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Overview

Genetic Resistance to Chemical Hepatocarcinogenesis in the DRH Rat Strain

Ken Higashi, MD, PhD,[1,*] Ayumi Denda, PhD,[2] Taneaki Higashi, PhD,[3] and Hiroshi Hiai, MD, PhD[1]

The carcinogen-resistant inbred rat strain DRH established from closed-colony Donryu rats by use of selective brother-sister mating over 20 generations under continuous feeding of 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) maintains a highly resistant phenotype without carcinogen exposure for many years. We reported that the clonal expansion of preneoplastic glutathione S-transferase-P(GST-P)-positive foci induced by 3'-Me-DAB was less extensive in the liver of DRH rats than in the liver of susceptible strains, such as Donryu and F344, although levels of DNA adducts were comparable among these rats. Comparative studies of the events after initiation indicate that DRH rats are constitutionally less prone to cellular damage caused by continuous administration of 3'-Me-DAB than are parental Donryu rats. Consequently, the reduced growth response of the liver during the promotion stage may contribute to the low susceptibility to development of liver tumors. Genetic analysis of (F344 × DRH)F2 rats identified two quantitative trait loci, *Drh1* on chromosome 1 and *Drh2* on chromosome 4, which provide resistance to the development of GST-P-positive preneoplastic foci induced by 3'-Me-DAB during the early stage of its administration. The resistance to progression to hepatocellular carcinoma is affected solely by *Drh2*. These observations indicate that at least two genetic loci are critically involved in the steps leading to chemical hepatocarcinogenesis. The DRH rat is a useful experimental model with which to study genetic susceptibility and resistance to chemically induced liver cancers.

Origin of the Carcinogen-Resistant DRH Rat Strain

In 1983, Taneaki Higashi noticed that, in a closed-colony of Donryu rats (Charles River Japan, Inc., Tokyo, Japan), some animals were not susceptible to induction of γ-glutamyltransferase (GGT) by adminstration of 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) and remained healthy after feeding of the carcinogen (35). To generate a carcinogen-resistant inbred strain, selective brother-sister matings of resistant Donryu rats were repeated under continuous feeding of 3'-Me-DAB. Reduced GGT induction, healthy growth, and the absence of any nodular lesions in the liver were monitored as resistance phenotypes (17). After 20 generations of inbreeding, a pair of littermates was found to be homozygous for all the available genetic markers (40), and from them, the inbred DRH strain was established. After the 20th generation, the DRH rats were maintained without the feeding of 3'-Me-DAB (Seac Yoshitomi Co. Ltd., Fukuoka, Japan). The inbred strain is different from DON/Kyo and DON/Ham rats, and has been designated DRH/Seac. The growth curves of DRH/Seac rats are similar to those of their parental strain, Crj:Donryu. While consuming a normal diet, spontaneous tumors were not observed in the lungs, liver, or uterus by 57 weeks of age (40). At present, the animals and their fertilized eggs are maintained in the National Bio Resource Project-Rat (nbrprat@anim.med.kyoto-u.ac.jp).

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[1]Department of Pathology and Biology of Diseases, Kyoto University Graduate School of Medicine, Yoshida-konoe-cho, Sakyo-ku, Kyoto 606-8501, [2]Department of Oncological Pathology, Cancer Center, Nara Medical University, Kashiwara, Nara 634-8522, and [3]College of Nutrition, Koshien University, Takarazuka 665-0006, Japan.
[*]Corresponding author.

Compared with the parental Donryu rats, DRH rats have resistance to a wide variety of structurally different chemical carcinogens (Table 1). They are highly resistant to hepatocarcinogens such as 3'-Me-DAB, as well as to chemically related aminoazo derivatives, 2-acetylaminofluorene (28), and N-nitrosodimethylamine (41). Moreover, DRH rats are resistant to mammary cancers induced by administration of 7,12-dimethylbenz(a)anthracene (28), which is metabolically activated by a mechanism different from that of aminoazo carcinogens. Subcutaneous administration of N-nitrosomethylbenzylamine induces esophageal tumors in F344 rats after activation of the procarcinogen by the liver (25). The induction of esophageal tumors is also less extensive in DRH than susceptible F344 rats (Hiai and Soma, unpublished observation). On the other hand, DRH rats do not have resistance to cancers of the tongue and oral cavity induced by oral administration of 4-nitroquinoline (Hiai and Tanuma, unpublished data).

