# Different susceptibility to peroxisome proliferator-induced hepatocarcinogenesis in rats with polymorphic glutathione transferase genes

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**Although peroxisomal bifunctional enzyme (enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase; BE) is a positive marker for peroxisome proliferation, it is completely absent or expressed very weakly in rat hepatic preneoplastic and neoplastic lesions induced by peroxisome proliferators (PP). After administration of PP for 8–15 weeks, some rats exhibit BE-negative preneoplastic foci but other rats do not. In the present study, to investigate the involvement of glutathione S-transferase (*GST*) M1 gene polymorphism in interindividual differences in susceptibility to PP, we developed a method to determine the genotypes of rats. We then examined whether rats with one type encoding $^{198}$Asn-$^{199}$Cys (NC-type) or another encoding $^{198}$Lys-$^{199}$Ser (KS-type) exhibit differences in clofibrate (CF) susceptibility. After administration of 0.3% CF for 6 weeks or more, BE-negative foci were found immunohistochemically in KS/KS-type rats, but not in NC/NC-type rats. The number of BE-negative foci in KS/KS rats was 15.3 ± 9.0 foci/cm$^2$ of liver section after 6 weeks of CF administration, and the values did not alter thereafter. The mean areas of BE-negative foci in KS/KS rat livers increased during the period from 6 to 60 weeks. At weeks 30 and 60, almost all BE-negative foci exhibited a clear cell phenotype, a type of preneoplastic hepatic lesion. BE-negative foci were devoid of peroxisome proliferator-activated receptor α, whereas surrounding tissues were positive for the receptor. These results indicate that rats that are polymorphic for the *GST M1* gene exhibit different susceptibilities to CF *in vivo*. (*Cancer Sci* 2006; 97: 703–709)**

Peroxisome proliferators, including CF, induce hepatomegaly, proliferation of peroxisomes and expression of several peroxisomal enzymes in rodent livers, such as acyl-CoA oxidase, BE and 3-ketoacyl-CoA thiolase, which participate in β-oxidation of fatty acids and result in the production of hydrogen peroxide.[1–5] Induction of BE expression in rat liver by PP is mediated by PPARα, which forms a complex with RXR. The PPARα–RXR complex binds to PP-responsive elements present in the 5′-flanking region of the BE gene and other target genes.[6]

Prolonged administration of PP to rats is associated with the development of hepatic preneoplastic and neoplastic lesions.[7–11] These lesions do not express GST-P, a reliable marker for preneoplastic lesions in rats induced by the great majority of carcinogens.[12,13] Using a rat liver bioassay system with GST-P as a marker, the carcinogenic potential of most chemicals, including mutagenic carcinogens, can be evaluated within 8 weeks.[13,14] As PP do not cause mutagenic effects in short-term *in vitro* assays,[15,16] administration of PP to rats for 60–100 weeks is required to evaluate their carcinogenic potential. PP include a broad spectrum of compounds of industrial, pharmaceutical and agricultural importance, such as phthalate ester plasticizers, lipid-lowering drugs and herbicides.[17] Thus, methods for early detection of the carcinogenic potential of PP are required. In our previous study, we demonstrated that peroxisomal enzymes in rat hepatic foci, nodules and carcinomas induced by PP were either completely absent or expressed very weakly, compared with the levels in the surrounding tissues.[18,19] Thus, although BE is a positive marker for peroxisome proliferation in the early stage of PP-associated hepatocarcinogenesis,[9–11] its expression is repressed in preneoplastic lesions in the late stage.[18] Loss of BE in PP-induced preneoplastic and neoplastic lesions has been confirmed by other investigators.[20,21] After administration of PP to SD rats for 8–15 weeks, some rats exhibit BE-negative foci but other rats do not.[18] It remains to be clarified what factors are involved in interindividual differences in susceptibility to PP.

