Technical Advance

Liver Gene Expression Profiles of Rats Treated with Clofibric Acid

Comparison of Whole Liver and Laser Capture Microdissected Liver

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Clofibric acid (CLO) is a peroxisome proliferator (PP) that acts through the peroxisome proliferator activated receptor α , leading to hepatocarcinogenesis in rodents. CLO-induced hepatocarcinogenesis is a multi-step process, first transforming normal liver cells into foci. The combination of laser capture microdissection (LCM) and genomics has the potential to provide expression profiles from such small cell clusters, giving an opportunity to understand the process of cancer development in response to PPs. To our knowledge, this is the first evaluation of the impact of the successive steps of LCM procedure on gene expression profiling by comparing profiles from LCM samples to those obtained with non-microdissected liver samples collected after a 1 month CLO treatment in the rat. We showed that hematoxylin and eosin (H&E) staining and laser microdissection itself do not impact on RNA quality. However, the overall process of the LCM procedure affects the RNA quality, resulting in a bias in the gene profiles. Nonetheless, this bias did not prevent accurate determination of a CLO-specific molecular signature. Thus, gene-profiling analysis of microdissected foci, identified by H&E staining may provide insight into the mechanisms underlying non-genotoxic hepatocarcinogenesis in the rat by allowing identification of specific genes that are regulated by CLO in early pre-neoplastic foci. (*Am J Pathol* 2003, 163:2191–2199)

Clofibric acid (CLO) is the principal metabolite of the hypolipidaemic drug clofibrate and is the pharmacologically active form.^{1,2} It belongs to the broad class of chemicals known as PPs, which act through the peroxisome proliferator activated receptor α (PPAR α). The activation of PPAR α induces cell proliferation and suppresses apoptosis, (for review see³), and mediates the hepatocarcinogenic properties of PPs in rodents since PPAR α knock-out mice are non-responsive and do not develop hepatocarcinogenesis after long-term treatment with PPs.^{4,5} However, genes modulated by PPs to regulate cell proliferation and apoptosis suppression remain to be determined and the exact cascade of molecular events leading to the transformation of normal hepatocytes to altered hepatocellular foci and/or hepatocellular neoplasms remains unclear.

To elucidate the mechanism of the CLO-induced hepatocarcinogenic process, it would help to define the variation of gene expression at different stages, particularly in the early pre-neoplastic foci. To facilitate this type of study, we first needed to evaluate the feasibility and reproducibility of monitoring gene expression in microdissected cells, by combining laser capture microdissection (LCM) with gene expression profiling. Indeed, no accurate and exhaustive comparison of the gene expression profile between LCM processed and unprocessed samples has been performed to date. Specifically, we aimed to carry out an objective assessment of the effect of LCM on gene expression measurement by comparing the gene expression profiles of liver samples obtained after key steps of the LCM procedure to that obtained from whole liver. Here we report the outcome of such a technical evaluation performed on liver obtained from a dose-range finding toxicity study on CLO, in preparation

Accepted for publication August 5, 2003.

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of a long-term hepatocarcinogenesis study. We showed that although the time required for processing LCM samples impacts, to some extent, on RNA quality, laser capture microdissection did not prevent the characterization of a CLO-specific molecular signature.

Materials and Methods

Animals and Dosing

Six to seven-week-old Fisher F344 male rats (Iffa-Credo, L'Arbresle, France) received clofibrac acid (Sigma Aldrich, Saint Quentin Fallavier, France) (0%, designated treatment control group, and 0.29% (v/v) or 0.54% (v/v), designated CLO-treated groups) for 4 weeks via powdered diet. Selected doses (0.29 and 0.54% in diet) were known to induce tumors after long-term treatment in rodents.^{6,7} The animals were kept under standard conditions of temperature ($20 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$) with a 12-hour light-dark cycle.

Necropsy

Rats were anesthetized by intraperitoneal injection of pentobarbital (0.7%, w/v) and culled by exsanguination. Livers were immediately excised under sterile conditions and liver weights were recorded for each animal. Portions of liver from all animals were flash-frozen in liquid nitrogen for total RNA extraction (sample W for whole liver). Other liver specimens were taken from the left, right, and median lobes and fixed in 10% formalin-phosphate-buffered saline for histopathological examination. The remaining liver was embedded in OCT (Labonord, Templemars, France), carefully frozen in liquid nitrogen for further LCM and RNA processing and stored at -80°C . All these steps were performed for all of the CLO-treated and treatment control groups. The formalin-fixed samples were routinely processed, embedded in paraffin, and sectioned at $6 \mu\text{m}$. Liver sections were stained with a classical hematoxylin, eosin, and saffron (HES) and examined by light microscopy.

