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# $\alpha_2$-Macroglobulin: A Novel Cytochemical Marker Characterizing Preneoplastic and Neoplastic Rat Liver Lesions Negative for Hitherto Established Cytochemical Markers

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**We tried to identify a novel marker characteristic for rat hepatocellular preneoplastic and neoplastic lesions, undetectable by well established cytochemical markers. Glutathione *S*-transferase placental (GST-P)-negative hepatocellular altered foci (HAF), hepatocellular adenoma (HCA), and hepatocellular carcinoma (HCC) were generated by two initiation-promotion models with *N*-nitrosodiethylamine (NDEN) and peroxisome proliferators, Wy-14,643 and clofibrate. Total RNAs isolated from laser-microdissected GST-P-negative HAF (amphophilic cell foci) and adjacent normal tissues were applied to microarray analysis. As a result, five up-regulated genes were identified, and further detailed examinations of the gene demonstrating most fluctuation, ie, that for $\alpha_2$-macroglobulin ($\alpha_2$M) were performed. In reverse transcriptase-polymerase chain reaction, $\alpha_2$M mRNA was overexpressed not only in amphophilic GST-P-negative HAF but also in amphophilic GST-P-negative HCA and HCC. *In situ* hybridization showed accumulation of $\alpha_2$M mRNA to be evenly distributed within GST-P-negative HAF (predominantly amphophilic cell foci). Distinctive immunohistochemical staining for $\alpha_2$M could be consistently demonstrated in GST-P-negative HAF, HCA, and HCC induced not only by peroxisome proliferators but also *N*-nitrosodiethylamine alone. Thus our findings suggest that $\alpha_2$M is an important novel cytochemical marker to identify hepatocellular preneoplastic and neoplastic lesions, particularly amphophilic cell foci, undetectable by established cytochemical markers and is tightly linked to rat hepatocarcinogenesis. *(Am J Pathol 2004, 165:1479–1488)***

During the process of hepatocarcinogenesis, some small proportion of hepatocellular altered foci (HAF) develop into hepatocellular adenomas (HCAs) and hepatocellular carcinomas (HCCs). Many preneoplastic lesions regress after withdrawal of a proliferative stimulus,[1–4] whereas others persist and demonstrate stable growth.[5,6] The molecular mechanisms underlying these phenomena are unclear. The phenotypic diversity demonstrated by foci[7] means that markers detectable for all foci have advantages for elucidating the process of hepatocarcinogenesis. Although γ-GT[3,4,6–11] and particularly glutathione *S*-transferase placental (GST-P)[12,13] have become established as marker enzymes for hepatocarcinogenesis in rats, they have shortcomings regarding detection of some types of HAF, predominantly amphophilic cell foci induced by peroxisome proliferators.[14–16] Amphophilic cell foci, characterized by increased granular acidophilia and randomly scattered cytoplasmic basophilia, demonstrate alterations in mitochondrial enzymes.[16,17] However, none of the cytochemical markers that are widely used such as γ-GT or GST-P are positive particularly in the small foci not readily evident in routine hematoxylin and eosin (H&E) staining.

Recent technical development and advances in tools for molecular biology such as cDNA microarrays and the availability of laser microdissection now allow us to monitor gene expression comprehensively in pure cell populations such as those in histopathological lesions in tissue sections. In addition, application of RNA linear amplifica-

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tion techniques for very limited quantities of RNA isolated from microdissected cells greatly facilitates examination of transcripts specific for microlesions and nonhomogeneous tissues. In the present study, we made use of these techniques to identify a novel marker specific for hepatocellular preneoplastic or neoplastic lesions undetectable with the hitherto available cytochemical markers for rat hepatocarcinogenesis.

Rat $\alpha_2$-macroglobulin ($\alpha_2$M), a homotetrameric major acute-phase glycoprotein[18,19] is a typical member of the pan-proteinase inhibitors of the $\alpha_2$M family, capable of inhibiting a wide spectrum of proteinases from all classes by means of steric entrapment and covalent binding.[20] It also plays a role as a carrier protein and regulator for various growth factors, polypeptide hormones, and cytokines.[21–26]

A number of authors have reported up-regulation of serum $\alpha_2$M in association with HCC in humans, being significantly raised as compared to liver cirrhosis and amoebic liver abscess.[27] Poon and colleagues[28] included $\alpha_2$M as a candidate serological marker for the diagnosis of HCC and a recent investigation revealed $\alpha_2$M to be overexpressed in HCCs with a background of hepatitis C virus as compared to nontumorous liver tissues.[29] On the other hand, although elevated concentrations of $\alpha_2$M have been also found in the sera of rats bearing HCC[30] or exposed to hepatocarcinogens,[31] Hudig and colleagues[32] concluded that this was not correlated with tumor development. Rather, they suggested that the previously observed increases in serum $\alpha_2$M concentrations during hepatocarcinogenesis and in animals bearing hepatic tumors were because of secretion by the host liver of $\alpha_2$M as an acute-phase reactant in response to inflammatory injury. Therefore, as contrast to the case in humans, so far it remains equivocal whether up-regulation of serum $\alpha_2$M is linked to hepatocarcinogenesis in rats.

We hereby demonstrate that $\alpha_2$M is a novel candidate cytochemical marker for identification of hepatocellular preneoplastic and neoplastic lesions, undetectable by hitherto established cytochemical markers, and may be tightly linked to rat liver lesion development from the initial stage through to tumor progression.

## Materials and Methods

### Animals and Chemicals

*N*-nitrosodiethylamine (NDEN) and clofibrate (>98%) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), and Wy-14,643 (>98%) was obtained from ChemSyn Laboratories (Lenexa, KS). A total of 54 male 5-week-old F344 rats were purchased from Charles River Japan, Inc. (Atsugi, Japan) and housed in suspended aluminum cages (three rats in a cage) in a room kept at 24 ± 2°C temperature and 40 to 70% humidity with a 12-hour light/dark cycle. They received CRF-1 laboratory chow (Charles River Japan, Inc.) as basal diet in experiments 1 and 2 *ad libitum*. The animals were observed daily and were used after a 1-week acclimation period for the experiments. Body weights were measured every week.