Glutathione S-transferases are a family of multifunctional dimeric proteins that catalyze the conjugation of glutathione with many electrophiles, including carcinogens.[22] Many molecular forms identified so far have been grouped into seven classes: Alpha, Mu, Pi, Theta, Sigma, Zeta and Omega.[23] GST-P is a member of the Pi-class[12] and Mu-class forms are involved in detoxification of carcinogens.[22] One form of the human Mu-class (M1-1) is not expressed in approximately one-half of the population, and hereditary differences in its expression are due to deletion of the encoding gene.[24] Loss of the gene has been suggested as a possible marker for greater susceptibility to the development of lung cancer, is well as other cancers.[25,26] The gene encoding rat GST M1 is polymorphic in SD rats, with one type encoding the NC type and another encoding the KS type. Rats with the NC genotype

Abbreviations: ABC, avidin–biotin–peroxidase complex; BE, L-bifunctional enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase; CF, clofibrate; GST, glutathione S-transferase; GST-P, P-form of GST; KS, $^{198}$Lys-$^{199}$Ser type of rat GST M1 subunit; NC, $^{198}$Asn-$^{199}$Cys type of rat GST M1 subunit; PCR, polymerase chain reaction; PP, peroxisome proliferator; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SD, Sprague–Dawley; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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exhibit enhanced susceptibility to the hepatotoxin carbon tetrachloride *in vivo*.[27,28] In the present study, we examined whether polymorphic rats exhibit different susceptibilities to CF *in vivo*. KS/KS-type rats showed enhanced susceptibility and BE-negative foci developed after CF administration for 4–6 weeks.

## Materials and Methods

### Animals

Male SD rats maintained in our Department and those purchased from Charles-River (Atsugi, Japan) and Clea Japan (Tokyo, Japan), aged 7 weeks and weighing 180–210 g initially, were used in the present study. The rats were housed in plastic cages in an air-conditioned room with a 12 : 12 h L : D cycle in the Institute for Animal Experiments of Hirosaki University School of Medicine, and had free access to water and food. All procedures and treatments that involved live animals were approved by the Animal Care and Use Committee, Hirosaki University.

### Determination of *GST M1* genotypes by PCR and restriction enzyme digestion

DNA was isolated from the tails of rats using the DNeasy Tissue Kit (Qiagen, Tokyo, Japan). The PCR was carried out in 20 μL of a reaction mixture consisting of 500 nM forward and reverse primers, 10 × PCR buffer, 200 μM each dNTP, 0.5 units of *Taq* DNA polymerase (Takara, Siga, Japan) and 0.06 μg of template DNA. The forward and reverse primers for the amplification of *GST M1* were 5′-AG GGC CTG AAG AAG ATC TCT GCC-3′ and 5′-GGA TCC AAT GTG GAC AGG TCC TCT-3′,[23] respectively. Amplification was carried out with 30 cycles of denaturation at 94°C for 30 s and annealing/extension at 65°C for 50 s. After amplification, restriction enzyme digestion was carried out in 10 μL of a digestion mixture consisting of 10 × NE buffer, 5 units of *Ear*I (New England BioLabs, Ipswich, MA, USA) and 4 μL PCR products with incubation at 37°C for 1 h. PCR products before and after restriction enzyme treatment were electrophoresed in a 3% agarose gel and visualized by ethidium bromide staining. The integrity of the PCR product was confirmed by sequencing.

### Induction of rat hepatic foci

In one experiment, 0.3% w/w CF (a product of Tokyo Kasei Kogyo, Tokyo, Japan; purity > 98%) in the basal diet (Oriental M; Oriental Yeast, Tokyo, Japan) was given to KS/KS and NC/NC-type SD rats for 2, 4, 6, 8, 15, 30 and 60 weeks, as reported previously.[18] Five rats were used at the individual time points. In another experiment, 0.6% w/w CF was given for 4 weeks to KS/KS and NC/NC rats obtained from Charles-River and Clea.

### Necropsy

Rats were killed by decapitation under ether anesthesia. Livers were excised immediately and liver weights were recorded for each animal. Portions of the same lobes of livers were fixed in cold acetone. The remaining livers were stored at −80°C. The acetone-fixed samples were processed routinely, embedded in paraffin, and sectioned at 4–6 μm.