The DNA Adducts of 3'-Me-DAB Metabolites

Comparative studies of several hepatic pathways that produce active metabolite(s) from 3'-Me-DAB and/or of detoxification have been carried out extensively in Donryu and DRH rats (28, 44, 45). Some results are consistent with the low susceptibility of DRH rats to 3'-Me-DAB, although the mechanism of tumor resistance still remains to be elucidated. However, more recently, we reported that DNA adducts of 3'-Me-DAB metabolites were detected at similar amounts and with indistinguishable profiles in DRH and Donryu rats at several time points (42). These results indicate that formation of DNA adducts of 3'-Me-DAB and their

Table 1. Comparison of tumor incidence in Donryu and DRH rats induced by use of various carcinogens (28)

Carcinogen	Donryu			DRH		
	Total no.	Tumor bearing	%	Total no.	Tumor bearing	%
3'-CH_3-DAB	15	15	100	33	1	3.0
3'-CH_2OH-DAB	7	7	100	8	0	0
DAB	16	16	100	11	0	0
2-AAF	7	7	100	8	1	12.5
DMBA	7	5	71	6	0	0

Carcinogenic activities of 3'-methyl-4-dimethylaminoazobenzene (3'-CH_3-DAB), 3'-hydroxymethyl-DAB (3'-CH_2OH-DAB), DAB, and 2-acetylaminofluorene (2-AAF) in the liver of male, and of 7,12-dimethylbenz(a)anthracene (DMBA) in the mammary glands of female carcinogen-sensitive Donryu and carcinogen-resistant DRH rats. From 4 weeks of age on, male rats were given diets containing 0.06% 3'-CH_3-DAB, 0.064% 3'-CH_2OH-DAB, 0.57% DAB, or 0.06% 2-AAF, and were killed after 20, 15, 60, and 16 weeks, respectively. Each 66.7 mg of DMBA was given intragastrically to female rats at 7 and 8 weeks of age. DMBA-treated rats were given basal diet and were killed after 15 weeks. Tumors were observed macroscopically. These data were taken from reference 28, with permission.

excision repair do not differ significantly between the carcinogen-resistant DRH and carcinogen-sensitive Donryu rat strains. It is unknown at present which DNA adduct is responsible for initiation of chemical carcinogenesis in the rat liver.

Resistance of DRH Rats to Cytotoxic and Genotoxic Effects of 3'-Me-DAB

Although the amounts of DNA adducts in DRH rats are indistinguishable from those in susceptible Donryu rats, transcription of genes, the products of which are associated with DNA damage such as *GADD45* (growth arrest and DNA damage-inducible [11]) and *O^6-methylguanine DNA methyltransferase* (putatively DNA damage-inducible [12]) in the liver, is higher in Donryu than DRH rats during 3'-Me-DAB administration (42). Levels of *heme oxygenase* (due to degradation of heme-protein [19]) and *hepatocyte growth factor* (HGF: cell death and regeneration of hepatocytes [38]) mRNAs were also higher in F344 than DRH rats (42), suggesting that the former are more sensitive to genotoxic and cytotoxic effects of 3'-Me-DAB than are the latter.

It is generally considered that the regulation of p53 content during cellular damage is carried out at a posttranslational level (i.e., by proteolysis [16, 22]). We found that the p53 protein content in the liver of Donryu rats increased remarkably during 1-5 weeks of 3'-Me-DAB administration, whereas that in the liver of DRH rats increased little under the same conditions (14).

Another notable difference between these strains is the significant induction of cytochrome p450 2E1 mRNA expression in Donryu rat liver during 3'-Me-DAB administration (42). This may contribute to generation of reactive oxygen intermediates (ROI) (2, 21, 31) Hammad and co-workers (15) reported that hepatic microsomal lipid peroxidation in vitro in the presence of exogenous NADPH was lower in DRH than Donryu rats. Hirano and co-workers (18) reported that 3'-Me-DAB increased the repair enzyme activity for 8-hydroxyguanine (8-OH-Gua) in the Donryu, but not the DRH rat liver, because there was no increase in 8-OH-Gua after 3'-Me-DAB treatment. These results suggest that, in the liver, generation of ROI and response to them differs between DRH and Donryu rats during 3'-Me-DAB treatment. Since DRH rats have resistance to various chemical carcinogens activated via different metabolic pathways, it is conceivable that ROI generation by carcinogenic treatment is one of the factors inducing injury in the carcinogenic process especially during the promotion stage (27, 31). We could not detect any difference between DRH and Donryu rats in the mismatch repair system for DNA damage (Higashi, unpublished data) or in any of the several multidrug resistance systems in the liver (14). Comparisons of various characteristics, including immune response (29), between DRH and Donryu rats are shown in Table 2. It is still unknown how DRH rat liver is protected from the cytotoxic effects of xenobiotics.