### Experimental Protocol

All experiments were performed in accordance with the Guide for Animal Care and Use of Sumitomo Chemical Co. Ltd. In experiment 1, an *in vivo* medium-term bioassay[13] with a minor modification as to the treatment period for the peroxisome proliferators was used. Briefly, at the age of 6 weeks, 24 male F344 rats were divided into four groups (six animals per group). Animals were given a single intraperitoneal injection of NDEN (200 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis and after a 2-week recovery period, received clofibrate (3000 ppm, group 1) or Wy-14,643 (1000 ppm, group 2) in the basal diet. The rats were subjected to two-thirds partial hepatectomy at week 3. Animals in group 3 were given NDEN and partial hepatectomy in the same manner as for groups 1 and 2 without administration of any other chemicals, and animals in group 4 were treated in the same manner as for group 3 except injection of saline instead of NDEN. All animals were sacrificed at week 12.

In experiment 2, an initiation-promotion model in which multiple administrations of NDEN were given in place of partial hepatectomy in experiment 1 was used. In light of our experience, however, the dose of NDEN was set at 100 mg/kg not to impose too heavy a burden on the rats. Briefly, at the age of 6 weeks, 30 male F344 rats were divided into three groups (10 animals per group). Animals in groups 1 and 2 were injected with NDEN (100 mg/kg body weight) intraperitoneally once a week for 2 weeks, and after a 1-week recovery period, received clofibrate (3000 ppm, group 1) or the basal diet (group 2). Animals in group 3 were injected with saline instead of NDEN solution without subsequent administration of any chemicals. Sacrifice was at weeks 26 and 36. In both experiments, all animals in the each group were exsanguinated and sacrificed under ether anesthesia, and the liver tissues were obtained and treated with some appropriate procedures for the following examinations.

### Laser Microdissection and Total RNA Isolation

Frozen liver tissues embedded in OCT compound (Sakura Finetech, Tokyo, Japan) were sectioned at ~8 $\mu$m to get several sets of seven serial sections, and the first and last sections in each suite were applied to routine H&E staining and immunohistochemical staining for rat GST-P to identify lesions histopathologically for microdissection. The remaining sections were applied to casual H&E staining to block RNA from degradation, ie, the sections were 70% ethanol-fixed for 1 minute, immersed in RNase-free hematoxylin for 5 minutes, rinsed by RNase-free water several times, immersed in RNase-free phosphate-buffered saline for 5 minutes, and immersed in RNase-free eosin for 1 minute. In experiment 1, GST-P-positive and -negative HAF, and corresponding adjacent normal tissues were microdissected with the use of

a laser microdissection system (Leica Microsystems Japan, Tokyo, Japan) from the following groups: GST-P-positive HAF (clear cell foci) in groups 1 and 3, and GST-P-negative HAF (amphophilic cell foci) in group 2. In experiment 2, GST-P-negative lesions [HAF, HCA, and HCC (amphophilic phenotype)], GST-P-positive lesions [HCA and HCC (clear and acidophilic phenotype)], and corresponding adjacent normal tissues were microdissected in the same manner from group 1. Subsequently, total RNAs were isolated from the microdissected tissues in accordance with the protocol for RNeasy Protect mini kit (Qiagen, Tokyo, Japan) with a minor modification using poly(C) (Amersham Bioscience, Buckinghamshire, UK) as a carrier. The total RNA pool was used for the following microarray analysis and reverse transcriptase-polymerase chain reaction (RT-PCR) assays.

### *High-Density Oligonucleotide Microarray Analysis*

Rat genome U34A arrays, which contained 9000 probes for known rat genes or expressed sequence tags, were purchased from Affymetrix (Santa Clara, CA). For microarray probing, reverse transcription, second-strand synthesis, and probe generation were all accomplished following the technical notes of the Small Sample Labeling Protocol version 2 (Affymetrix). Briefly, from 100 ng of total RNA, first-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Groningen, The Netherlands) and a T7-(dT)24 primer (Amersham Bioscience) and then double-strand cDNA was synthesized with *Escherichia coli* RNase H, *E. coli* DNA polymerase I, and *E. coli* DNA ligase (Invitrogen). From the double-strand cDNA, cRNA was prepared using a MEGAscript T7 kit (Ambion, Austin, TX). After a second cycle of amplification and biotin labeling with a BioArray high-yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY), 20 $\mu$g of labeled cRNA was fragmented. The RGU34A arrays were hybridized as described in the Gene Chip Expression Analysis Technical Manual (Affymetrix) and stained for use with a GeneArray scanner (Agilent Technologies, Palo Alto, CA). The derived signal value was globally normalized and targeted to all probe sets equal to 100 before comparative analysis.

### *Microarray Data Analysis*

To examine gene expression differences between GST-P-negative or -positive HAF, and the corresponding adjacent normal tissue, we performed comparison analysis using the Affymetrix data suite system, MAS 5.0. The genes (probe sets) showing greater than twofold alteration in value with a change of I or D were chosen as changed genes.

### *Semiquantitative RT-PCR Assay*

Adequate amounts of total RNA that had not been amplified were reverse-transcribed in a 20-$\mu$l reaction mixture using the ThermoScript RT-PCR system (Invitrogen). Semiquantitative PCR conditions were optimized to obtain reproducible and reliable amplification within the logarithmic phase of the reaction. The cycling amplifications were conducted with 10-$\mu$l of reaction mixture using a Program Temp control system PC-800 (Astec, Fukuoka, Japan) programmed for 95°C for 5 minutes and then 23 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 30 seconds (for 5 minutes in a final cycle). Alignments of primers were as follows: $\alpha_2$M forward, 5′-GGCCATTGCCTATCTCAATACG-3′, reverse, 5′-TTATCCCCAAAGGCGCTGTA-3′; $\beta$-actin forward, 5′-GACAGGATGCAGAAGGAGATTACTG-3′, reverse, 5′-AGAGCCACCAATCCACACAGA-3′. Signals stained by GelStar (FMC, Rockland, ME) were detected using a luminescent image analyzer (LAS-1000 plus; Fuji Photo Film, Tokyo, Japan).