### Immunohistochemical staining

Paraffin sections were passed through xylene and a graded alcohol series and stained for BE and PPARα using the ABC method,[29] with their respective antibodies. Affinity-purified, biotin-labeled goat antirabbit immunoglobulin G and an ABC (Vectastain ABC kit, PK4001) were obtained from Vector Laboratories (Burlingame, CA, USA). The binding sites of peroxidase were determined using diaminobenzidine as the substrate. Sections were then counterstained with hematoxylin for microscopic examination. As a negative control, pre-immune rabbit serum was used instead of antiserum. Sections were also stained with hematoxylin and eosin. Histological typing of liver tumors was carried out according to the criteria proposed by the Institute of Laboratory Animal Resources, National Research Council.[30] The numbers of BE-negative or PPARα-negative foci were counted directly under a microscope. Areas of liver section and BE-negative foci were measured with the aid of a digital camera interfaced with a computer (VL 100/4, NEC, Tokyo, Japan) using NIH Image software 1.62. Anti-BE antibody was raised in a rabbit as described previously.[18] To prepare anti-PPARα antibody, a 15-amino acid peptide of PPARα (amino acid residues 454–468) synthesized by a peptide synthesizer (model 432 A; Applied Biosystems, Foster City, CA, USA), was coupled to keyhole limpet hemocyanin with *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester[31] and used to immunize a rabbit as described for the anti-BE antibody. The specificity of anti-PPARα antibody was confirmed by immunoblotting using crude liver extract.

### SDS-PAGE and immunoblotting

Using an 8% polyacrylamide gel, SDS-PAGE was carried out according to the method of Laemmli.[32] Protein was stained with Coomassie Brilliant Blue. Immunoblotting was carried out according to the method of Towbin *et al*.[33] using anti-BE antibody.

## Results

### Differentiation of the KS-type and NC-type rat *GST M1* gene

We reported a polymorphism of the GST M1 subunit in rats: one type (KS-type) has lysine and serine at amino acid residues 198 and 199, respectively, whereas the other (NC-type) has asparagine and cysteine. The gene encoding KS-type GST possesses the sequence GAAGAG,[27] the recognition site of *Ear*I, whereas the NC-type gene does not. To differentiate the KS-type and NC-type *GST M1* gene, PCR was carried out using common primer sets to include the *Ear*I site. PCR products were treated with the restriction enzyme and then subjected to gel electrophoresis (Fig. 1). Both KS-type and NC-type genes provided a band of 141 bp before *Ear*I treatment. After treatment, the KS-type gene was cleaved into a 117 bp band, but the NC-type gene remained at 141 bp. A heterozygous KS/NC-type exhibited with two bands of 141 and 117 bp. Thus, the individual genotypes can be identified by PCR followed by *Ear*I digestion.

### BE-negative foci in KS/KS-type but not in NC/NC-type rat livers

After administration of 0.3% CF to KS/KS-type rats for 6 weeks or more, foci lacking BE expression were found


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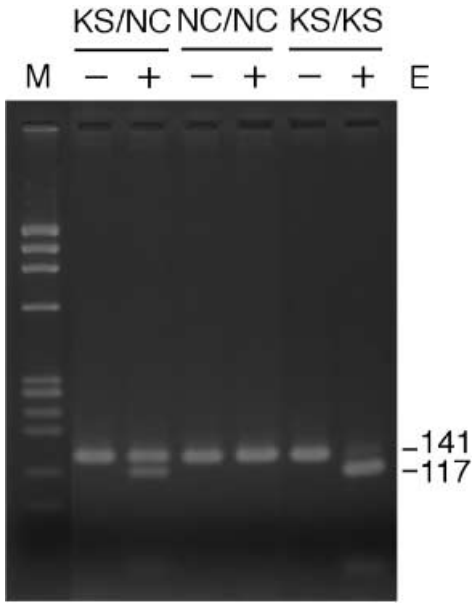


**Fig. 1.** Determination of the $^{198}$Lys-$^{199}$Ser (KS) and $^{198}$Asn-$^{199}$Cys (NC) types of *GST M1* genotypes in Sprague–Dawley (SD) rats by polymerase chain reaction (PCR) and subsequent digestion with a KS-type-specific restriction enzyme. Genomic DNA prepared from the tails of SD rats was amplified by PCR using common primers as described in the text. PCR products were then digested with (+) or without (–) restriction enzyme *Ear*I (E), subjected to electrophoresis in a 3% agarose gel, and visualized with ethidium bromide. KS/KS and NC/NC represent the respective homozygous genotypes, and KS/NC denotes the heterozygous genotype. For size markers (M), ϕX174 DNA was digested with *Hae*III. 141, PCR product of NC type and PCR product of KS type before digestion (141 bp); 117, PCR product of KS type after digestion (117 bp).