LCM Tissue Preparation

The main steps of a classical LCM experiment and the different sample types of the experiment are depicted in Figure 1. The experimental conditions that were used to study the influence of two critical steps in this process (staining and microdissection) are summarized in Table 1. Eight to 10 μm serial frozen sections were cut with a cryostat at -20°C , mounted on LLR2 RNase-free slides (CML, Nemours, France) and kept at -80°C until staining (Figure 1 and Table 1). Immediately before use, the slides were thawed at room temperature for 30 seconds and fixed in 70% ethanol (30 seconds). Then they were stained with 75% Mayer's hematoxylin (30 seconds), briefly rinsed in diethylpyrocarbonate (DEPC)-treated water, dehydrated in graded alcohols (30 seconds each). They were stained with 0.75% alcoholic eosin (20 seconds) and dehydration was completed by rinsing the slides in

Figure 1. Schematic outline of the experiment. Summary of preparation of the different sample types.

95% ethanol (30 seconds), 100% ethanol (7 minutes), and xylene (7.5 minutes, samples S for stained). Six replicates were performed for each treatment group (0, 0.29% or 0.54% CLO). To study the influence of staining, other slides were thawed at room temperature for 30 seconds, fixed in 70% ethanol (1 minute), and directly dehydrated in 95% and 100% ethanol (30 seconds each) without staining. Dehydration was completed by rinsing the slides in pure ethanol (1.30 minutes) and xylene (5.5 minutes) (samples D for dehydrated, 1 replicate per treatment group) to evaluate the impact of this key step. All slides were finally air-dried for at least 1 minute. LCM (on samples S) or direct RNA extraction on the slide (on samples D and S) was performed immediately after staining (see below). To get enough starting RNA material to perform the labeling step without amplification (see RNA processing), total RNA extracted from 27 liver sections of around 1 cm^2 each were pooled per replicate.

LCM

After staining and dehydration, some stained slides were immediately microdissected by the Pixcell II LCM instrument (Arcturus, Mountain View, USA), using Capsure transfer film (Arcturus). Although sections had been stained by a process that would allow for identification of foci, cells were microdissected randomly such that the microdissected cell population and whole liver sample were comparable. The procedure took no more than 20 minutes per section and the samples (samples M for microdissected) were immediately taken for total RNA extraction (see below). To study the influence of duration time of microdissection, some LCM time reference slides were left aside during the procedure and then taken for

Table 1. Summary of the Different Experimental Conditions

	Dehydration	Staining	Microdissection
Whole liver sample	—	—	—
Dehydrated sample	+	—	—
Stained sample	+	+	—
LCM-time reference	+	+	no, but left-aside for the duration of LCM
Microdissected sample	+	+	+

total RNA extraction (samples L for LCM time reference, one replicate per treatment group).

Total RNA Extraction

Total RNA was extracted from rat liver (samples W) using the Qiagen Rneasy Maxi kit (Valencia, USA). Total RNA from samples S, D, and L was extracted by adding the extraction buffer directly onto the slice. Samples M, collected on LCM Caps (Arcturus), were transferred to RNase-free microtubes containing extraction buffer. Microtubes were incubated for 30 minutes at 42°C for cell lysis and RNA solubilization and extraction was performed using the Qiagen Rneasy Mini kit (Valencia, USA). Around 50 μg of total RNA was obtained from a total number of 27 stained liver sections. A similar amount of RNA was obtained from 27 dehydrated liver sections. Around 15 μg of total RNA were obtained from 20 LCM time reference liver sections (samples L). To be able to perform gene profiling on microdissected liver without amplification (only using the standard Affymetrix protocol, starting with 15 μg total RNA), 20 caps were microdissected on 20 liver sections. This is the reason why only one replicate per treatment group was performed. Quality of total RNA was checked on the Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). Quantitation was performed using an Uvikon 860 spectrophotometer (Secomam, Domont, France) at $\lambda = 260 \text{ nm}$.