### In Situ *Hybridization for $\alpha_2$M mRNA*

*In situ* hybridization of $\alpha_2$M mRNA was performed in 4% paraformaldehyde-fixed liver sections consecutive to those applied to H&E staining and immunohistochemical staining for $\alpha_2$M and GST-P. Briefly, at first, cRNA probe for $\alpha_2$M mRNA was prepared by *in vitro* transcription of the cDNA fragment (~1000 bp; 3315 to 4349) generated from the same cDNA pool as for the above-described semiquantitative RT-PCR assay, and labeled using a fluorescein RNA labeling mix (Roche Molecular Biochemicals, Mannheim, Germany). After several pretreatments including target retrieval using Target Retrieval Solution (DAKO Co., Carpinteria, CA), 4-$\mu$m sections were hybridized with cRNA probe overnight at 60°C, and signals were detected using the *In Situ* Hybridization Detection system (DAKO Co.). The numbers of foci positive for $\alpha_2$M mRNA signals (>0.1 mm in diameter) were counted under a light microscope in the groups of animals sacrificed at week 26 in experiment 2 (all of the sections counted were applied to *in situ* hybridization for $\alpha_2$M mRNA simultaneously). The total area of the liver sections was measured using an IPAP image analyzer (Sumika Technoservice, Osaka, Japan). Counts of the numbers of the positive foci for mRNA signals were repeated at least three times for confirmation in a blinded manner and an average value of the three counts was used as the final data.

### *Immunohistochemistry for $\alpha_2$M and GST-P*

In the present study, immunohistochemical staining for $\alpha_2$M was conducted only for a qualitative analysis, because there was concern that this serum protein might be released from the lesions because of an inadequate fixation as mentioned in the Discussion. Immunohistochemical examination of localization of $\alpha_2$M and GST-P proteins was performed respectively in 4% paraformaldehyde-fixed liver sections consecutive to those applied for *in situ* hybridization, using the avidin-biotin complex method. Briefly, after deparaffinization (and target retrieval using Target Retrieval Solution in the case of

**Table 1.** Histopathological Findings in Liver

| Histopathological findings* | Breeding term Group† | Experiment 1 12W 1 | 2 | 3 | 4 | Experiment 2 26W 1 | 2 | 3 | 36W 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of animals examined | | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Centrilobular hepatocellular hypertrophy | + | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2+ | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Hepatocellular altered foci | | 5‡ | 5§ | 5¶ | 0 | 5‖ | 5** | 0 | 5‖ | 5** | 1†† |
| Hepatocellular adenoma | | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 2 | 2 | 0 |
| Hepatocellular carcinoma | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 4 | 1 | 0 |

*Grading: +−, slight; +, mild; 2+, moderate; 3+, severe.
†Experiment 1: 1, NDEN + clofibrate; 2, NDEN + Wy-14,643; 3, NDEN alone; 4, no treatment. Experiment 2: 1, NDEN + clofibrate; 2, NDEN alone; 3, no treatment.
‡Predominantly clear cell foci, and small number of amphophilic cell foci.
§Amphophilic cell foci and clear cell foci.
¶Predominantly clear cell foci and small number of amphophilic cell foci (basophilic).
‖Clear and acidophilic cell foci and amphophilic cell foci.
**Predominantly clear and acidophilic cell foci, and small number of amphophilic cell foci (basophilic).
††Clear cell foci.

$\alpha_2$M), the sections were treated sequentially with 3% $H_2O_2$, normal goat serum, primary antibody, ie, goat anti-rat GST-P (1:2000, room temperature, 1 hour; MBL Co., Ltd., Nagoya, Japan) or goat anti-rat $\alpha_2$M (1:2000, room temperature, 2 hours; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), biotin-labeled goat anti-rabbit IgG and avidin-biotin-peroxidase complex (ABC kit; Funakoshi Co., Ltd., Tokyo, Japan). The sites of peroxidase binding were demonstrated by the diaminobenzidine method and light counterstaining with hematoxylin was performed to facilitate orientation. The numbers of GST-P-positive foci (>0.1 mm in diameter) were counted under a light microscope in experiments 1 and 2. Additionally, the numbers of GST-P-negative foci (>0.1 mm in diameter), which were detected by routine H&E staining and subsequently checked stainability against antibody to GST-P by immunohistochemical staining of the sections consecutive to those stained with H&E, were also counted by the same manner. The total area of the liver sections was measured using an IPAP image analyzer (Sumika Technoservice). Counts of the numbers of GST-P-positive or -negative foci were repeated at least three times for confirmation in a blinded manner and an average value of the three counts was used as the final data.

## Results

### Histopathology and Laser Microdissection

Histopathological examination and quantitative analysis of GST-P-positive and -negative foci were conducted with reference to earlier articles[16,33] and a diagnostic guide[34] and the results are shown in Tables 1 and 2. Several frozen cell populations were harvested specifically from GST-P-positive and/or-negative HAF, HCAs, and HCCs using laser microdissection, and total RNA was isolated from each lesion in experiments 1 and 2. GST-P-negative HAF, ie, amphophilic cell foci, were not clearly detectable in the frozen sections stained by H&E in group 1 of experiment 1, so that amphophilic GST-P-negative HAFs were microdissected from group 2 in experiment 1 and group 1 in experiment 2 (Table 3).