among normal hepatocytes exhibiting strong expression by immunohistochemical study (Fig. 2A), but these were not in NC/NC-type rats (Fig. 2B). The staining intensity of normal hepatocytes in NC/NC rats was less than that for KS/KS rats. When pre-immune rabbit serum was used, both NC/NC and KS/KS rat livers showed negative staining results (data not shown).

The development of BE-negative foci with time is summarized in Fig. 3. The number of BE-negative foci in KS/KS rat livers was $15.3 \pm 9.0$ foci/cm$^2$ of liver section after 6 weeks of CF administration, and the values did not alter thereafter with statistical significance. In contrast, BE-negative foci were hardly observed in NC/NC rat livers even after 60 weeks of administration. Thus, there was a significant difference in the number of BE-negative foci between KS/KS and NC/NC rat livers ($P < 0.05$) (Fig. 3A). The mean areas of BE-negative foci in KS/KS rat livers increased during the period from 6 to 60 weeks, although the value at week 30 was similar to that at week 15 (Fig. 3B). Histological examination of quasi-serial sections stained with hematoxylin and eosin revealed that almost all BE-negative foci (94.8%) at weeks 30 and 60 exhibited a clear cell phenotype (Fig. 4), along with lower proportions of BE-negative foci, 33.0%, 34.3% and 68.3% at 6, 8 and 15 weeks, respectively.

### Repressed PPARα expression in BE-negative foci

Peroxisome proliferator-activated receptor α is a transcription factor for PP-induced BE gene expression. To examine whether loss of BE expression is due to alterations in PPARα expression, serial liver sections of CF-administered KS/KS rats were examined immunohistochemically with the anti-PPARα antibody. The typical staining pattern for PPARα is shown in Fig. 2C. In general, PPARα was stained homogeneously in the cytoplasm and nuclei in the surrounding tissues expressing BE, but was hardly stained in BE-negative foci. Thus, the shapes of most PPARα-negative foci coincided with those of BE-negative foci. Control staining using pre-immune serum proved negative (data not shown). PPARα was hardly stained in control KS/KS rats without CF administration. In KS/KS rats, the numbers of PPARα-negative foci were similar to those of BE-negative foci at 6–60 weeks of CF administration and such foci were not found at 4 weeks (data not shown).

### BE-negative foci in KS/KS rats from commercial sources

After administration of 0.3% CF for 6 weeks, BE-negative foci were not observed in NC/NC rats but in KS/KS rats maintained in our Department. Next, we investigated whether such a relationship also existed in SD rats from commercial sources. Male KS/KS and NC/NC rats selected by PCR and subsequent *Ear*I digestion received 0.6% CF for 4 weeks. Immunohistochemical analysis revealed that BE-negative foci were detected in KS/KS rats but not in NC/NC rats (Table 1), confirming the data presented in Fig. 3. The number of BE-negative foci was comparable to that in rats treated with 0.3% CF for 6 or 8 weeks. These results support the selective induction of BE-negative foci in KS/KS rats after CF administration and a high concentration of CF results in the early appearance of negative foci.

### Enhanced BE induction in KS/KS rat livers after CF administration

Immunohistochemical analysis suggested induction of BE in both KS/KS and NC/NC rat livers after CF administration,