RNA Processing

Fifteen μg of total RNA samples were labeled using standard Affymetrix protocol to generate complementary RNA (cRNA), which were hybridized on Affymetrix rat RG-U34A GeneChips (8799 full-length cDNA + Expressed Sequence Tags, Affymetrix, Santa Clara, USA). The arrays were scanned using the GeneArray scanner (Affymetrix) and the scanned image were quantitatively analyzed with the software MicroArray Suite 4.0 (Affymetrix). The quality of hybridization as well as the RNA preparation were checked with the following Affymetrix

quality control criteria: mean average difference, which is an absolute indicator of the expression level of a transcript, raw intensity of the housekeeping genes β -actin and GAPDH to assess RNA sample and assay quality, and their 3'/5' ratio, which is representative of the quality of the initial total RNA and of the elongation process during the labeling protocol. High quality cRNA commonly exhibit a 3'/5' ratio around 1. Genes are designated as present when their raw signal intensity is regarded as significant by the Affymetrix Scanning procedure.

Gene Expression Data Analysis

Gene Expression data were analyzed using an in-house data-mining tool (GECKO 2). The software performed global normalization across the various GeneChips, using a reference chip and the 75th percentile of the median. Reproducibility and similarity between all of the processes were evaluated by calculating a Pearson correlation coefficient on normalized raw data. "CLO-treated" normalized intensities were divided by "treatment-control" normalized intensities of each process to calculate fold-changes and associated confidence indices (p value) for each modulation.⁸ The p value for differential gene expression selected for statistical significance was 0.05. Graphic representations as scatter plot for Principal Component Analysis (PCA) as well as Heat Map were performed using Spotfire (Spotfire, Somerville, USA).

Results

Histopathological Findings Were Similar between Both CLO Doses

Liver weight variations and microscopic changes were evaluated in all animals from both groups. Mean absolute and relative (to body weight) liver weights increased with the treatment as compared to controls. Absolute liver weights increased significantly ($P < 0.05$) and reached a value of +74 and +55% of the mean control value for the

Figure 2. Paraffin section of liver (HES stain). **a:** Liver section of control animal. **b:** Liver section of 0.54% CLO-treated animal. Note the diffuse hepatocellular hypertrophy characterized by enlarged hepatocytes with a glassy eosinophilic appearance. **Bars,** 100 μm

Figure 3. Analysis of RNA quality and size. **a:** Agilent 2100 Bioanalyzer profiles of total RNA extracted from: W, whole liver; S, stained slices; D, dehydrated slices; L, LCM time-reference liver samples; M, microdissected liver tissue. **b:** Agilent 2100 Bioanalyzer electrophoregram gel of cRNA obtained after labeling of the total RNA of the samples W, S, D, L, and M.

animals exposed by diet containing 0.29% (w/w) and 0.54% (w/w) clofibric acid, respectively. The relative liver weights reached statistically significant ($P < 0.05$) values when compared to the mean control value: +97 and +110% for animals on the 0.29% (w/w) and 0.54% (w/w) clofibric acid diets, respectively.

Clofibric acid-related microscopic findings (Figure 2) were noted in the liver at both dose levels and consisted of diffuse hepatocellular hypertrophy characterized by enlarged hepatocytes with a glassy eosinophilic cytoplasm. An example is shown in Figure 2b, which represents a liver section of rat treated with 0.54% CLO. This finding correlated with increased liver weights. All liver lobes were similarly affected microscopically. The incidence of hepatocellular hypertrophy was 5 of 6 rats affected in the 0.29% diet group and 6 of 6 animals affected in the 0.54% diet group. The severity varied from minimal to mild and was distributed similarly between both groups.

RNA Quality Decreases during the LCM Process

The quality of the total RNA was first evaluated on the RNA electrophoregrams generated by the Agilent 2100 Bioanalyzer. The flat baseline of the profiles indicated a high quality RNA (Figure 3a). Twenty-eight S ribosomal

peaks were higher than the 18S peaks in each profile. Taken together, these profiles suggested a good quality of total RNA extracted from all samples, irrespective of processing. Examination of the cRNA smears (Figure 3b) obtained after labeling showed an increase in small molecular weight cRNA in all of the processed samples (D, S, L, and M) compared to samples from whole liver (W). Size of transcripts obtained from whole liver samples ranged from 1500 to 4000 nucleotides. For dehydrated and stained samples, it ranged from 200 to 4000 nucleotides, while for LCM time reference and microdissected samples, the range went from 200 to 2000 nucleotides. Our results suggested a decrease in the cRNA transcript size obtained after labeling and hence a decreased cRNA quality.