**Table 2.** Quantitative Analysis of GST-P-Positive and -Negative Foci Larger than 0.1 mm in Diameter

| No. of group | Treatment | No. of foci (No./cm$^2$)* GST-P-Positive | GST-P-Negative† |
|---|---|---|---|
| Experiment 1 | | | |
| 1 | NDEN + clofibrate | 9.61 ± 2.82 | 1.21 ± 0.85 |
| 2 | NDEN + Wy-14,643 | 8.81 ± 2.65 | 11.74 ± 2.65 |
| 3 | NDEN alone | 16.15 ± 7.46 | 1.74 ± 1.11 |
| 4 | No treatment | 0.09 ± 0.21 | 0.00 ± 0.00 |
| Experiment 2 (26W) | | | |
| 1 | NDEN + clofibrate | 33.17 ± 5.31 | 10.30 ± 2.10 |
| 2 | NDEN alone | 41.09 ± 9.84 | 5.69 ± 2.40 |
| 3 | No treatment | 0.24 ± 0.22 | 0.00 ± 0.00 |
| Experiment 2 (36W) | | | |
| 1 | NDEN + clofibrate | 52.02 ± 11.33 | 14.07 ± 2.84 |
| 2 | NDEN alone | 75.66 ± 10.19 | 9.34 ± 2.72 |
| 3 | No treatment | 0.25 ± 0.37 | 0.00 ± 0.00 |

*Except hepatocellular adenoma and carcinoma.
†The foci, predominantly amphophilic cell foci, were detected by staining with H&E, and subsequently checked stainability against antibody to GST-P by using the serial sections.

**Table 3.** Area of the Lesion Microdissected and Quantity of the Isolated Total RNA

| No. of group | Treatment | Microdissected lesion* | GST-P | Area of lesion microdissected† ($mm^2$) | Total area microdissected ($mm^2$) | Quantity of RNA (ng)‡ |
|---|---|---|---|---|---|---|
| Experiment 1 | | | | | | |
| 1 | NDEN + clofibrate | HAF | Positive | 0.32‖ | 20.44 | 588 |
| 2 | NDEN + Wy-14,643 | HAF | Negative¶ | 0.08‖ | 18.44 | 492 |
| 3 | NDEN alone | HAF | Positive | 0.23‖ | 20.00 | 644 |
| Experiment 2 | | | | | | |
| 1 | NDEN + clofibrate | HAF | Negative¶ | 0.62‖ | 18.72 | 881 |
| 1 | NDEN + clofibrate | HCA§ | Positive | 1.59 | 23.11 | 591 |
| 1 | NDEN + clofibrate | HCA§ | Negative¶ | 2.51 | 21.87 | 606 |
| 1 | NDEN + clofibrate | HCC§ | Positive | 3.84 | 23.06 | 759 |
| 1 | NDEN + clofibrate | HCC§ | Negative¶ | 3.95 | 21.34 | 482 |

*HAF, hepatocellular altered foci; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma.
†In HCA and HCC, the tissue was microdissected from the same one lesion. In HAF, however, several lesions were pooled, since the size of each lesion was too small to get sufficient quantity of RNA from one lesion. Namely, 4 foci from group 1, 23 foci from group 2, and 6 foci from group 3 in experiment 1, and 3 foci from group 1 in experiment 2 were pooled, respectively.
‡One hundred ng of total RNA was used for linear amplification.
§Total RNA isolated from the lesions were used only in RT-PCR assay.
¶Amphophilic phenotype.
‖The values show average area of the lesions microdissected.

### *Microarray Analysis*

GST-P-negative HAF (amphophilic cell foci) induced by NDEN + Wy-14,643 or NDEN + clofibrate and GST-P-positive HAF (clear cell foci) induced by NDEN alone or NDEN + clofibrate were compared with the corresponding adjacent normal tissues. The numbers of up- and down-regulated genes obtained from those comparisons are listed in Table 4. In the up-regulated genes extracted from amphophilic GST-P-negative HAF, five genes were commonly overexpressed in the lesions induced by two different peroxisome proliferators (Table 5). That demonstrating greatest fluctuation, the $\alpha_2$M gene, showed overexpression specific for amphophilic GST-P-negative HAF (not up-regulated in GST-P-positive HAF). On the other hand, of the down-regulated genes extracted from amphophilic GST-P-negative HAF, two demonstrated a decrease in common in HAF induced by two different peroxisome proliferators (Table 5), but none were specific only for amphophilic GST-P-negative HAF. In the present study, microarray analysis revealed increased $\gamma$-GT mRNA in GST-P-positive HAF but not amphophilic GST-P-negative HAF.

### *Semiquantitative Analysis of $\alpha_2$M mRNA*

To validate expression of $\alpha_2$M mRNA observed in microarray analysis, semiquantitative RT-PCR was conducted. As shown in Figure 1, clear increased $\alpha_2$M mRNA expression was detected in amphophilic GST-P-negative HAF induced by NDEN + Wy-14,643 and NDEN + clofibrate as compared to the respective adjacent normal tissues, and also pronounced in amphophilic GST-P-negative HCA and HCC induced by NDEN + clofibrate. In the present study, however, elevation of $\alpha_2$M mRNA was less pronounced in amphophilic GST-P-negative HAF induced by NDEN + Wy-14,643 as compared to the lesions after exposure to NDEN + clofibrate. We consider that this variability is because of differences in progression of the lesions, because amphophilic GST-P-negative HAF microdissected in the NDEN + Wy-14,643 group were much smaller than the lesions microdissected in the group receiving NDEN + clofibrate (ie, 0.08 $mm^2$ in the former and 0.62 $mm^2$ in the latter as shown in Table 3).

**Table 4.** The Number of Dysregulated Genes

| No. of group | Treatment | Microdissected lesions | GST-P | No. of dysregulated genes* | |
|---|---|---|---|---|---|
| | | | | Up | Down |
| Experiment 1 | | | | | |
| 1 | NDEN + clofibrate | Hepatocellular altered foci† | Positive | 279 | 67 |
| 2 | NDEN + Wy-14,643 | Hepatocellular altered foci‡ | Negative | 67 | 7 |
| 3 | NDEN alone | Hepatocellular altered foci† | Positive | 137 | 71 |
| Experiment 2 | | | | | |
| 1 | NDEN + clofibrate | Hepatocellular altered foci‡ | Negative | 110 | 51 |

*Number of dysregulated genes in the lesion as compared to adjacent normal tissue.
†Clear cell foci.
‡Amphophilic cell foci.

