Integration of Woodchuck Hepatitis and N-myc Rearrangement Determine Size and Histologic Grade of Hepatic Tumors

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Integrations of woodchuck hepatitis virus (WHV) DNA and rearrangements of the N-myc 2 gene have been detected frequently in hepatocellular carcinoma (HCC) of Eastern woodchucks (*Marmota monax*) chronically infected with WHV. Fifty-five hepatocellular neoplasms and matched nontumor hepatic tissue specimens obtained postmortem from 13 chronic WHV carriers were analyzed and the frequency of WHV DNA integrations and of N-myc rearrangements compared in tumors of different size and histologic grade. Four small tumor nodules were classified histologically as adenomas and integrated sequences of WHV DNA were detected in two of the four tumor nodules. In one of the two nodules, there was evidence of N-myc rearrangement. Fifty-one neoplasms were classified as HCC. Seven were grade 1 HCCs. WHV DNA integrations were demonstrated in 43% but none had N-myc rearrangements. Twenty grade 2 HCCs had WHV DNA integrations in 80% and in 38% N-myc rearrangements were present. Twenty-four grade 3 HCCs had integrations of WHV DNA in 79% and N-myc rearrangements in 74%. In two other grade 3 HCCs, rearrangements of N-myc were detected in the absence of WHV DNA integrations. The 12 largest tumors in the series all were grade 2 or 3 HCCs, and in 83%, both WHV DNA integrations and N-myc rearrangements were demonstrated. In conclusion, molecular changes observed in this study suggest a progression of genetic alterations providing either a significant proliferative stimulation and/or a growth advantage in hepatocarcinogenesis of woodchucks with chronic WHV infection. (HEPATOLOGY **2004;39:1008–1016.**)

Primary liver cancer is one of the most common human malignancies worldwide.[1,2] Epidemiologic studies have shown that hepatitis B virus (HBV) infection is a significant risk factor for the development of hepatocellular carcinoma (HCC).[3,4] Development of HCC in HBV-infected individuals characteristically requires several decades, suggesting that malignant transformation of hepatocytes is a complex process possibly involving multiple genetic and epigenetic alterations.[5] The multistage process of hepatocarcinogenesis is suggested by histologic changes in the liver that are presumed to be related to molecular events that lead to aberrant gene expression including activation of protooncogenes, inactivation of tumor suppressor genes, and loss of heterozygosity.[5–7]

Woodchucks (*Marmota monax*) chronically infected with the woodchuck hepatitis virus (WHV) now serve as a useful animal model for investigation of viral hepatocarcinogenesis.[8,9] WHV is a member of the family Hepadnaviridae, for which HBV is the prototype virus.[10] Experimental infection of newborn woodchucks with WHV results in a high rate of chronic infection that almost always leads to development of HCC.[8,9] Moreover, the precancerous hepatic lesions observed in WHV-infected woodchucks are similar to those of other animal models of hepatocarcinogenesis.[9,11–14]

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; WHV, woodchuck hepatitis virus; GGT, gamma glutamyltranspeptidase; WHcAg, woodchuck hepatitis virus core antigen; FAH, foci of altered hepatocytes; GSHV, ground squirrel hepatitis virus.

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Integration of hepadnaviral DNA into the host cell genome results in insertional mutations that are associated with the malignant transformation of hepatocytes.[5,15–17] The sites of HBV integration that lead to aberrant gene expression appear to be random in the HCCs of humans. In the woodchuck model of hepadnaviral-induced HCC, however, WHV integrations affecting the *myc* family of protooncogenes, especially the N-myc 2 retroposon, have been demonstrated frequently.[15,16,18,19]

Reports of integrated WHV DNA sequences in small neoplastic nodules and in apparently nonneoplastic hepatic tissue of chronic WHV carrier woodchucks have suggested that integration occurs early in the process of hepatocarcinogenesis and possibly before clonal expansion of individual tumors.[20,21] It also has been proposed that epigenetic changes associated with the integration of viral trans-activators maintain hepatocytes in a proliferative state that predisposes to malignant transformation.[6,16,22] The mechanism by which N-myc 2 expression is enhanced in woodchuck HCC is known to involve enhancer insertional activation,[23] but the stage at which this occurs during progression to the malignant phenotype has not been fully established. The purpose of this study was to determine the relation between the size and histologic grade of hepatic neoplasms of woodchucks and the presence of WHV DNA integrations and N-myc 2 rearrangements.