Dehydrated and stained samples differed only by the addition of a hematoxylin and eosin (H&E) staining step. Analysis of total RNA profile (Figure 3a, D and S) as well as the cRNA electrophoregram (Figure 3b, D and S) showed no differences in term of transcript size between these two samples, suggesting that the staining process did not impact the RNA quality. Similarly LCM time reference and microdissected samples were similar, indicating that the LCM itself did not have an effect on RNA quality.

Confirmation of the degradation of cRNA during the processing was provided by examination of different Affymetrix quality controls (Table 2). Even though the total RNA degradation was not visible using Agilent 2100 Bioanalyzer technology, hybridization of the cRNA obtained from these "processed" total RNA showed an increase in the 3'/5' ratio, a decrease in the global intensity of the chip, and a decrease in the intensity of the two house-keeping reference genes GAPDH and β -actin. Similarly, a decrease of the number of present genes was observed, correlated to the mean average difference drop. Here again, there were no real differences between quality controls from chips hybridized from stained and dehydrated samples, confirming that the H&E staining step did not impact on RNA quality. Similarly, there were no differences between quality controls obtained from LCM time reference and microdissected samples, suggesting that laser microdissection is not detrimental to RNA quality. The most important decrease of these quality control criteria was observed between samples D/S and L/M, thus indicating that the 20-minute duration time for microdissection had a major impact on RNA quality.

Table 2. Quality Control of the Various Sample Types

Sample type	Mean average difference $\pm$ S.D.	GAPDH		β -Actin		Number of present genes $\pm$ S.D.
		Raw intensity $\pm$ S.D.	3/5 ratio $\pm$ S.D.	Raw intensity $\pm$ S.D.	3/5 Ratio $\pm$ S.D.	
Whole liver sample	208 $\pm$ 67	2376 $\pm$ 706	1.76 $\pm$ 0.71	1257 $\pm$ 361	1.91 $\pm$ 0.66	3810 $\pm$ 414
Dehydrated sample	130 $\pm$ 51	1178 $\pm$ 760	10.6 $\pm$ 5.9	217 $\pm$ 193	5.90 $\pm$ 2.76	3259 $\pm$ 588
Stained sample	221 $\pm$ 100	1148 $\pm$ 699	13.6 $\pm$ 5.23	188 $\pm$ 134	7.66 $\pm$ 5.5	3250 $\pm$ 431
LCM-time reference	44.5 $\pm$ 26	394 $\pm$ 327	4.47 $\pm$ 1.16	63 $\pm$ 38	2.00 $\pm$ 0.47	2067 $\pm$ 588
Microdissected sample	47 $\pm$ 30	321 $\pm$ 309	8.84 $\pm$ 5.11	67 $\pm$ 78	4.98 $\pm$ 2.39	2163 $\pm$ 580

Table 3. Pearson Correlation Coefficients on Raw Data Intensities Evaluating the Influence of Each Step of the Process on the Similarity between Gene Expression Profiles from Processed Liver Samples and from Non-Processed Liver Samples

Pearson correlation coefficient	Same whole liver sample from the same experiment Intramanipulation reproducibility	Same whole liver sample from another experiment Intermanipulation reproducibility	Same liver sample, dehydrated within the same experiment	Same liver sample, stained within the same experiment	LCM time reference sample within the same experiment	Same liver sample, microdissected within the same experiment
Whole liver sample	0.991	0.963	0.930	0.907	0.829	0.834
Stained liver sample			0.96	0.972		
Microdissected sample					0.978	

The Pearson correlation coefficient was calculated for each replicate of each treatment group and averaged.