**Table 5.** Common Dysregulated Genes in Amphophilic GST-P-Negative Foci Induced by Two Peroxisome Proliferators

| | | GST-P-negative foci | | | | GST-P-positive foci | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | NDEN + WY* | | NDEN + CF† | | NDEN alone | | NDEN + CF | |
| Access no. | Gene name | Signal‡ | Ratio§ | Signal | Ratio | Signal | Ratio | Signal | Ratio |
| Up-regulated genes | | | | | | | | | |
| M23566 | $\alpha_2$-macroglobulin | 385.7 | 1.4 | 3507.5 | 5.3 | ND¶ | – | ND | – |
| X61654 | ad1-antigen | 568.0 | 1.3 | 1019.5 | 1.8 | ND¶ | – | ND | – |
| X60767 | cdc2 promoter | 30.1 | 1.3 | 19.4 | 1.2 | 18.7 | 1.0 | 18.3 | 1.1 |
| AA860039 | EST | 22.6 | 1.1 | 7.8 | 2.9 | 28.1 | 2.1 | 16.7 | 1.8 |
| AI070295 | EST | 88.2 | 3.1 | 58.8 | 1.2 | ND | – | 58.0 | 0.8 |
| Down-regulated genes | | | | | | | | | |
| J00738 | $\alpha$-2u globulin | 3.1 | −1.4 | 175.0 | −1.9 | 1110.8 | −0.6 | 54.9 | −1.8 |
| M18363 | P-450(M-1) | 3.9 | −2.7 | 512.0 | −1.4 | 403.8 | −1.2 | 233.7 | −1.6 |

*WY; Wy-14,643.
†CF; clofibrate.
‡Signal represents intensity of gene expression.
§Ratio represents the $\log_2$ of the ratio between the experimental (GST-P-negative or -positive foci) and the control (adjacent normal liver tissue) samples.
¶ND represents "not detectable."

## *Localization of $\alpha_2$M mRNA*

To examine the localization of $\alpha_2$M mRNA in the GST-P-negative HAF, *in situ* hybridization was performed (Figure 2). Specific increased accumulation of $\alpha_2$M mRNA was consistently and evenly distributed within amphophilic GST-P-negative HAF, including small number of foci composed of homogeneous cells whose cytoplasms were poor in glycogen and diffusely and relatively intense basophilic, induced by NDEN alone [described as amphophilic cell foci (basophilic) in Table 1]. Furthermore, the distinctive accumulation was observed not only in large but also very small foci, for which diagnosis only by H&E staining was difficult. Subsequently, the total number of foci positive for $\alpha_2$M mRNA signals was counted to investigate sensitivity as a marker of GST-P-negative foci. *In situ* hybridization was conducted for the animals sacrificed at week 26 in experiment 2, in which relatively large and many GST-P-negative foci were induced. As shown in Table 6, the total number of foci positive for $\alpha_2$M mRNA signals was higher than the total number of GST-P-negative foci (predominantly amphophilic cell foci) detected only by H&E staining shown in Table 2 ($\alpha_2$M mRNA-positive foci [19.06 ± 3.63 and 10.80 ± 2.26] versus GST-P-negative foci detected by H&E staining [10.30 ± 2.10 and 5.69 ± 2.40] in the groups of NDEN + clofibrate and NDEN alone, respectively).

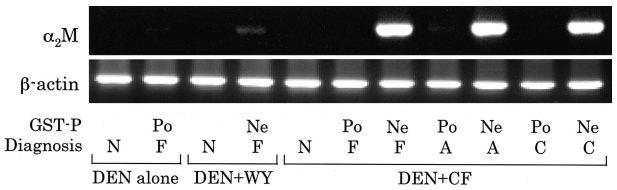


**Figure 1.** Semiquantitative RT-PCR for $\alpha_2$M mRNA in HAF, HCA, HCC, and adjacent normal liver tissues. Po, GST-P-positive; Ne, GST-P-negative (amphophilic phenotype); N, normal tissue; F, hepatocellular altered foci; A, hepatocellular adenoma; C, hepatocellular carcinoma; WY, Wy-14,643; CF, clofibrate.