Materials and Methods

Experimental Animals. The 13 chronic WHV-carrier woodchucks used in the study ranged in age from 24 to 39 months (median age, 30 months). Three were female and 10 were male. The body weights ranged from 2.38 kg to 4.31 kg. No abnormalities in physical appearance or body condition were apparent at the time of euthanasia. The animals were born, reared, and maintained in laboratory animal facilities of the College of Veterinary Medicine, Cornell University (Ithaca, NY). Water and laboratory chow based on a rabbit formulation were provided *ad libitum.* The animals were inoculated at birth with 100 μL of a 1:10 dilution of the WHV7P1 inoculum containing approximately 5 million woodchuck infectious doses of WHV. Protocols for animal studies were approved by the Cornell University institutional animal care and use committee.

The antemortem diagnosis of HCC was based initially on increasing serum activity of gamma-glutamyltransferase (GGT) measured colorimetrically using L-gamma-glutamyl-3 carboxy-4 nitroamilade and glyclyglycine as substrates (Sigma, St. Louis, MO). Ultrasound examinations of the liver were used to confirm the presence of hepatic tumors before euthanasia (HP Sonos 100, Hewlett Packard). Characteristically, the smallest tumors detected by this method are greater than 1 cm in diameter. Tumors of some woodchucks were examined two or more times and changes in tumor volume were used to calculate tumor volume doubling time.[24] Woodchucks were euthanized when serum GGT levels reached persistently high levels or when ultrasound examination demonstrated the presence of significant tumor masses. The animals were anesthetized initially with ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) and euthanized with an overdose of pentobarbital administered by intracardiac injection.

Liver samples were obtained at necropsy from the 13 chronic WHV-carrier woodchucks. Postmortem examinations were performed, macroscopically observed hepatic neoplasms were removed and measured, and tumor volumes were calculated on the basis of mean tumor diameter. Tumor burden was defined as the sum of all individual neoplasms (identified at necropsy) that were greater than1 cm^3 in volume. Aliquots of hepatic tissue specimens that appeared macroscopically to be tumor free and aliquots of each tumor were snap frozen in liquid nitrogen and stored at $-70°C$ until molecular analyses were performed. Aliquots of each neoplasm and of nontumor liver tissue specimens adjacent to the tissue specimens used for molecular studies were fixed in phosphate-buffered formalin, embedded in paraffin, sectioned, and stained for histologic examination.

Histopathologic Examination of Hepatic Neoplasms. Specimens of formalin-fixed, nontumor liver tissue and of hepatic tumor tissue were sectioned and stained with hematoxylin-eosin or for WHV core antigen (WHcAg) expression as described previously.[25] Nontumorous hepatic tissue specimens were evaluated histologically for the presence of preneoplastic foci of altered hepatocytes (FAH). Hepatic tumor tissue specimens were classified either as hepatocellular adenoma or as HCC of grades 1, 2, or 3 based on increasing degrees of anaplasia. The histologic classification used was based on the established classification of preneoplastic liver lesions of animals[11–13] and the grading system for HCC.[26]

Molecular Analysis of Hepatic Tumors. DNA isolation and Southern blot analysis were performed by standard molecular methods.[27] Total cellular DNA samples were extracted from tumor and from nontumor liver tissue specimens. Cellular DNA (30 μg) specimens were digested with the restriction enzyme HindIII (Roche, Indianapolis, IN) and the resultant DNA fragments were separated by electrophoresis through 0.9% agarose gels, followed by capillary transfer to nylon membranes (GeneScreenPlus, NEN, Boston, MA). Hybridization was performed in Church buffer

consisting of 0.5 mol/L $NaPO_4$, 7% sodium dodecyl sulfate, 1 mmol/L ethylenediaminetetraacetic acid, and 100 μg/mL yeast *t*RNA at 60°C. The 3.3-kb EcoRI fragment of pWHV-2 and the 2.6-kb BglII/HindIII fragment of pKSE[18] were used to generate WHV-specific and N-myc–specific probes, respectively, by random-primed, DNA synthesis (LifeTechnologies, Gaithersburg, MD) incorporating [^{32}P]dCTP (3,000 Ci/mmol, NEN). Hybridization signals were detected by Phosphor-Imaging (Amersham Biosciences Corp, Piscataway, NJ). Using a specimen of cloned plasmid DNA as a standard, the limit of detection for this technique was 5 pg of homologous DNA. To detect a single copy of the gene, approximately 8 μg of total cellular DNA was required. In our assays, 30 μg of cellular DNA was analyzed. Each sample was analyzed in two separate experiments, and in some cases reported a third time to ascertain the presence or lack of integration or rearrangement.