The Staining and Microdissection Steps of the LCM Process Do Not Alter the Reproducibility of Gene Expression Data

Table 3 shows the average of Pearson correlation coefficients calculated between the gene expression profiles obtained from a sample after different steps of the LCM process (or non-processed, ie, from the corresponding whole liver). To evaluate the impact of LCM, Pearson correlation coefficients were calculated, as references, between different whole liver samples. The Pearson correlation coefficient obtained by comparing the experimental replicates of the same sample was high (0.991), showing good reproducibility within each experiment. Comparison of the same sample between different experiments gave a 0.963 correlation coefficient.

The influence of sample processing was evaluated by comparing the gene expression profiles obtained from the same source material either processed or not, whatever the CLO treatment. The Pearson correlation coefficient decreased with increased complexity of the process (0.930 and 0.907 when comparing respectively dehydrated and stained samples *versus* whole liver samples). Comparison of dehydrated sample *versus* stained sample lead to a 0.96 correlation coefficient, showing that the staining dyes do not alter the gene profile of the sample. The Pearson correlation coefficient obtained from the comparison of the expression profile from whole liver sample *versus* microdissected samples was 0.834. Similar results were obtained with LCM time reference samples (0.829). The 0.972 Pearson coefficient obtained by comparing stained samples showed that the slight decrease in RNA quality undergone by stained samples was consistent, confirming a good reproducibility of the process and of its effect. The main decrease of the Pearson coefficient correlation was observed by comparing the L/M samples with the corresponding whole liver sample, highlighting the influence of the time required for microdissection. However, the Pearson coefficient correlation between the expression profile from LCM time reference *versus* microdissected samples was 0.978 (Table 3), confirming again that laser microdissection itself did not induce additional changes in gene expression profiles.

Gene Expression Analysis Emphasizes the Bias Induced by the LCM Process

Figure 4 represents a scatter plot obtained by performing a PCA on the raw gene expression data. The gene expression profiles from the various treatment controls (LCM processed or not, Δ) clustered together in a group distant from the treated samples. Irrespective of the LCM process, changes in gene expression profiles induced by CLO were distinguishable using PCA. However, further examination of treatment controls (Δ) revealed a clear separation into two groups (orange circles), the whole liver and the processed treatment controls, suggesting that the LCM process does induce an experimental bias on gene expression profile.

In the CLO-treated cluster, data were again separated into two groups (green circles), according to the LCM process. Gene expression profiles from the two treated groups (0.29% CLO and 0.54% CLO) overlapped. Comparison of the gene expression profiles from whole liver samples between 0.29% and 0.54% CLO groups showed

Figure 4. Scatter plot after PCA of raw data. Raw data intensities were reduced to the top 1000 most differentially expressed genes as determined by a $\chi^2 = 0.05$ test and were submitted to a PCA. The initial 1000 dimensions of the data set were reduced into three dimensions accounting for most of the differences,³⁸ before visualizing it by a scatter plot using Spotfire. Each symbol of the graph represents a sample. The process stage is color-coded and each treatment group is represented by a specific form. Treatment controls clustered together in an **orange circle**. CLO-treated samples clustered together in a **green circle**.

