidation reduction and generation of reactive oxygen species from free iron, directly through the Fenton reaction and indirectly through the acceleration of lipid peroxidation [reviewed in (2)].

In an iron–nitroliotriacetic acid rat model of hemochromatosis, renal samples showed an increase in the levels of several reactive intermediates, including 4-hydroxy-2-nonenal and malondialdehyde (3,4), both of which are known to be cytotoxic and genotoxic (5,6). These increases were accompanied by decreased availability of systems that protect against oxidation by iron, such as vitamin E levels, the glutathione system, thiol-specific antioxidants, and superoxide dismutase (7,8). In addition, excess iron provides a strong growth-promotion advantage in human hepatoma cell lines and chemically induces carcinomas in experimental animals (9,10).

Previously, we have reported higher frequencies of p53 mutations, including G:C to T:A transversions at codon 249 and C:G to A:T and C:G to T:A changes at codon 250 in liver tissue samples from cancer-free patients with either hemochromatosis or Wilson’s disease (11). We present here a detailed analysis of 14 cases of hemochromatosis-associated HCC (Fig. 1, A), including the p53 mutation spectrum, Prussian blue stain for iron (Fig. 1, B), and immunohistochemical analysis of p53, nitric oxide synthase-2 (NOS-2), and cyclooxygenase-2 (COX-2). The study protocol was submitted to the human subjects committee at our institution but was exempted, because the tissues were obtained in the course of patients’ treatment and without the knowledge of patients’ identities. Histologically, all tumors, except three, were well differentiated. Nuclear p53 overexpression was observed in eight of 14 HCC cases (Fig. 1, C) with varying intensity, including two (+1), two (+2), three (+3), and one (+4), according to the following
scoring scheme: less than 10% nuclear reactivity = 0, greater than 11% to less than 25% = +1, greater than 25% to less than 50% = +2, greater than 50% to less than 90% = +3, and greater than 90% = +4 (12,13). Cytoplasmic NOS-2 immunoreactivity was seen in one HCC and in eight adjacent non-neoplastic regenerative nodules (Fig. 1, D). COX-2 overexpression was not seen in these cases of HCC.

p53 mutations in nine of 14 HCCs were identified by p53 microarray analysis (14) and confirmed by automated DNA sequencing. Two possible hotspots were identified in exon 7 (codon 275, three tumors), with A:T to C:G transversions, and in exon 8 (codon 298, two cases), with G:C to C:G transversions. Overall, G:C to C:G transversions were present in four (44%) of nine cases, and three (33%) were A:T to C:G transversions. The two other mutations included a G:C to A:T transition and a G:C to T:A transversion (Fig. 2, A). Substantial differences were apparent in p53 mutation spectra from these HCC when compared with those reported by Vautier et al. (15). Although the prevalence of p53 mutations in both studies was similar (71% versus 64%), 60% of the tumors displayed A:T to G:C transitions, and 40% showed A:T to T:A transversions in the report by Vautier et al. (15) (Fig. 2, B). Some of the differences may be attributable to possible differences in the prevalence of other contributing factors between the two studies, such as viral hepatitis and alcoholic liver disease. In addition, both the incidence and spectra are vastly different from HCC of other causes (16) (compare panels A–C with panel D, Fig. 2). These differences suggest that multiple underlying mechanisms can lead to HCC, and that these mechanisms are different in hemochromatosis-associated HCC and HCC associated with other etiologic factors, such as aflatoxin B1 and viral hepatitis (17). Furthermore, although we have previously reported higher frequencies of G:C to T:A transversions at codon 249 and C:G to A:T transversions in codon 250 in non-neoplastic liver samples from patients with hemochromatosis and Wilson’s disease (17), we were not able to detect similar p53 genetic alterations in neoplastic samples. It is possible that mutational spectra in p53 similar to those observed here would be detectable in non-neoplastic specimens from the same patients. However, mutational load assays could not be performed because no fresh-frozen tissues were available. On the other hand, if this were the case and similar p53 mutational spectra were also detected in non-neoplastic tissues in codons 275 and 298, it is possible that these mutations could provide cellular growth advantage over mutations elsewhere in the p53 gene. In vitro studies have shown “gain of function” such as transactivation, allowing a cellular growth advantage over mutations elsewhere. Increased NOS-2 expression has been associated with several malignancies (19–22) and preneoplastic chronic inflammatory conditions (20,23–26). The overexpression of NOS-2 and NO in the non-neoplastic hepatic regenerative nodules—that are both preneoplastic and associated with increased risk of HCC—as presented here indicates a possible role in hepatic carcinogenesis. Although the frequency of NOS-2 over-expression in the non-neoplastic hemochromatosis tissues adjacent to the tumors is higher in our study than reported.