The restriction enzyme HindIII cuts once within the WHV genome and cuts outside both the N-myc alleles. Southern blot analysis of cellular DNA digested with HindIII and hybridized with a WHV-specific probe reveals a WHV-specific fragment that migrates at 3.3 kb and represents the double-stranded, linear form of the covalently closed circular WHV genome found in the nucleus of WHV-infected cells. A smear smaller than 3 kb represents partially double-stranded or single-stranded WHV DNA, characteristic of WHV replicative intermediates. WHV-specific fragments migrating greater than 3.3 kb were interpreted as viral integrations. The WHV-specific fragment detected at approximately 2.8 kb represents the partially double-stranded linear form of WHV DNA found in WHV particles. Southern blot analysis with the N-myc–specific probe identified two fragments with molecular sizes of 7.4 kb and 3.4 kb, which represent the N-myc 1 and N-myc 2 alleles, respectively. An N-myc–related gene migrating at 3.0 kb also was detected. N-myc–specific fragments migrating differently from these molecular species were interpreted as rearrangement of an N-myc allele.

Statistical Methods. Wilcoxon rank-sum analysis was used to determine the significance of differences between groups of tumors categorized either on the basis of histologic grade or genetic alteration. Correlation coefficients and significance were determined using the Spearman rank order correlation.

Results

Serum GGT Activity and Hepatic Tumor Burden. Progressive increases in serum GGT activity were detected in all the chronic WHV carrier woodchucks during development of HCC. Serum GGT activity before development of HCC ranged from 2 to 4 IU/L. At the time of euthanasia, GGT activity reached values of 55 to 1052 IU/L (Table 1). Terminal GGT activity was directly related to tumor burden. In the seven woodchucks with the highest tumor burdens (20-114 cm^3), the mean serum GGT of 394 $\pm$ 324 IU/L was significantly higher than that of 85 $\pm$ 23 IU/L in the six woodchucks with the lowest tumor burdens (3-16 cm^3).

There was a significant correlation between the antemortem measurements of hepatic tumor volume made by ultrasonography and the measurements made postmortem. Some larger tumors were observed by ultrasound to grow rapidly. Based on ultrasound examinations before euthanasia, the tumor volume doubling time was 16 days for the largest tumor of woodchuck 9050 (54.4 cm^3) and 22 days for the largest tumor of woodchuck. 4355 (95.3 cm^3). Rapid tumor growth was associated with progressive increases in the serum GGT in both of these woodchucks (Fig. 1).

Relation of Tumor Volume and Histologic Grade. Nonneoplastic tissue specimens showing normal hepatic architecture is shown in Fig. 2A. Chronic portal and parenchymal hepatitis were characteristic lesions in the nontumor hepatic tissue specimens of all 13 woodchucks. FAH were observed in the nontumor hepatic tissue specimens of these 13 woodchucks. These were primarily amphophilic, basophilic, or clear cell FAH. In one sample of hepatic tissue specimens in which no tumor had been recognized macroscopically, an adenoma was identified histologically, which contained a microscopic grade 1 HCC.

Fifty-five hepatic neoplasms identified postmortem in the 13 woodchucks were examined histologically (Table 1). Four were classified as adenomas and the remaining 51 tumors were classified as HCC. Seven well-differentiated tumors were classified as grade 1 HCC (Fig. 2B). Histologically, three of these seven contained components that were more characteristic of adenoma and the HCCs appeared to arise from the adenomas (tumor within a tumor). As with all other tumors in this series, the final histologic classification was based on the least differentiated (most anaplastic) characteristics of the tumor.

Twenty less well-differentiated tumors with wider trabeculae and mitotic figures were classified as grade 2 HCC (Fig. 2C). Their mean tumor volume was significantly greater than that of the adenomas ($P < .05$) but not significantly larger than grade 1 HCCs. The 24 least differentiated tumors were classified as grade 3 HCC (Fig. 2D). Their mean volume was significantly greater than that of tumors of histologic grade 2 and of adenomas ($P = .05$).

Table 1. Tabulation of Age, GGT, Size, and Histologic Grade of Tumors for Individual Woodchucks

Animal I.D.	*Sex †Age	GGT (IU/L)		Tumors 1	2	3	4	5	6	7	8
3842	F	91	‡Volume	8.2	0.065	0.014	0.91	0.042			
	33		§Grade	3	2	3	2	Ad			
3904	M	55	Volume	1.9	3.2	4.2	2.7	0.27	0.11	5.6	3.1
	37		Grade	3	3	3	2	Ad	3	3	1
3919	M	103	Volume	1.4	1.0	0.034	1.9	0.087	0.087	1.2	
	37		Grade	3	3	2	2	2	3	2	
3951	M	1052	Volume	23.1	3.2	51					
	27		Grade	2	2	3					
4134	M	70	Volume	6.4	4.6	2.4	0.83				
	28		Grade	2	3	3	2				
4151	M	116	Volume	0.76	2.7	0.18	0.76				
	28		Grade	1	2	1	1				
4338	F	305	Volume	51	12.3						
	25		Grade	3	2						
4355	M	111	Volume	95.3	17.7	1.4					
	27		Grade	3	3	3					
4388	F	105	Volume	29.5	15.6	0.18	0.98				
	24		Grade	3	3	2	3				
4771	M	275	Volume	13.7	20	1.4	4.4				
	24		Grade	2	3	2	3				
4944	M	407	Volume	1.2	51	6.1					
	26		Grade	2	3	2					
5158	F	73	Volume	nm	4.2	0.3	1.8	1.8	0.52	0.065	
	33		Grade	3	2	1	1	Ad	1	Ad	
9050	M	505	Volume	54.4	1.2	0.47					
	38		Grade	3	2	2					