Cellular Pathway		Gene Name	Description	0,29%			0,54%		
				whole liver	stained samples	M&L samples	whole liver	stained samples	M&L samples
Cellular metabolism	Other	ATRC	ATC carboxylase						
		ASAL	argininosuccinate lyase						
		Es2	Esterase 2						
		NDUFB9	NADH-ubiquinone oxidoreductase B9 su						
		Nme2	nucleoside diphosphate kinase						
	Glycolysis/ gluconeogenesis	T2	Acetoacetyl-CoA thiolase						
		THP	Uromodulin precursor						
		Me1	Malic enzyme 1						
		ALDA	Aldolase A						
		G6P	Glucose-6-phosphatase						
	Amino acid metabolism	GCK	Glucokinase						
		GRP75	Glucose regulated protein						
		Me1	Oxidative decarboxylation of malate						
		Ddah1	Dimethylarginine 2CH3aminohydrolase 1						
		Cbs	Pseudo-cystathionine beta-synthase						
		Csad	Cysteine-sulfinate						
		Hal	Histidine ammonia-lyase						
		Oat	Ornithine aminotransferase						
		SDH2	Serine dehydratase						
		Sds	Serine dehydratase						
		C1s	Serine protease						
		HDC	Histidine decarboxylase						
		KST	Kallistatin						
		Mug1	α -1-inhibitor III precursor						
	β oxidation	Ehhadh	Enoyl-CoA- hydratase-3-hydroxyacyl-CoA						
		Acaa	3-ketoacyl-CoA thiolase						
		COT	carnitine O-octanoyltransferase						
		Decr1	2,4-dienoyl-CoA reductase						
		PECI	δ 3, δ 2-enoyl-CoA isomerase						
	Fatty acid synthesis	Dci	dodecenoyl-coenzyme A d isomerase						
		Hpd2	2-hydroxyphytanoyl-CoA						
		KS	Acetyl-coenzyme A synthetase						
		Mte1	Malic enzyme 2						
		Scd1	Stearoyl-CoA desaturase 1						
	Triglycerides catabolism	Scd2	Stearoyl-CoA desaturase 2						
		CPT 1	Carnitine palmitoyltransferase 1						
		LPL	Lipoprotein lipase						
		Fabp7	Fatty acid binding protein 7						
		FABP	Fatty acid binding protein						
	Fatty acid Transport	CYP2J3P2	Oxidation of arachidonic acid						
		PGCL4	α -2u globulin						
		SREBP-1	Sterol regulatory element-binding transcription f 1						
		FPPS	farnesyl diphosphate synthase						
		ApoA4	Apolipoprotein A-IV						
Cholesterol	Steroid metabolism	ApoC3	Apolipoprotein C-III precursor						
		ApoC4	Apolipoprotein C4 precursor						
		ApoM	Apolipoprotein M precursor						
		CYP17	17- α -hydroxylase						
		Hsd11b1	Hydroxysteroid dehydrogenase, 11 β type 1						
	Effects on HDL, Lipid transport	Smp2a	Senescence marker protein 2A						
		SRD5A	Rat steroid 5 α -reductase						
		SRD5A1	3-oxo-5- α -steroid 4-dehydrogenase 1						
		STA	Hydroxysteroid sulfotransferase subunit						
		UGT-21	Phase II biotransformation enzyme						
	Detoxification metabolism	CYP4A1/P452	Cytochrome P450, Lauroic acid ω - hydroxylase						
		Dig1	Dithiolethione-inducible gene-1						
		Sth	Hydroxysteroid sulfotransferase						
		Sth2	Hydroxysteroid sulfotransferase						
		GSTa	Glutathione S-transferase						
		AFAR	Aflatoxin B1 aldehyde reductase						
		Cyp1A2	Cytochrome P450						
		Cyp2C11G	Cytochrome P450						
		Cyp2C22	Cytochrome P450						
		Cyp2C39	Cytochrome P450						
Other steroid metabolism		CYP3A23	Cytochrome P450						
		CYP3A9	Cytochrome P450						
		Cyp4A10	Cytochrome P450						
		Cyp1A2	Cytochrome P450						
		Cyp2C7	Cytochrome P450						
	Oxidative stress	Fmo1	Dimethylamine monooxygenase						
		Tfrc	Rat transferrin receptor						
		Phb	Prohibitin						
		IGFBP-1	IGF binding protein-1						
		4F2hc	4F2 heavy chain						
	Growth regulation	GADD45	Growth arrest and DNA damage-inducible gene						
		Dri42	ER transmembrane protein						
		GPIV	Platelet glycoprotein IV						
		Tmsb10	Thymosin β -10						
		Cd36	Platelet glycoprotein IV						
Cell differentiation	Cell adhesion	Ela1	Preproelastase						
		Alcam	CD166 antigen precursor						
		Pdlim1	DZ and LIM domain 1						
		RAP	RAS-related protein P23						
		SMP30	Regucalcin						
		Map2k2	Mitogen activated protein kinase 2						
		Atp1b1	ATPase Na ⁺ /K ⁺ transporting beta 1 polypeptide						
		IOR	Indolepyruvate oxidoreductase subunit						
		P2RY2	Purinergic receptor P2Y, G-protein coupled 2						
		AQP9	Aquaporin 9						
	Cell cytoskeleton	Sgk	Serum and glucocorticoid-regulated kinase						
		Syngr1	Synaptogyrin 1						
		Atp1b1	Sodium/potassium-transporting ATPase β -1 chain						
		PSTI	ATPase inhibitor, mitochondrial precursor						
		IFP	Interferon inducible protein						
Transcription variation	Intracellular protease	Hes1	Transcription factor HES-1						
		Prkcdp	Protein kinase C δ -bindg protein						
		Mbl2	Mannose-binding protein C						
		Nupr1	Protein p8						
	Acute-phase protein								