Fig. 2. p53 mutation spectra in hepatocellular carcinoma (HCC). A) p53 mutation spectrum in 14 cases of hemochromatosis-associated HCC. Although the incidence of p53 mutations was similar in our case series and in those cases reported by Vautier et al. (15) (64% versus 71%), marked differences were observed. Forty-five percent of the samples exhibited G:C to C:G transversions and another 33% showed A:T to C:G transitions compared with these data (B), whereas 60% of the tumors (a total of 10 mutations among 14 samples) showed A:T to G:C and another 40% showed A:T to T:A. Methods: Slides were deparaffinized with xylene and ethanol, microdissected, and digested at 37 °C in a solution of sodium dodecyl sulfate/Proteinase K for several days until tissues were dissolved completely. DNA extraction was performed by use of the phenol–chloroform method as described elsewhere (35). Then, the p53 status was established by use of sample DNA and p53 gene chip methodology according to the manufacturer’s protocol (Affymetrix, Santa Barbara, CA). Exons 2–11 of the p53 gene were amplified by use of a multiplex polymerase chain reaction, fragmented and labeled with fluorescein adenosine monophosphate tag, and hybridized onto a GeneChip® with 2 nM control oligonucleotide, supplied by the manufacturer, for 30 minutes at 45 °C (Affymetrix). The resultant chips were scanned on a Hewlett-Packard scanner (Santa Clara, CA). The scanned results were compared with a wild-type p53 sequence. With the aid of a detection algorithm, mutations in the test samples were detected and assigned a scoring number based on the differences in hybridization intensity of every mutation. DNA from cell lines with known p53 gene mutation status was used as a control with each run. Twenty percent of the samples were repeated twice as a quality-control measure. Only mutations with a hybridization followed by scanning score of at least 13 were considered to be valid; a score of less than 13 indicates no evidence of mutation (14). To eliminate any false-positive results, confirmatory standard automated sequencing was performed on each mutation. Del = deletion; Ins = insertion.
previously (57% versus 28%), the disease grade is different, because all of our samples were cirrhotic compared with 33% in our previous work (11). NO generated by NOS-2 has been reported to induce mutations in vitro and in animal models (27,28), and DNA damage by several mechanisms, such as nitrosative deamination or induction of lipid peroxidation (28,29), as well as under-
mine cellular DNA repair processes per-
formed by DNA glycosylase (30). In ad-
dition, ionic iron has been shown to modulate NO-mediated cell death and apoptosis (31). We have reported higher NOS-2 enzymatic activity in colonic adenomas than in colonic carcinomas (19). Furthermore, high levels of NOS-2 are associated with p53 mutations in colorectal neoplasms, particularly those with G:C to A:T transitions at CpG di-
nucleotides by a mechanism of NO-increased deamination of 5-methylcyto-
sine (19,32). However, the p53 mutation spectrum in HCC associated with hemochromatosis reported here and else-
where (15) is more consistent with some other unknown endogenous mutagenic mechanism that is mediated by lipid per-
oxidation, based on the types of muta-
tions detected. The increase in etheno →dG and →dA DNA adducts, caused by lipid peroxidation in liver tissues from hemochromatosis patients (33,34), and the p53 mutational load (11) in liver tis-
 sue from individuals with hemochrom-
atosis are both consistent with the hy-
pothesis of generation of oxynitrals as a
underlying mechanism of cancer in heavy metal overload diseases (16).

REFERENCES

(1) Hoising AW, McLaughlin JK, Olsen JH, Mel-
lemkjar L, Wacholder S, Fraumeni JF Jr. Cance-
rrisk following primary hemochroma-
tosis: a population-based cohort study in

(2) Meneghini R. Iron homeostasis, oxidative stress, and DNA damage. Free Radic Biol

(3) Toyokuni S, Uchida K, Okamoto K, Hattori-
nakakuki Y, Hiai H, Stadtmann ER. Forma-
tion of 4-hydroxy-2-nonalenal-modified pro-
teins in the renal proximal tubules of rats
reated with a renal carcinogen, ferric nitro-
litrocatechate. Proc Natl Acad Sci U S A 1994;

(4) Fukuda A, Osawa T, Oda H, Tanaka T, To-
yokuni S, Uchida K. Oxidative stress re-
sponse in iron-induced acute nephrotoxicity:
enhanced expression of heat shock protein
90. Biochem Biophys Res Commun 1996;
219:76–81.

(5) Esterbauer H, Eckl P, Ortuor A. Possible mu-
tagenic origins from lipids and lipid precu-

(6) Esterbauer H, Schaur RJ, Zollner H. Chem-
istry and biochemistry of 4-hydroxynonalen,
malonaldehyde and related aldehydes. Free

(7) Cheng KC, Cahill DS, Hasai H, Nishimura S,
Loeb LA. 8-Hydroxyguanine, an abundant
form of oxidative DNA damage, causes C→T and A→C substitutions. J Biol Chem

(8) Dabbagh AJ, Mannion T, Lynch SM, Frei B.