*Sex: M = male, F = female.
†Age in months at time of death.
‡Volume expressed as cm^3, nm = not measured.
§Histologic grade of hepatocellular carcinoma (grade 1 most differentiated, grade 2 less differentiated, grade 3 least differentiated); Ad = adenoma.

Expression of WHcAg. The immunohistologic expression of WHcAg was examined in nontumor liver specimens and in all tumor tissue specimens of the 13 woodchucks. WHcAg expression was directly related quantitatively to the presence of replicative WHV DNA intermediates. In all cases, histologic expression of WHcAg was highest in nonneoplastic liver tissue specimens (Fig. 2E, F) in which the highest levels of replicative intermediates were observed in Southern blots (Fig. 3). WHcAg expression was invariably diminished in the FAH in nontumor liver specimens. WHcAg was detected in three of four (75%) adenomas, although at remarkably

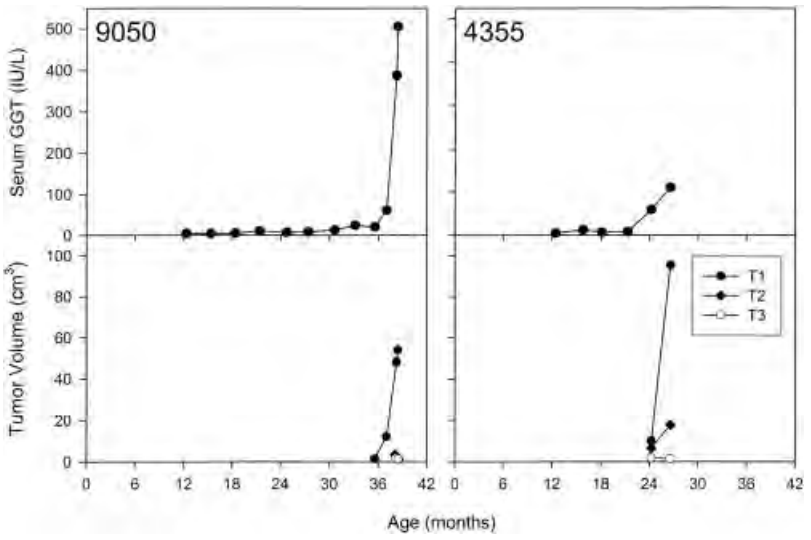

Fig. 1. Relation between hepatic tumor volume and serum GGT activity in two chronic WHV-carrier woodchucks with rapidly growing HCC.