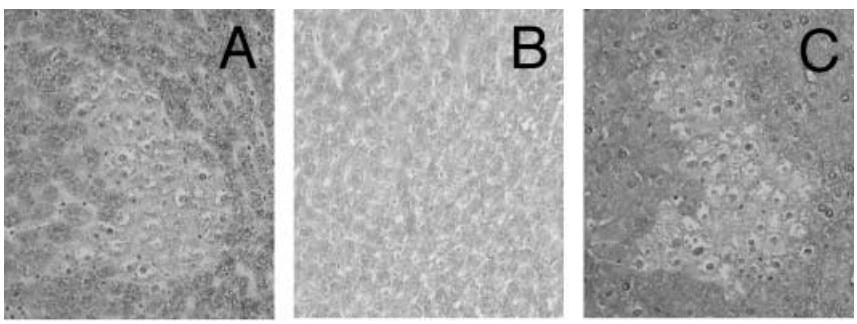


**Fig. 2.** Immunohistochemical staining of L-bifunctional enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (BE) in the livers of (A) $^{198}$Lys-$^{199}$Ser (KS)/KS-type and (B) $^{198}$Asn-$^{199}$Cys (NC)/NC-type rats administered clofibrate (CF) for 8 weeks. Magnification, ×200. A focus lacking BE expression is observed in a KS/KS rat. (C) The liver of a KS/KS rat administered CF for 8 weeks (serial section of part A) was stained immunohistochemically for peroxisome proliferator-activated receptor (PPAR) α. Magnification, ×200. PPARα expression is repressed in a BE-negative focus.

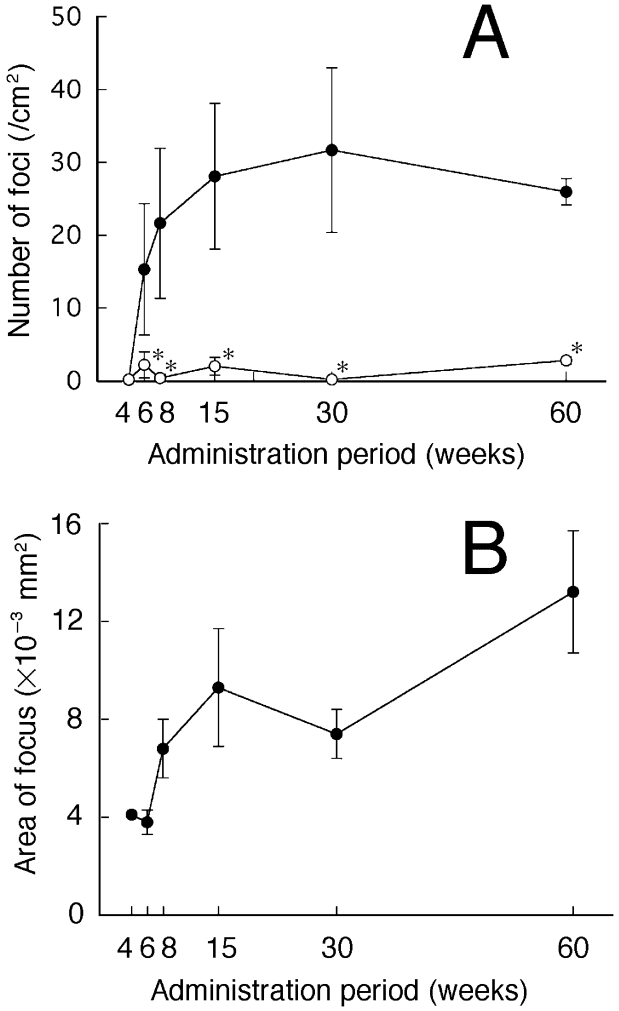


**Fig. 3.** (A) Changes in the number of L-bifunctional enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (BE)-negative foci in the livers of $^{198}$Lys-$^{199}$Ser (KS)/KS (●) and $^{198}$Asn-$^{199}$Cys (NC)/NC (○) rats administered clofibrate (CF) for up to 60 weeks. Five rats were analyzed at each time point and standard deviations are indicated by bars. The number of BE-negative foci is expressed as value per unit area (cm$^2$) of liver section. Asterisks represent statistical significance at $P < 0.05$ between KS/KS and NC/NC rats. (B) Changes in the area of BE-negative foci in the livers of KS/KS rats administered CF for up to 60 weeks are also presented. Measurement of the area was carried out as described in the text and the value is expressed as the area in $10^{-3}$ mm$^2$.

but its induction level was higher in KS/KS than in NC/NC. To evaluate BE levels in both types, precipitate fractions of liver homogenate from the rats with and without 0.3% CF treatment for 4 weeks were subjected to electrophoresis followed by immunoblotting with anti-BE antibody. At week 4, BE-negative foci were not yet formed. A BE protein band at 72 kDa was increased in both KS/KS and NC/NC rats by CF administration. When BE levels were compared in CF-treated livers, KS/KS rats exhibited higher level than NC/NC rats (Fig. 5).