Table 2. Comparison of phenotypic parameters between Donryu and DRH rats

Phenotypic parameters	Donryu	DRH
Normal liver		
Metabolic activation	++	+
Multidrug resistance	+	+
Mismatch DNA repair	+	+
Ploidy	4N > 2N	2N > 4N
Immune response	Weak	Normal
3'-Me-DAB treated		
DNA adduct	+	+
DNA damage	++	+ or -
Cellular injury	++	+ or -
Liver surface	Rough	Smooth
GST-P induction	+++	+
HCC	++	-

GST-P = glutathione S-transferase placental form; HCC = hepatocellular carcinoma.

Blunt Cell Growth Potential in Hepatocytes of DRH Rats

Lead nitrate is a potent mitogen for liver cells (8). A single intravenous injection of lead nitrate (100 μmol/kg of body weight) induced a transient wave of DNA synthesis in rat hepatocytes (3). However, the DNA synthesis in the liver of DRH rats treated with lead nitrate is greatly reduced compared with that in the liver of Donryu rats (7, 40). Furthermore, we observed delayed appearance of the onset and peak of DNA synthesis after partial hepatectomy in DRH, relative to Donryu rats (7). In the rat liver, growth during normal development is characterized by progressive polyploidization and a decrease in the fraction of diploid hepatocytes (13). Surprisingly, polyploidization of hepatocytes in DRH rats is significantly suppressed compared with that in Donryu rats of the same age (40), although the growth of DRH rats is similar to that of Donryu rats as described previously. It is likely that a balance between the formation and repair of DNA-adducts and extent of cell proliferation in the liver of rats exposed to hepatocarcinogens determines the probability of neoplasia, since the fixing of DNA damage may require DNA replication. After treatment with 3'-Me-DAB, a significant increase in the fraction of diploid hepatocytes was found in Donryu rats, which is commonly observed during hepatocarcinogenesis in rodents, probably due to the proliferation of small round cells containing diploid nuclei (34, 37). In contrast, little change in the ploidy pattern is observed in the DRH rat liver under the same conditions (40), which is also supported by the fact that few histologic changes to liver lobules are detected in DRH rats even

Table 3. Effects of *Drh1* and *Drh2* on the parameters of preneoplastic and neoplastic stages (43, 46)

Weeks	Parameters	*Drh1*	*Drh2*
7	Number of EAF	+++	+++
	Area of EAF	+	++
	GST-P mRNA	+++	+++
20	Number of tumors	-	++
	Area of tumor	-	++
	GST-P mRNA	++	-

EAF = enzyme-altered foci; weeks = period of 3'-Me-DAB treatment.
See Table 1 for key.

after treatment with 3'-Me-DAB for 7 weeks (data not shown). The reduced growth response of DRH rat liver may somehow contribute to the low susceptibility to liver tumors.

Genetic Resistance of DRH Rats to GST-P-positive Foci Induced by 3'-Me-DAB

It has been well established that 3'-Me-DAB induces multiple enzyme-altered foci (EAF) of liver cells with a high level of expression of GST-P and/or GGT (10, 30). Such foci have been assumed to be preneoplastic lesions for HCC (9, 33). Results of our previous study indicated that the mean area of GST-P-positive foci in the liver of DRH rats at 7 weeks of 3'-Me-DAB administration was about 4%, whereas that of Donryu rats was 23% (7). This resistance is inherited in an autosomal semi-dominant manner because reciprocal F1 rats from crosses between DRH and F344 rats express an intermediate amount of GST-P mRNA, relative to that expressed by the parental strains (7).