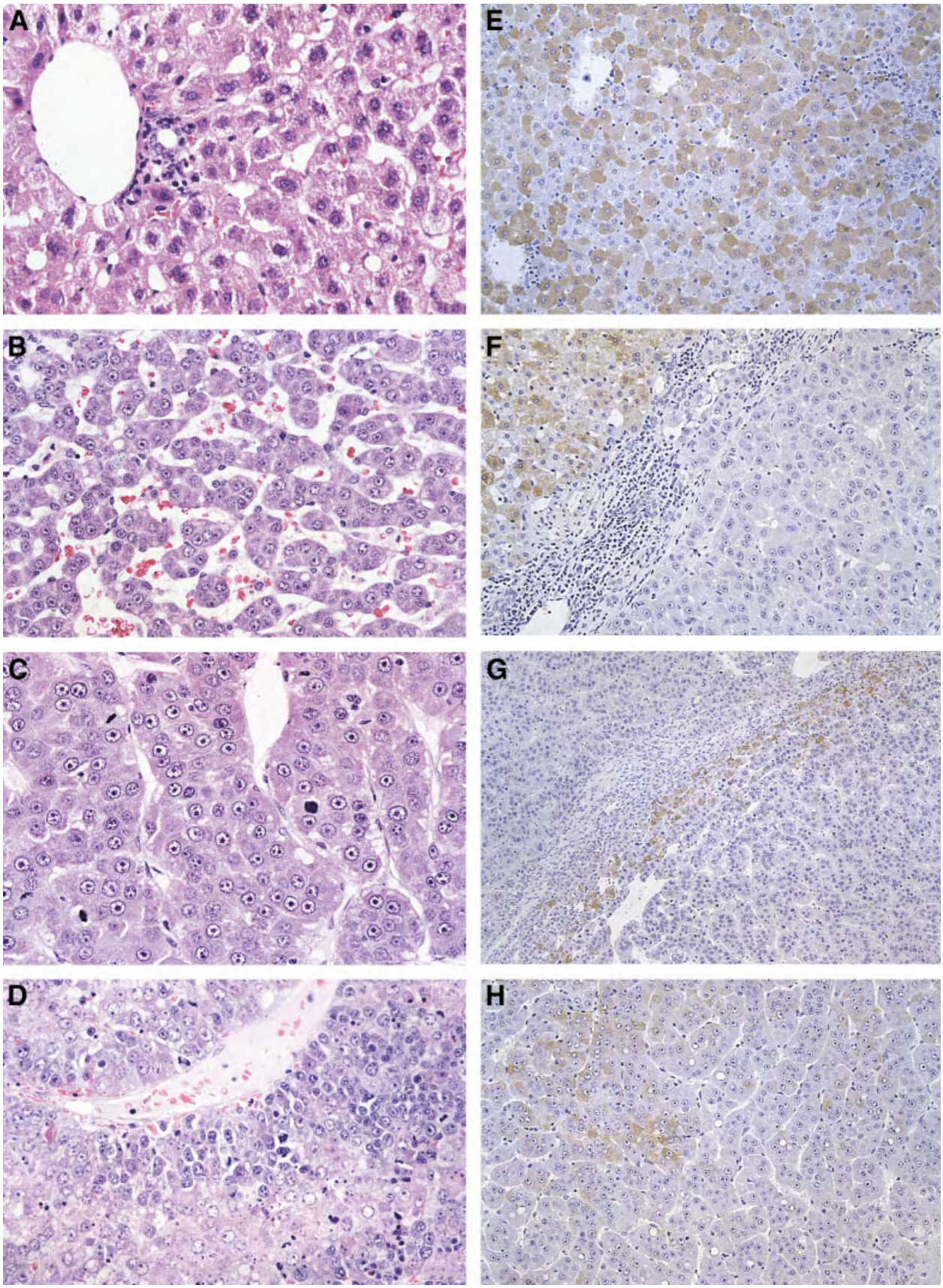

Fig. 2. Photomicrographs of woodchuck liver sections stained with hematoxylin-eosin. (A) Nonneoplastic tissue specimen exhibiting normal hepatic architecture. (B) Well-differentiated grade 1 HCC showing increased nuclear-to-cytoplasmic ratio, basophilic cytoplasm, prominent nucleoli, and thick hepatic plate. (C) Grade 2 HCC with mitotic figures and thick trabecules. (D) Grade 3 HCC showing more malignant features. Photomicrographs of liver sections stained immunohistochemically for WHcAg expression. (E) Nonneoplastic tissue exhibiting a mosaic pattern of WHcAg expression in distribution and intensity. (F) Nonneoplastic cells (**upper left**) expressing WHcAg in contrast to adjacent HCC cells negative for WHcAg expression. (G) WHcAg-positive cells along connective tissue between two neoplastic nodules. (H) Small group of WHcAg-positive neoplastic cells within an otherwise WHcAg-negative HCC.