## Discussion

In the present study we have developed a method to determine the *GST M1* genotypes of SD rats by PCR and *Ear*I digestion

**Table 1. Induction of L-bifunctional enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (BE)-negative foci by 0.6% clofibrate (CF) in $^{198}$Lys-$^{199}$Ser (KS)/KS rats from commercial sources**

| *GST M1* genotype | No. rats | CF administration (weeks) | No. BE-negative foci (/cm$^2$ liver) | Area of BE-negative focus ($10^{-3}$ mm$^2$) |
|---|---|---|---|---|
| KS/KS | 4 | 4 | 18.8 ± 5.9 | 4.2 ± 2.9 |
| NC/NC | 4 | 4 | 0 | 0 |

Values are mean ± SD.

and have examined whether polymorphic rats exhibit different susceptibilities to CF *in vivo*. Rat Mu-class GST include six genes (*M1–M6*), each sharing highly homologous base sequences.[23] As not only the KS-type of *M1* gene but also the *M4* (Yb4) gene has the recognition site for *Ear*I, the primers for PCR were selected to amplify specifically the *M1* gene. Thus, the *M1* genotypes can be determined without interference from the *M4* or other Mu-class genes.

In KS/KS rats, BE-negative foci were found after CF administration for 4–8 weeks, whereas they were not in NC/NC rats even after 60 weeks of administration. The number of BE-negative foci was not further increased thereafter in KS/KS rats but the areas of the foci were increased during the period from 6 to 60 weeks. Almost all BE-negative foci showed a clear cell phenotype at weeks 30 and 60. Clear cell foci are considered to be a type of early lesion in rat hepatocarcinogenic processes, induced by many carcinogens, including PP,[34,35] which then progress to neoplastic lesions.[36–38] These results suggest that BE-negative foci observed at week 6 lead to clear cell foci at weeks 30 and 60, and that rats with polymorphic *M1* genes exhibit different susceptibilities to CF-induced hepatocarcinogenesis. KS/KS rats exhibited enhanced susceptibility to CF and BE-negative foci developed after CF administration for 4–6 weeks. Thus, by using the sensitive KS/KS rats, the carcinogenic potentials of PP may be evaluated by observing the number of BE-negative foci after 4–6 weeks administration.

The induction level of BE in the surrounding tissues was higher in KS/KS rats than in NC/NC rats. As induction of BE expression in the rat liver by PP is mediated by PPARα, altered BE levels between the polymorphic rats may reflect differences in the degree of PPARα activation. Although it remains to be clarified how GST polymorphisms result in the different extent of PPARα activation, our recent study has revealed that the level of steroid receptor coactivator, a coactivator of PPARα,[39] is much higher in KS/KS rats than in NC/NC rats (data not shown). CF administration causes enhanced production of hydrogen peroxide through the induction of BE and other peroxisomal enzymes for fatty acid β-oxidation.[1,4] The KS-type enzyme is activated by reactive oxygen species whereas the NC-type enzyme is not.[27] Thus, high coactivator levels and the properties of the KS-type enzyme under oxidative conditions may be involved in enhanced PPARα activation in KS/KS rats. Further studies are needed to establish the molecular mechanism of PPARα activation in the polymorphic rats.

The strong induction of BE by CF and subsequent development of BE-negative foci in the KS/KS rats (Fig. 6) suggest