A genetic linkage mapping analysis was carried out on the GST-P-positive foci in the liver of (F344 × DRH)F2 rats during an early stage (7 weeks) of 3'-Me-DAB administration. For this analysis, we selected inbred F344 as a susceptible strain because the Donryu rat is a closed colony and is expected to share a large fraction of its genome with the DRH rat.

For the analysis of preneoplastic liver lesions, several quantitative parameters were selected, namely, the number of GST-P-positive foci per unit area of liver section, average size of foci, and GST-P mRNA levels (46). A composite interval mapping analysis of 108 (F344 × DRH)F2 rats revealed two remarkably significant clusters of quantitative trait loci (QTL) affecting preneoplastic liver lesions on rat chromosomes (RNO) 1 and 4 (Table 3). These clusters were designated collectively as *Drh1* and *Drh2*, respectively (46). Both *Drh1* and *Drh2* suppressed the number and size of GST-P-positive foci and GST-P mRNA expression semidominantly. In the (F344 × DRH)F2 rats, the number of EAF, their size, and GST-P mRNA levels were closely correlated to each other ($r > 0.7$) (46).

Genetic Resistance of DRH Rats to HCC Induced by 3'-Me-DAB

When fed 3'-Me-DAB for 20 weeks, all 11 F344 rats developed macroscopically evident HCC, but none of the 5 DRH rats developed any tumors. Seven of 12 (F344 × DRH)F1 rats developed comparable numbers of nodules/tumors to those in F344 rats, but the nodules/tumors were far smaller (43). These results indicate that the development of HCC is also under genetic control.

We tried to determine whether the QTL affecting preneoplastic lesions are the determinants of the later stage of hepatocarcinogenesis as well and whether there are any additional QTL in the progression stage by analyzing five quantitative parameters in 99 (F344 × DRH)F2 males (i.e., GST-P mRNA levels, ornithine decarboxylase activity, number of tumors and/or neoplastic nodules macroscopically detectable on the liver surface, and their size) (43). Genome-wide screening and composite-interval mapping for quantitative parameters revealed two major QTL peaks that coincided with the map positions of *Drh1* on RNO1 and *Drh2* on RNO4 (Table 3). Other significant QTL were not noted. The newly mapped QTL on RNO1 affected the GST-P mRNA levels, but not the number and size of HCC developing after 20 weeks of 3'-Me-DAB feeding. In contrast, the QTL on RNO4, co-mapped to *Drh2*, affected all parameters examined except for the levels of GST-P mRNA. Therefore, in the later stage of carcinogenesis, the locus on RNO4 (possibly *Drh2*) predominantly affected the progression of EAF to HCC. The candidates for this locus are genes encoding a growth hormone-releasing hormone receptor (36) and tumor growth factor alpha (24). Studying the genetic resistance to N-nitrosodiethylamine (DEN)-induced hepatocarcinogenesis in BN rats, DeMiglio and co-workers (5, 6) found a resistance QTL Hcr2 mapped close to *Drh2* on RNO4. In the later stage, GST-P expression was solely under control of the locus on RNO1. Therefore, the induction and clonal expansion of preneoplastic lesions are affected by *Drh1* and *Drh2*, but only a small fraction of these lesions progress to HCC (20). A variety of events during the progression may further complicate matters; therefore, the effects of the host genes are frequently not straightforward in the analysis of genetic predisposition to HCC. To further characterize the function and role of each locus, the construction of reciprocal congenic strains between DRH and F344 for *Drh1* and *Drh2* will be useful.

Effect of Partial Hepatectomy on HCC Development

The F1 generations from crosses between DRH and susceptible F344 rats are resistant to liver lesions induced by 3'-Me-DAB, but not to the same extent as are the parental resistant DRH rats, indicating an incomplete mode of inheritance. Accordingly, small liver tumors/nodules are occasionally induced in (F344 × DRH)F1 rats after administration of 3'-Me-DAB for 20 weeks, but without significant increase in serum AFP concentration (43). We evaluated the effect of partial hepatectomy on genetic resistance to development of HCC in (F344 × DRH)F1 rats, since hepatic regeneration could be expected to induce preneoplastic lesions (4, 23). Forced proliferation caused by partial hepatectomy was applied to F1 rats at 8 weeks from the start of treatment with 3'-Me-DAB after the formation of small preneoplastic foci. At 20 weeks after surgery, increases in the number and size of tumors were achieved by use of this modified protocol, although the incidence of liver tumors in these (F344 × DRH)F1 rats was still not the same as that in the parental F344 rats under the same conditions (26). It is likely that a regulatory mechanism of compensatory cell growth overcomes the block of the cell cycle in F1 rat liver. It is reminiscent of the lower or no increases in the expression of several cell cycle-related genes in nodules of carcinogen-resistant Wistar and Brown Norway rats that are induced by an initiation and selection protocol after DEN treatment (32).