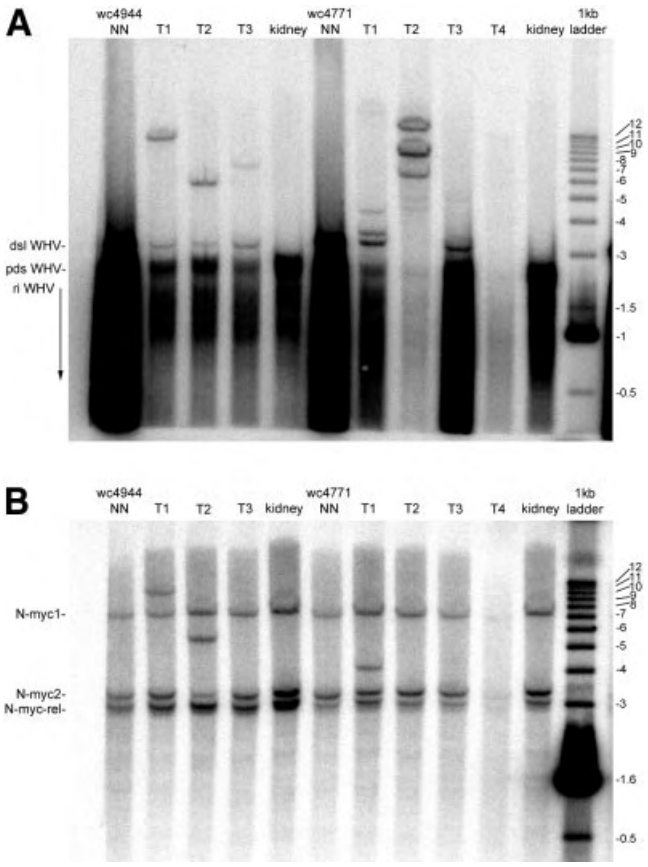

Fig. 3. Southern blots demonstrating WHV integrations and N-myc rearrangements in genomic DNA isolated from nonneoplastic tissue specimens and HCCs of chronic WHV-carrier woodchucks. (**Top**) Membrane probed for WHV sequences: double-stranded, linear (dsl) WHV DNA at 3.3 kb; partially double-stranded (pds) virion WHV DNA at 2.8 kb; and replicative intermediate (ri) WHV DNA smearing below 3 kb. Fragments migrating greater than 3.3 kb are indicative of integrated WHV DNA. (**Bottom**) Membrane probed for N-myc: N-myc 1 at 7.4 kb, N-myc 2 at 3.4 kb, and N-myc–related (rel.) gene at 3.0 kb. Fragments migrating differently from normal pattern are indicative of N-myc gene rearrangements. DNA isolated from woodchucks 4944 and 4771, nonneoplastic (NN), tumor (T), and kidney (KID) tissue specimens. A ^{32}P-labeled DNA ladder in 1 kb increments provided for reference.

diminished levels. In some HCCs, WHcAg was expressed in nonneoplastic hepatocytes located within connective tissue septa or adjacent to blood vessels within the HCC (Fig. 2G). In comparison to nonneoplastic tissue specimens, marked reductions in WHcAg expression were detected in neoplastic hepatocytes in 71% and 70% of grade 1 and grade 2 HCCs, respectively, and no expression of WHcAg was detected in neoplastic hepatocytes in the remaining grade 1 and grade 2 tumors. In grade 3 HCC, very low levels of WHcAg expression were found in neoplastic hepatocytes in 38%. When present within tumor hepatocytes of grade 3 HCCs, expression was confined to single cells or to small groups of neoplastic cells (Fig. 2H). In the 12 largest tumors of this series (12.3-95.3 cm^3), WHcAg was expressed in one and in that tumor only in rare single neoplastic hepatocytes.

WHV DNA Integration and N-myc Rearrangement. Hepatic tissue specimens in which no tumors were macroscopically observed were analyzed for WHV DNA integrations and N-myc rearrangements. In all 13 specimens, FAH were identified histologically. WHV DNA integrations were identified in 2 of 13 nontumor specimens of liver. In one of these specimens, a N-myc rearrangement also was observed. Another specimen identified as nontumorous at the time of postmortem examination contained an adenoma in which a microscopic grade 1 HCC was present. In the specimen analyzed by Southern blot that was taken adjacent to this lesion, no genetic alterations were detected. This data was not included in the analyses of the other 55 neoplasms, all of which had been recognized as tumors macroscopically.

In 13 of the 55 tumor specimens examined, no WHV DNA integrations or N-myc rearrangements were identified (Table 2, Fig. 4). These 13 tumor specimens generally were small (0.065-5.6 cm^3), but 7 of the 13 were classified either as grade 2 or 3 HCC. In 19 tumor specimens, WHV DNA integrations were present alone without evidence of N-myc rearrangement. Two of these were among the largest studied (12.3 and 20 cm^3), but the other 17 were among the smallest tumors in the series (0.034-6.1 cm^3). Fifteen were classified as grade 2 or grade 3 HCCs.

Twenty-three tumor specimens contained N-myc rearrangements. Two tumor specimens (0.11 and 2.4 cm^3) that were classified histologically as grade 3 HCC contained rearrangements of N-myc without detectable integrations of WHV DNA. In the other 21 tumor specimens, WHV DNA integrations and N-myc rearrangements were demonstrated together. In all 21 tumor specimens, cohybridization of at least one rearranged N-myc fragment and a WHV DNA integration was demonstrated. One of the group of 21 tumor specimens was classified histologically as an adenoma and 20 were classified as grade 2 or grade 3 HCC.

Seventeen neoplasias were less than 1.0 cm^3 in volume and four (24%) had both WHV DNA integration and N-myc rearrangements. Of the remaining 38 tumor specimens, 17 (45%) contained N-myc rearrangements, 15 of which were associated with detectable WHV DNA integrations. Of the 12 largest HCCs studied (12.3-95.3 cm^3), 10 (83%) had WHV DNA integrations associated with N-myc rearrangements. Three of the 12 HCCs were classified as grade 2 and nine were classified as grade 3.