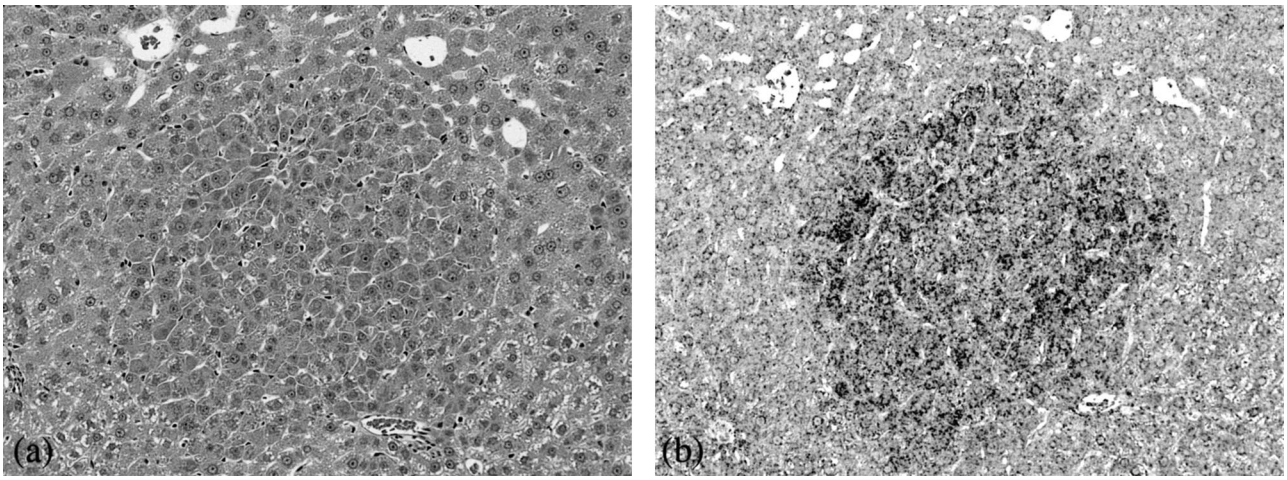


**Figure 2.** *In situ* hybridization for $\alpha_2$M mRNA in GST-P-negative HAF (amphophilic cell foci) induced by NDEN + clofibrate. **a:** H&E staining. **b:** Accumulation of $\alpha_2$-M mRNA. Original magnifications, ×125.

**Table 6.** Quantitative Analysis of the Foci Positive for $\alpha_2$M mRNA Signals Larger than 0.1 mm in Diameter (Animals Sacrificed at Week 26 in Experiment 2)

| No. of group | Treatment | Total number of foci positive for $\alpha_2$M mRNA (No./cm$^2$) |
|---|---|---|
| 1 | NDEN + clofibrate | 19.06 ± 3.63 |
| 2 | NDEN alone | 10.80 ± 2.26 |
| 3 | No treatment | 0.00 ± 0.00 |

## *Localization of $\alpha_2$M Protein*

Strong immunohistochemical staining of $\alpha_2$M protein was observed characteristically in amphophilic GST-P-negative HAF, HCAs, and HCCs induced by NDEN + PPs (Figures 3 and 4). Interestingly, GST-P-negative HAF induced by NDEN alone also showed distinctive reactivity to anti-$\alpha_2$M antibody (Figure 3B). Furthermore, in contrast to amphophilic GST-P-negative HAF and HCAs, heterogeneous staining was shown within amphophilic GST-P-negative HCC, ie, the invasive tumor cells were more distinctly stained especially as compared to the other tumor cells (Figure 4c, arrow). Detailed observation, however, revealed that nearly same sized GST-P-negative HAF did not necessarily take on equivalent staining properties, and some foci with $\alpha_2$M mRNA signals showed no clearly positive staining.

## *Discussion*

In the present study, microarray analysis revealed $\alpha_2$M to be characteristically overexpressed in amphophilic GST-P-negative HAF, in which mRNA overexpression for γ-GT was also undetectable as compared to adjacent normal tissues. Further detailed examinations demonstrated the same characteristics observed not only in amphophilic GST-P-negative HAF induced by peroxisome proliferators, but also amphophilic GST-P-negative HCAs and HCCs induced by peroxisome proliferators, and small number of foci composed of homogeneous cells whose