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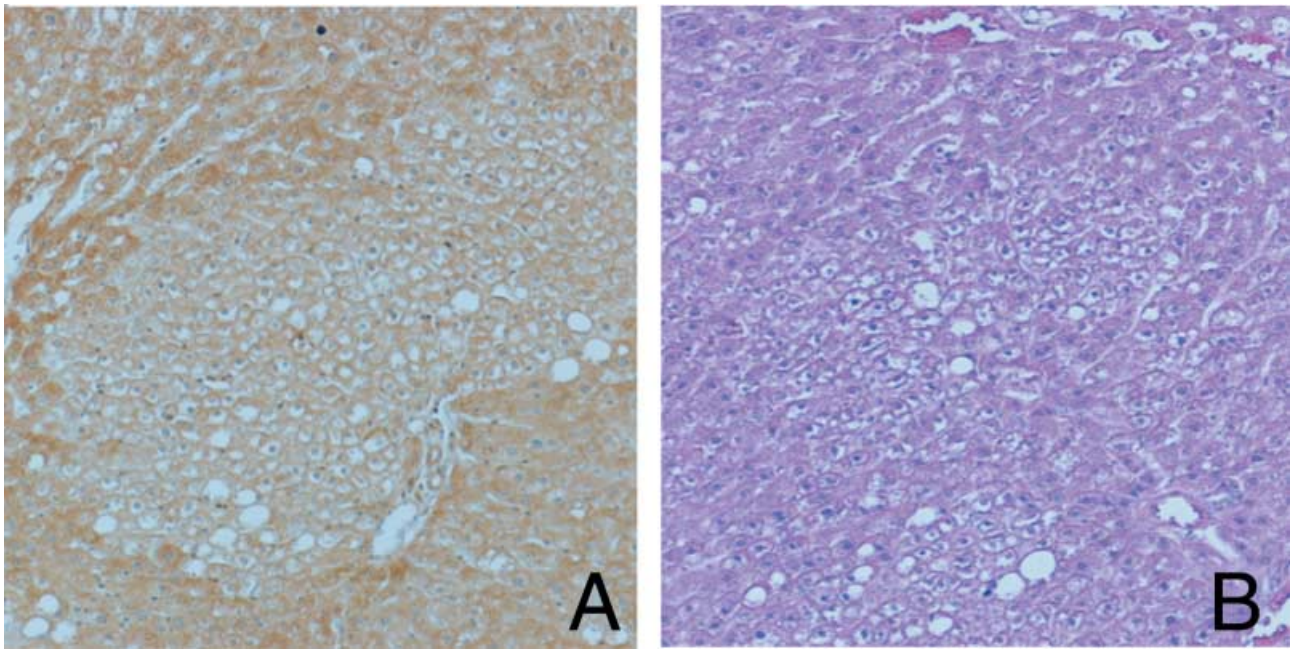


**Fig. 4.** Clear cell phenotype of L-bifunctional enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (BE)-negative foci induced by clofibrate (CF). The liver sections of $^{198}$Lys-$^{199}$Ser (KS)/KS-type rats administered CF for 60 weeks were stained with (A) anti-BE antibody and (B) hematoxylin and eosin. Magnification, ×100. A BE-negative focus exhibits clear cell phenotype by hematoxylin and eosin staining.