In none of the 13 woodchucks was there evidence of distant metastasis to the lung or other tissues. Intrahepatic metastasis in the form of multiple satellite tumors adja-

Table 2. Relationship of Volume, Histologic Grade, and Genetic Alteration of Hepatic Neoplasms of Woodchucks With Chronic WHV Infection

	Histologic Grade			
	Adenomas	**Grade 1 HCC**	**Grade 2 HCC**	**Grade 3 HCC**
No. of Tumors	4	7	20	24
Mean vol. (cm^3) ± SD	0.28 ± 0.33	1.35 ± 0.97	4.1 ± 5.9	17.71 ± 24.96
Median	0.15	1.05	1.39	4.31
Range	0.042–0.76	0.3–3.1	0.034–23.1	0.014–95.3
Genetic Alteration	Number (%)	Number (%)	Number (%)	Number (%)
None	2 (50)	4 (57)	4 (20)	3 (13)
WHV integration only	1 (25)	3 (43)	10 (50)	5 (21)
N-myc rearrangement only	0 (0)	0 (0)	0 (0)	2 (8)
Both WHV and N-myc	1 (25)	0 (0)	6 (30)	14 (58)

cent to a large primary tumor also was not observed. Single, small tumor nodules sometimes were located adjacent to large grade 3 HCCs. In two of these, the patterns of WHV DNA integration and N-myc rearrangement were identical, suggesting their clonal identity, and they were metastatic in origin (data not shown).

Discussion

Activation of members of the myc family of protooncogenes is a recognized characteristic of HCC in woodchucks.[18,19,21,23,28–30] In most cases, increased gene expression is related to rearrangements of myc genes associated with integrations of subgenomic sequences of WHV DNA. By far the most frequently altered member of the myc family is N-myc 2, an intronless homolog of the conventional N-myc gene of woodchucks (N-myc 1), other species of rodents, and humans.[18,23,30] N-myc 2 is a retroposon that has been identified in the woodchuck, the Alpine marmot *(M. marmota)*, and the California ground (*Spermophilus beecheyi*), all members of the ground squirrel family, Sciuridae. Unlike most processed genes, N-myc 2 is functional but is expressed in normal adult woodchucks only in brain tissue.[31] N-myc 2 is not expressed in normal adult liver specimens, but significant expression has been detected in almost all precancerous FAH, in adenomas, and in HCC.[18,19,21,23,30]

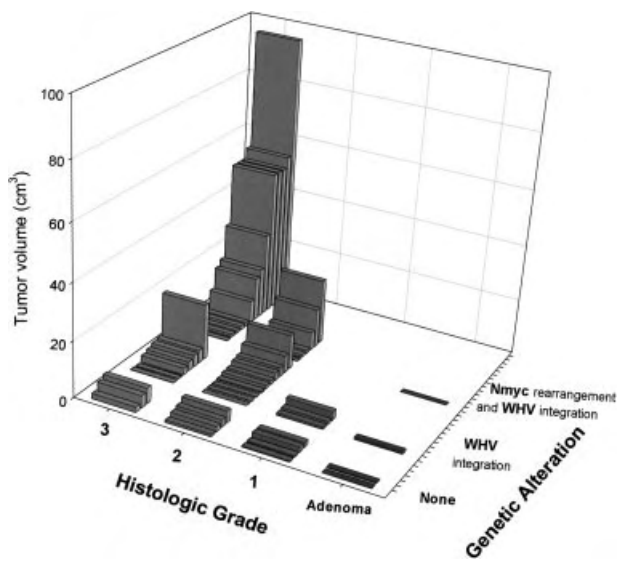

Fig. 4. Relation between histologic grade of tumor (X-axis), the calculated volume (Y-axis), and the status of WHV integrations and N-myc rearrangements (Z-axis) of 55 hepatic tumors. Tumors exhibiting both WHV integration and N-myc rearrangement were predominately large, grade 3 HCC.

A high frequency of transcriptional activation of N-myc 2 has been associated with N-myc rearrangements similar to those described in the current study. In hepatic tumor specimens described by Wei et al., N-myc 2 rearrangements either upstream of exon-1 or in the 3′ untranslated region of exon-3 were often detected.[29,30] In one series of 30 tumor specimens described by these authors, such rearrangements of N-myc were detected in 30%. In another group of 19 hepatic tumor specimens, rearrangements of N-myc were detected in 68%. It was suggested that the difference in the groups may have been related to differences in the stage of progression of the tumors when they were obtained. In almost all the tumor specimens, the abnormal N-myc 2 fragment comigrated with a WHV DNA integrant, an observation similar to other reports[19] and to the observations described in the current study.