Conclusions and Perspective

The DRH strain was established by inbreeding carcinogen-resistant rats among closed-colony Donryu rats under continuous feeding of 3'-Me-DAB. Such approach is a tedious but effective way of finding resistance gene(s). However, to the best of our knowledge, this is the first carcinogen-resistant animal model to be obtained by use of such protocol, the procedure taking more than 10 years. Despite their establishment in the presence of 3'-Me-DAB, DRH rats develop and reproduce normally for many years and do not exhibit spontaneous tumorigenesis in the liver or other organs when fed normal diets for more than one year (40), although the presence of some trivial mutations after such a selection protocol cannot be ruled out.

Several other rat strains resistant to liver carcinogenesis such as Copenhagen (Cop) and Brown Norway (BN) have been found (39). The Cop and BN rats have been reported to develop putative preneoplastic foci, but these lesions fail to develop into nodules (39). Precocious remodeling of preneoplastic liver lesions could account for the regression of Cop and BN lesions, but not cell death (39). Although there are several differences among Cop, BN and DRH rats, these carcinogen-resistant strains have several features in common concerning chemical carcinogenesis, including development of putative preneoplastic hepatocytes with the initiation of hepatocarcinogenesis is intact and preneoplastic lesions that fail to progress to HCC. In these resistant rats, the lesions fail to develop beyond the microscopic level, indicating that clonal expansion of enzyme-altered foci is suppressed. We are convinced that the clonal expansion of preneoplastic lesions in the liver of DRH rats is suppressed by their genetic background, which is controlled at least in part by modifier genes (1) located at *Drh2* on RNO4 (43).

Using the DRH model, several breakthroughs in clarifying the mechanism of tumor susceptibility/resistance are considered possible including: the search for candidate genes at *Drh1* and *Drh2* by narrowing of these loci with increased numbers of (F344 × DRH)F2 rats; characterization of the function and role of each locus through the construction of reciprocal congenic strains with DRH and F344 strains for *Drh1* and *Drh2*; a comparative study at the molecular level of several checkpoints of the cell cycle using DRH and either Donryu or F344 rats, since these strains have significant differences in the clonal expansion of preneoplastic lesions; examination of the possible participation of intrinsic blunt growth potential of DRH rats in tumor resistance and strong resistance to cytotoxic effects of chemical carcinogens, both of which protect hepatocytes from fixing DNA damage (i.e., DRH rats provide the opportunity to study the epigenetic effects of chemical carcinogens on the carcinogenesis of hepatocytes); and genetic analysis of (F344 × DRH)F2 rats, suggesting that enzyme-altered foci such as GST-P-positive foci are required to create conditions that favor the subsequent stage of carcinogenesis, but are not directly associated with the mechanism of progression. Further study is required to obtain a better understanding of cell lineage in enzyme-altered foci and HCC.

Recently, a number of attempts to obtain mice and/or rats with modification of genes that participate in or are related to carcinogenesis have been carried out (e.g., by gene targeting and/or knockout) to elucidate the process of carcinogenesis and develop treatments for human cancer. Individuals who are constitutively predisposed to cancer will suffer from common carcinomas if they are exposed to certain environmental factors (1). In general, inhibiting the initiation of carcinogenesis after exposure to these environmental factors is almost impossible, since a variety of chemicals and physical factors could act as carcinogenic agents. However, our studies of carcinogen-resistant DRH rats suggest that suppression of the growth of preneoplastic or even small-sized neoplastic lesions is a realizable step for preventing carcinogenesis. Elucidating the modifier genes that suppress tumor growth will be extremely important for cancer prevention (1). Studies of DRH resistance will provide an excellent foundation on which to unravel the elementary genetic steps of hepatocarcinogenesis conferring the basis of individual predisposition.

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