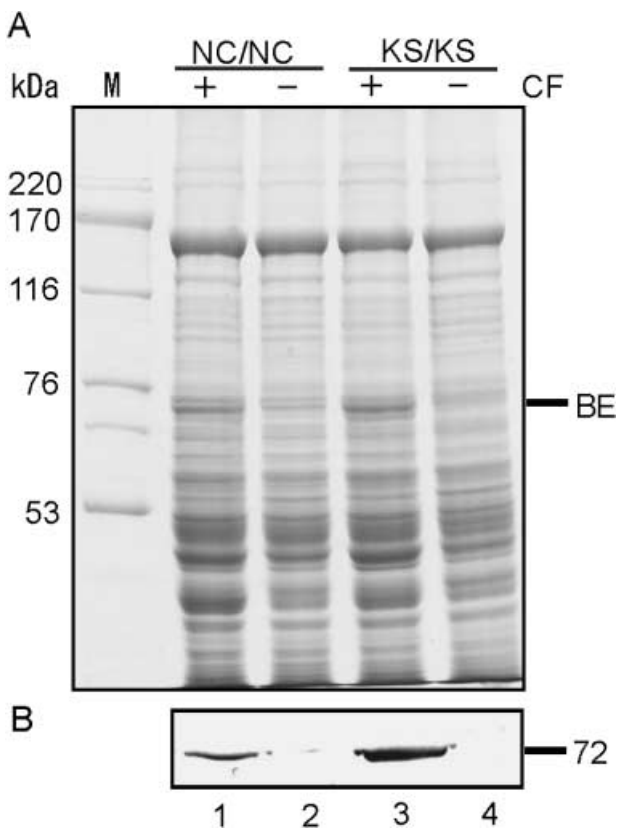


**Fig. 5.** (A) Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of liver proteins from $^{198}$Lys-$^{199}$Ser (KS)/KS and $^{198}$Asn-$^{199}$Cys (NC)/NC rats administered clofibrate (CF) for 4 weeks (+). CF was not administered to control NC/NC or KS/KS rats (–). Precipitate fractions centrifuged at 15 000*g* for 15 min were analyzed by SDS-PAGE (50 μg of each protein). After electrophoresis, proteins were stained with Coomassie Brilliant Blue. Molecular-mass size markers (M) were 220, 170, 116, 76 and 53 kDa. The L-bifunctional enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (BE) protein had a subunit molecular mass of 72 kDa. (B) Immunoblotting was carried out using an anti-BE antibody. Lanes were the same as in (A) except the lane M. The band of 72 kDa was stained with the antibody.

activation of PPARα in whole livers and negative conversion of PPARα in foci thereafter. In the NC/NC rats, activation of PPARα was weak and loss of PPARα expression did not occur. The induction of peroxisome proliferation and the peroxisomal fatty acid β-oxidation system has been suggested as a practical short-term *in vivo* test to evaluate the carcinogenic potentials of PP.[40] Fan *et al*. have reported that massive spontaneous peroxisome proliferation is observed in acyl-CoA oxidase-deficient mice at 6 months of age, whereas hepatic adenomas and carcinomas develop at 11–15 months.[40,41] These findings are similar to the enhanced induction of BE and subsequent BE-negative foci in KS/KS rats in the present study. BE-negative foci were devoid of PPARα expression and the areas of such foci were increased during weeks 6–60. This is contradictory to the findings that no neoplastic or preneoplastic lesions are formed in PPARα-null mice and PPARα is required for cell proliferation and carcinogenic response to PP.[42] The results of the present study suggest that PPARα is required for BE induction by PP in normal hepatocytes but not for the development of preneoplastic nodules from BE-negative foci in the late stage. A recent study has suggested that hepatocyte proliferation is not mediated by PPARα.[43]

Unlike rodent hepatocytes, human hepatocytes appear to be unresponsive to PP, displaying minimal peroxisome and

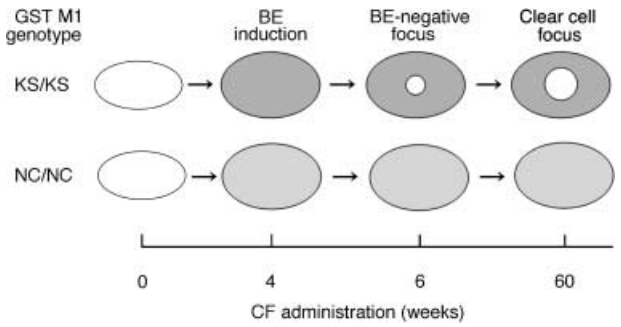


**Fig. 6.** Different susceptibility of Sprague–Dawley rats with polymorphic *GST M1* genes to clofibrate (CF). In $^{198}$Lys-$^{199}$Ser (KS)/KS rat livers L-bifunctional enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (BE) was highly induced by CF administration and BE-negative foci developed at week 6. In contrast, BE induction was low in $^{198}$Asn-$^{199}$Cys (NC)/NC rat livers and such foci were not induced. BE-negative foci were also devoid of peroxisome proliferator-activated receptor α expression, grew in size during the period from 6 to 60 weeks, and exhibited a clear cell phenotype at later stage.

hepatocyte proliferation.[44] Only approximately one-half of the population have the human *GST M1* gene[24,25] and there are additional polymorphisms in the gene: one type encoding $^{172}$Lys (*M1a*) and another encoding $^{172}$Asn (*M1b*). Both genotypes in the human case have a common sequence KS at residues 198 and 199,[25,45] suggesting that these KS residues of the human enzyme may not be involved in sensitivity to PP. In conclusion, rats with the polymorphic *GST M1* gene exhibit different susceptibilities to CF *in vivo*, and BE-negative foci observed at week 6 can be used as a marker for PP-induced preneoplastic hepatic lesions.

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