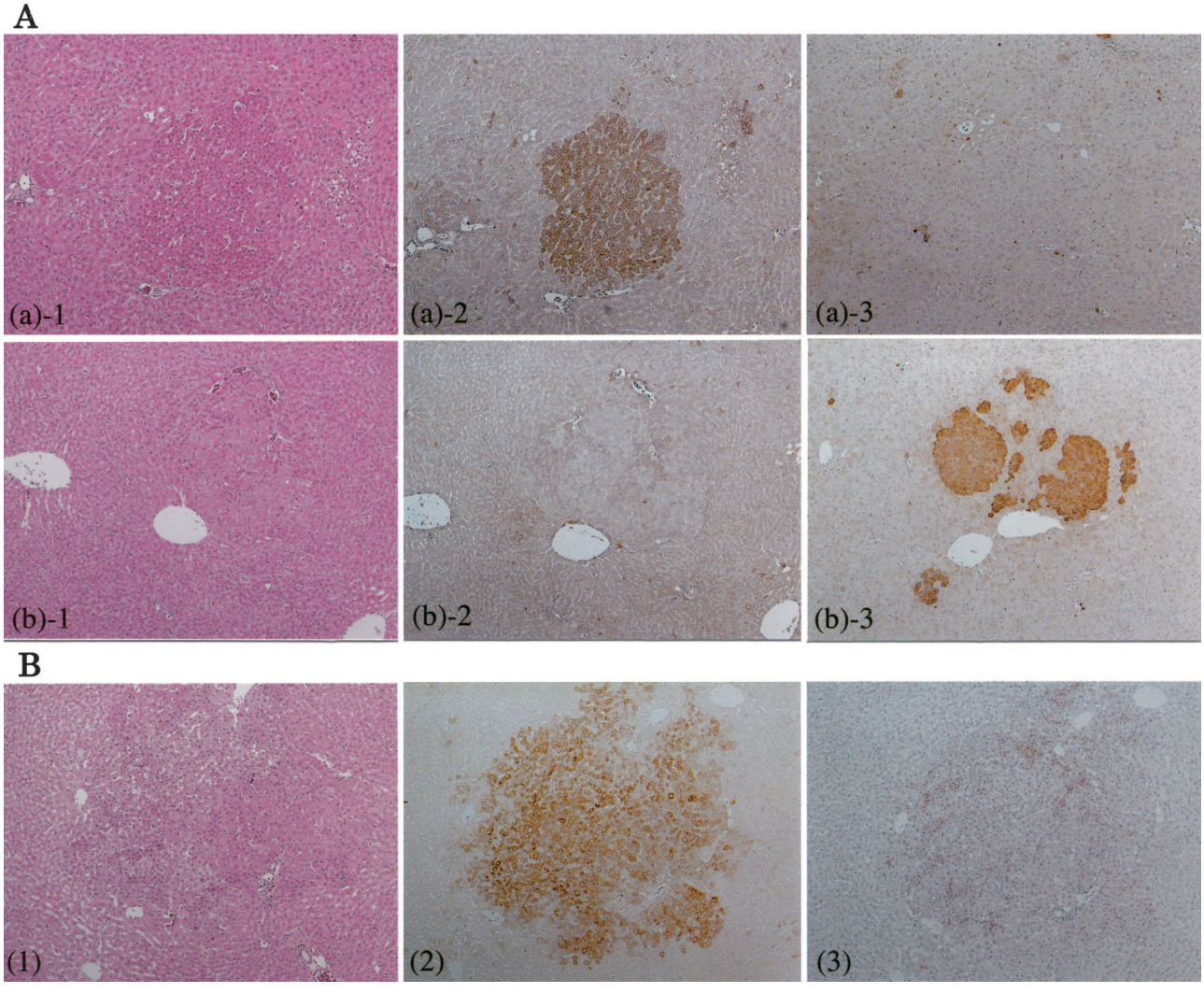


**Figure 3.** Immunohistochemistry for $\alpha_2$M and GST-P. **A:** HAF induced by NDEN + clofibrate. **a:** Amphophilic GST-P-negative HAF: **1**, H&E staining; **2**, $\alpha_2$-M; **3**, GST-P. **b:** GST-P-positive HAF: **1**, H&E staining; **2**, $\alpha_2$-M; **3**, GST-P. **B:** GST-P-negative HAF induced by NDEN alone. **1**, H&E staining; **2**, $\alpha_2$-M; **3**, GST-P. Original magnifications: ×40 (**A**); ×30 (**B**).

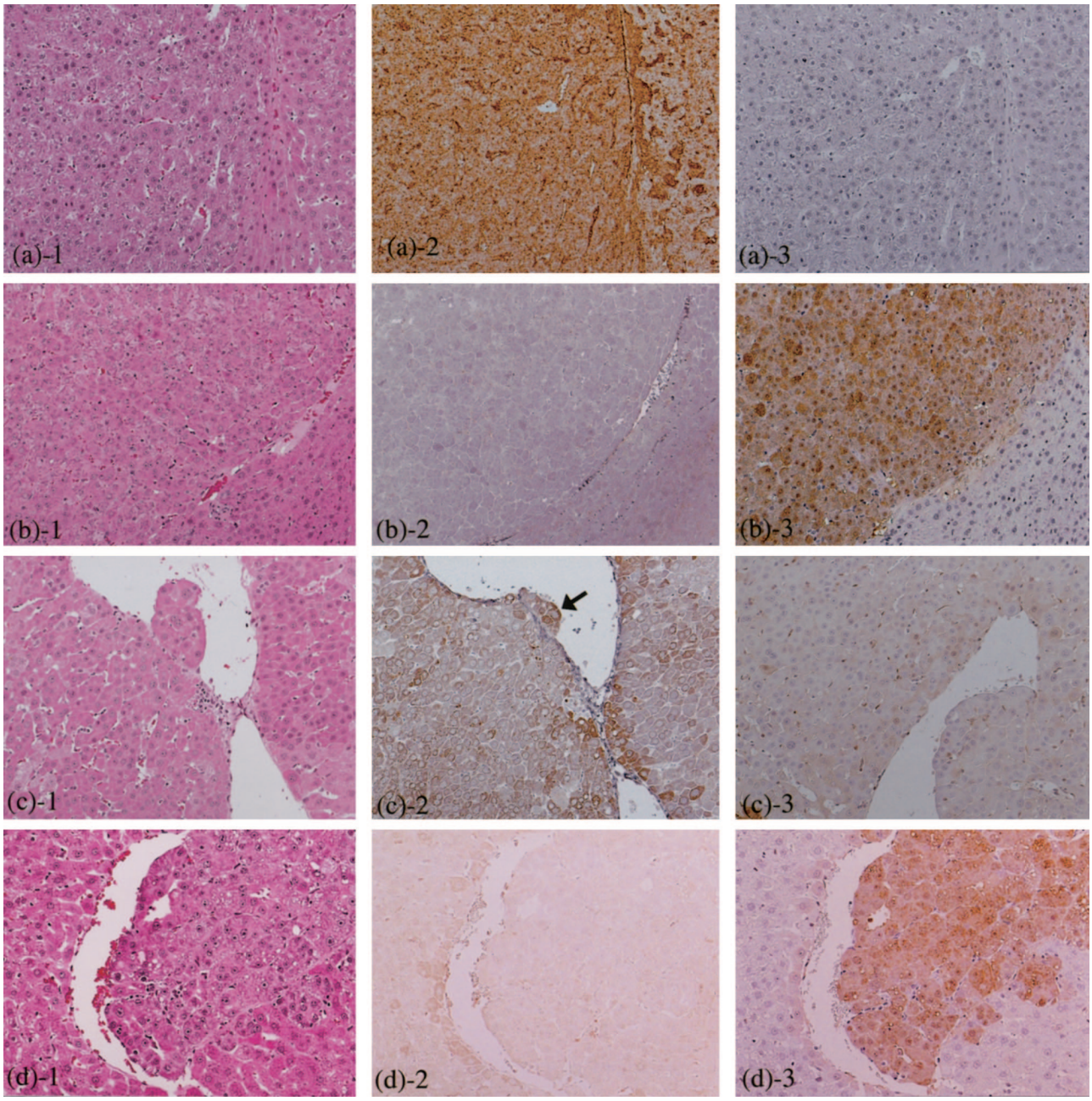


**Figure 4.** Immunohistochemistry for $\alpha_2$M and GST-P in liver tumors induced by NDEN + clofibrate. **a** and **c:** GST-P-negative HCA and HCC (amphophilic phenotype), respectively. **b** and **d:** GST-P-positive HCA and HCC, respectively. **1**, H&E staining; **2**, $\alpha_2$-M; **3**, GST-P. **Arrow** illustrates invasive tumor cells into blood vessel in **c2**. Original magnifications, ×85.