(9) Hann HW, Stahlhut MW, Hann CL. Effect of
iron and desferoxamine on cell growth and in vitro ferritin synthesis in human hepatoma

(10) Smith AG, Carthep P, Clother B, Constan-
tin D, Francis JE, Madra S. Synergy of iron in
the toxicity and carcinogenicity of polychlorinated biphenyls (PCBs) and related

(11) Hussain SP, Raja K, Amstad PA, Sawyer M,
Trudel LJ, Wogan GN, et al. Increased p53
mutation load in nontumorous human liver of
Wilson disease and hemochromatosis: oxy-
radical overload diseases. Proc Natl Acad Sci

(12) Marrogi AJ, Travis WD, Welsh JA, Khan MA,
Rahim H, Tazelaar H, et al. Nitric oxide
synthase, cyclooxygenase-2, and vascular en-
dothelial growth factor in the angiogenesis of
2000;6:4739–44.

(13) Przygodzki RM, Bennett WP, Guineen DG Jr,
P53 mutation spectrum in relation to GSTM1,
CYP1A1 and CYP2E1 in surgically treated
patients with non-small cell lung cancer.

(14) Ahrendt SA, Halachmi S, Chow JT, Wu L,
Halachmi N, Yang SC, et al. Rapid p53 se-
quence analysis in primary lung cancer using
an oligonucleotide probe array. Proc Natl

(15) Vautier G, Bombfod AB, Portman BC, Me-
tivier E, Williams R, Ryder SD. P53 muta-
tions in British patients with hepatocellular
carcinoma: clustering in aggressive hepa-

(16) Hussain SP, Harris CC. P53 mutation spec-
trum and load: the generation of hypotheses
linking the exposure of endogenous or exog-
enous carcinogens to human cancer. Mutat

(17) Hachiyi M, Chumakov A, Miller CW, Aka-
shi M, Said J, Koeffler HP. Mutant p53 pro-
teins behave in a dominant, negative fashion

(18) Murphy KL, Dennis AP, Rosen JM. A gain
of function p53 mutant promotes both geno-
ic instability and cell survival in a novel
p53-null mammary epithelial cell model.

(19) Amba S, Bennett WP, Merriam WG, Ogun-
fusika MO, Oser SM, Harrington AM, et al.
relationship between p53 mutations and in-
ducible nitric oxide synthase expression in
human colorectal cancer. J Natl Cancer Inst

(20) Wilson KT, Fu S, Ramanujam KS, Meltzer
SJ. Increased expression of inducible nitric
oxide synthase and cyclooxygenase-2 in Bar-
rett’s esophagus and associated adenocarcin-

(21) Goto T, Haruma K, Kitadai Y, Ito M, Yoshi-
hara M, Sumii K, et al. Enhanced expres-
sion of inducible nitric oxide synthase and
nitrotyrosine in gastric mucosa of gastric
cancer patients. Clin Cancer Res 1999:5:
1411–5.

(22) Marrogi A, Pass HI, Khan M, Metheny-
Barlow LJ, Harris CC, Gerwin BF. Human
mesothelioma serology: coexpress both cyc-
looxygenase-2 (COX-2) and inducible nitric
oxide synthase (NOS2): in vitro antiprolifera-
tive effects of a COX-2 inhibitor. Cancer Res

(23) Majano PL, Garcia-Monzon C, Lopez-Caberra
M, Lara-Pezzi E, Fernandez-Ruiz E, Garcia-
Iglesias C, et al. Inducible nitric oxide syn-
thease expression in chronic viral hepatitis.
Evidence for a virus-induced gene upregula-

(24) Vickers SM, MacMillan-Crow LA, Green M,
Ellis C, Thompson JA. Association of in-
creased immunostaining for inducible nitric
oxide synthase and nitrotyrosine with fibro-
blast growth factor transformation in pancre-

(25) Hussain SP, Amstad P, Raja K, Amba S, Na-
gashima M, Bennett WP, et al. Increased p53
mutation load in noncancerous colon tissue
from ulcerative colitis: a cancer-prone chronic
inflammatory disease. Cancer Res 2000:
60:3333–7.

(26) Tatemichi M, Ogura T, Nagata H, Esumi H.
Enhanced expression of inducible nitric ox-
ide synthase in chronic gastritis with intesti-
240–5.

(27) Zhuang JC, Wright TL, deRosas-Walker T,
Tannenbaum SR, Wogan GN. Nitric oxide-
induced mutations in the HPRT gene of hu-
man lymphoblastoid TK6 cells and in Salmo-
nella typhimurium. Environ Mol Mutagen

(28) Gal A, Wogan GN. Mutagenesis associated
with nitric oxide production in transgenic SJL
mice. Proc Natl Acad Sci U S A 1996:93:
15102–7.

(29) Alagel H, Erdem E, Sancak B, Turkmem G,
Camlibel M, Bugdayci G. Nitric oxide bio-
synthesis and malondialdehyde levels in ad-

(30) Wink DA, Vodovotz Y, Laval J, Laval F,
Dewhirst MW, Mitchell JB. The multifaceted
roles of nitric oxide in cancer. Carcinogene-

(31) Kim YM, Chung HT, Simmons RL, Bilier
TR. Cellular non-heme iron content is a de-
terminant of nitric oxide-mediated apoptosis,
necrosis, and caspase inhibition. J Biol Chem


NOTES

We thank Dorothea Dudek for editorial assistance. Manuscript received December 26, 2000; revised August 24, 2001; accepted September 4, 2001.