We have examined the relation of size and histologic grade of WHV-induced HCC to the presence of WHV DNA integrations and N-myc rearrangements. WHV DNA integrations were detected in 75% of 51 histologically confirmed HCCs and N-myc rearrangements were detected in 43%. Of 22 HCCs with N-myc rearrangements, 91% were associated with detectable WHV DNA integrations. Similar frequencies of N-myc rearrangement have been reported previously.[18,19,30] In almost all tumor specimens in the current study, comigration of the aberrant N-myc fragment and a WHV DNA integrant sug-

gested integration was responsible for the rearrangement as has been demonstrated by others using direct cloning methods.[18,30]

In the current study, one of four small adenomas contained both WHV DNA integration and an N-myc rearrangement. Overall, however, the presence of an N-myc rearrangement was directly related to tumor size and histologic grade. N-myc rearrangements were not found in grade 1 HCCs, but 30% of grade 2 HCCs and 67% of the larger, more anaplastic grade 3 HCCs exhibited rearrangements of N-myc.

In one of the woodchuck HCCs described by Wei et al., the N-myc-2 allele was rearranged without evidence of WHV DNA integration.[30] Two similar tumors were found in the current series. Woodchuck hepatic tumors with rearrangements and amplified expression of the N-myc 1 gene or c-myc have been described but are unusual.[18,19,23,28–30] Because we used a probe that did not differentiate between N-myc 1 and N-myc 2, it is not possible to be certain of the relative frequency of N-myc 1 or N-myc 2 rearrangements, but N-myc 1 alterations in our series are assumed to be infrequent.

Three related lines of experimental evidence support the direct role of myc family genes in woodchuck hepatocarcinogenesis. N-myc 2 has been shown to cooperate with activated *ras* by cotransforming rat embryo fibroblasts in tumorogenicity assays in a manner similar to c-myc.[18] Mice transgenic for the N-myc 2 allele under the control of WHV regulatory elements have a high rate of HCC despite hypermethylation and low expression of the transgene. A founder mouse carrying unmethylated N-myc 2 died at 2 months of age with a large HCC which further demonstrated the oncogenicity of woodchuck N-myc 2.[32] Finally, in a malignant woodchuck hepatocyte cell line in which the N-myc 1 gene was rearranged and overexpressed, Wang et al.[33] used an N-myc antisense vector to silence gene expression. The oncogenic capacity of the resulting phenotype was remarkably diminished compared with that of the parent cell line, indicating that N-myc overexpression was essential for maintenance of the malignant phenotype.

California ground squirrels (*S. beecheyi*) are naturally infected with the ground squirrel hepatitis virus (GSHV), a hepadnavirus related to WHV and HBV. In a study by Seeger et al.,[34] separate groups of neonatal woodchucks were inoculated with WHV or with GSHV and the infectivity and the oncogenic capacity of the viruses were compared in the same host species. In GSHV-infected woodchucks, the DNA of all HCCs examined contained GSHV DNA integrants and in this regard were similar to the HCCs of woodchucks with chronic WHV infection. Subsequent molecular analyses of tumor specimens from woodchucks infected either with WHV or GSHV demonstrated differences that were similar to those shown in their respective natural hosts.[19] Rearrangements of N-myc were identified in 41% from WHV carriers. In contrast, the 3′ rearrangement of the N-myc 2 locus was detected in only 6% of HCCs from GSHV carriers. It is noteworthy that the first tumor to develop in GSHV-infected woodchucks (31 months of age) was the largest tumor (40 mm diameter) reported in this group of animals. In addition, the N-myc transcription was up-regulated, suggesting that the N-myc rearrangement contributed to tumor progression.

In human HCC, HBV DNA integrations appear to be distributed randomly throughout the genome and are infrequently associated with specific growth regulatory genes. Initially, WHV DNA integration may be random. Evidence for random integration of WHV DNA has been provided by lamda cloning experiments.[35] When integrations are associated with rearrangement and increased expression of N-myc 2, selective cellular growth advantage results in clonal expansion. This mechanism is similar to that in avian leukosis in which initial proviral integrations into lymphocytes of the bursa of Capricious occurs in multiple sites.[36] Integrations of avian leukosis provirus that activate c-myc are clonally expanded and are characteristic of the malignant tumors that ultimately develop.[37]

Woodchucks with chronic WHV infection have been an important predictive model for development of antiviral therapies for chronic HBV infection in humans.[38] Almost all experimental WHV-infected chronic carriers develop HCC with a median life expectancy of 29 months.[9] This animal model of viral hepatocarcinoma is now available for use in the preclinical development of methods for chemopreventive and genetic therapy of HCC.[48–50]

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