cytoplasms were poor in glycogen and diffusely and relatively intense basophilic, induced by NDEN alone. Additionally, no obviously different immunohistochemical properties for $\alpha_2$M were observed among morphologically different types of GST-P-negative HAF, such as amphophilic cell foci (weakly basophilic or basophilic). Members in the group of Bannasch[16,17] earlier reported in detail that enzyme histochemical examination revealed alterations of some mitochondrial enzymes in amphophilic cell foci. In the present study, however, characteristic accumulation of $\alpha_2$M mRNA was observed even in HAF not evident on routine H&E staining because of the small size or equivocal morphological character. Quantitative analysis of the total number of foci positive for $\alpha_2$M mRNA signals showed quantitative assay using $\alpha_2$M as a marker is possibly sensitive as compared to one only by routine H&E staining in evaluation of GST-P-negative foci. At present, the quantitative assay using GST-P as the indicator for carcinogenicity, eg, *in vivo* medium-term bioassay for carcinogens,[13] is one of the most sensitive assays and makes it possible to evaluate earlier as compared to assessment only by routine H&E staining. Thus, in addition to hitherto markers such as GST-P, application of $\alpha_2$M to the above assay possibly accomplishes more

accurate safety assessment. On the contrary of characteristic accumulation of $\alpha_2$M mRNA, however, immunohistochemical staining of $\alpha_2$M protein was not necessarily observed at all of the corresponding lesions. Smorenburg and colleagues[35] reported only weak immunostaining of $\alpha_2$M protein present in cancer cells in the metastases of colon cancer, although high levels of the mRNA were found in cancer cells of all metastases, suggesting immediate release of $\alpha_2$M after being produced. Therefore, discrepancy between accumulation of mRNA and immunohistochemical staining of $\alpha_2$M in the HAF might be because of the same reason, so that if some appropriate procedures, eg, liver perfusion with an appropriate fixative, are conducted to prevent $\alpha_2$M being released, the inconvenience observed in the immunohistochemistry might be improved.

Furthermore, $\alpha_2$M increased with progression from HAF to HCA. As noted above, $\alpha_2$M functions as a carrier protein and regulator for various growth factors and cytokines such as transforming growth factor-$\beta$, interleukin-1, and tumor necrosis factor-$\alpha$.[21] transforming growth factor-$\beta$1 is known to be involved in the onset of hepatocyte apoptosis and that peroxisome proliferators can impinge on this cell death pathway.[36] Furthermore, $\alpha_2$M partially counteracts the inhibitory effects of transforming growth factor-$\beta$1 or -$\beta$2 on proliferation of neoplastic hepatocytes,[37] suggesting that under some conditions, $\alpha_2$M can promote hepatocarcinogenesis by perturbing transforming growth factor-$\beta$-induced apoptosis. Additionally, interleukin-1 and tumor necrosis factor-$\alpha$ mediate hepatocyte DNA synthesis and suppression of apoptosis induced by peroxisome proliferators,[38] suggesting that $\alpha_2$M can also exert an influence by up-regulating some growth factors and cytokines such as interleukin-1 and tumor necrosis factor-$\alpha$. Several investigators[32,39] reported no elevations of serum $\alpha_2$M level during rat hepatocarcinogenesis induced by NDEN, acetylaminofluorene, and 3′-methyl-4-dimethyl aminoazobenzene except at the late stage. We consider that this is probably because most of all lesions induced by these agents were GST-P-positive lesions.[13]

The preferential localization of $\alpha_2$M protein was observed in the invasive and/or perivascular tumor cells, as opposed to homogeneous accumulation of $\alpha_2$M mRNA observed in our preliminary experiment (data not shown). Several experiments have been undertaken to clarify what role $\alpha_2$M might play in invasion or metastasis of tumor cells and it has been found to inhibit invasive growth because of its function as pan-protease inhibitor.[40,41] However, Asplin and colleagues[42] recently suggested that receptor-recognized forms of $\alpha_2$M behave like a growth factor in highly metastatic human prostate carcinoma cell line, 1-LN, and the 1-LN metastatic phenotype may result, in part, from aberrant expression of $\alpha_2$M signaling receptor, indicating the possible involvement of receptor-recognized forms of $\alpha_2$M in tumor progression. In addition, Smorenburg and colleagues[35] indicated that $\alpha_2$M might be related to tumorigenicity, ie, they speculate $\alpha_2$M produced by metastatic colon cancer cells captures inflammatory cytokines, thereby inhibiting the defensive reaction against cancer cell spread, because colon cancer cells in metastases of all different stages were shown to produce $\alpha_2$M mRNA and seem to release the protein effectively. They further observed that although stromal cells surrounding metastases were low in $\alpha_2$M mRNA expression, they were positively stained for the protein. They speculated the stromal cells seem to be involved in endocytosis of $\alpha_2$M. In the present study, it seems likely that $\alpha_2$M produced by the tumor cells is trapped by the invasive tumor cells and tumor cells surrounding the blood vessels via the $\alpha_2$M receptor or low-density lipoprotein receptor-related protein.[43] Taking all of the findings together, however, the significance of specific overexpression of $\alpha_2$M protein in tumor cells at the invasive sites and surrounding blood vessels remains unclear.

In conclusion, the present study demonstrated that $\alpha_2$M is a novel and an important candidate cytochemical marker for identification of HAF, in particular amphophilic cell foci, which are undetectable by established cytochemical markers for rat hepatocarcinogenesis, and could be applied to *in vivo* medium term bioassay for carcinogens[13] in addition to GST-P. In addition, $\alpha_2$M may be tightly linked to the hepatocarcinogenesis from the initial stage through to tumor progression. However, it remains to be clarified why up-regulation of $\alpha_2$M is characteristic for GST-P-negative lesions and how $\alpha_2$M makes a contribution to rat hepatocarcinogenesis, invasion, or metastasis. Elevated $\alpha_2$M is also observed in humans with some type of HCC, so that it must give beneficial information for clinical diagnosis, prevention of liver tumor, and risk assessment for chemical carcinogens to elucidate what role $\alpha_2$M plays in rat hepatocarcinogenesis.

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