a 0.981 Pearson correlation coefficient (Table 3). Taken together, these results indicate that both doses induce very similar gene expression profiles. Whatever the treatment group, gene expression profiles from dehydrated samples clustered with profiles from stained samples. This confirms that H&E staining does not impact on the measurement of gene expression. In addition, gene expression profiles from LCM time reference and microdissected samples clustered together, arguing again in favor of the lack of impact of laser microdissection on gene expression evaluation.

LCM Preserves the CLO-Specific Molecular Signature

Figure 5 shows that no major differences were observed in the list of CLO target genes between both doses in whole liver samples, confirming the similarity of their effect at the gene expression level. In addition, the degree of modulation for the highly modulated genes ([fold-change] > 10) (eg, ornithine aminotransferase, histidine decarboxylase, enoyl-CoA-hydratase-3-hydroxyacyl-CoA, 3-ketoacyl-CoA-thiolase, apolipoprotein A-IV, and cytochromes P450 3A9 and 4A10) was similar in whole liver and LCM processed samples, irrespective of the stage of the process. However, some of the small variations ($-5 < \text{fold-change} < +5$) concerning genes associated with cell differentiation and gluconeogenesis (eg, platelet glycoprotein IV, indolpyruvate oxidoreductase, uromodulin precursor, and glucose-regulated protein) were lost during processing. This was highly noticeable for down-regulated genes (eg, arginosuccinate lyase, esterase 2, pseudo-cystathionine β -synthase, histidine ammonia-lyase, steroid- α -reductase, apolipoprotein M precursor, purinergic receptor, interferon inducible protein, transcription factor, and mannose-binding protein C). Finally, most of the genes that were not found modulated in whole liver samples also presented no expression modulation in the processed liver samples (eg, dimethylarginine-2CH₃-aminohydrolase, apolipoprotein C4 precursor, and aquaporin 9).

Discussion

The goal of this study was to evaluate the effect of the LCM process on the gene expression profile, by comparing profiles from processed liver tissue with their matching profile from whole liver. Previous studies^{9–11} showed good reproducibility of gene expression profiles between microdissected samples and good sensitivity for identifying genes functionally representative of the microdissected cells.¹² However, no accurate and exhaustive comparison of the gene expression profile between LCM

processed and unprocessed samples has been performed to date. This technical evaluation was performed on liver taken from CLO-treated rats to evaluate the accuracy of the technique in determining genes of interest. This was evaluated without using a RNA amplification protocol to allow a thorough and specific evaluation of the influence of the successive steps of LCM on gene expression profile. Examination of total RNA, cRNA, and Affymetrix quality controls showed that neither the dyes nor the LCM induced a bias. Although the quality of the cRNA obtained from LCM-processed samples was slightly decreased, this did not prevent the characterization of a CLO-specific molecular signature.

Despite the good quality of the total RNA, electrophoretic analysis of the cRNA showed a decrease in the cRNA transcript size obtained after labeling of the processed samples. This shows that, despite the apparent high quality of the total RNA, there was degradation during the LCM-processing, which can only be seen after labeling. This was confirmed by the Affymetrix quality controls: decrease in mean average difference, decrease in intensity of GAPDH and β -actin, and increase in 3'/5' ratio. Two general mRNA decay pathways exist: the deadenylation-dependent decapping pathway leading to a 5' to 3' degradation, and the exosome-mediated decay pathway which destroys mRNA from 3' to 5' end. In yeast, degradation occurs more rapidly via the first pathway and a little is known about the second pathway in mammals.¹³ So, it is assumed that degradation of mRNA mainly begins from the 5' end of the transcripts. This would explain the increase of the 3'/5' ratio along the process. Given that the Affymetrix probes are mostly located on the 3' end of the transcript, a degradation beginning at the 5' end would affect gene expression profiles to a lesser extent than if the probes were randomly distributed. This alteration of gene expression profiles has already been suggested by Srinivasan et al.¹⁴ These findings stress that in further studies, gene expression analysis of small cell clusters of interest will have to be performed by comparing microdissected samples with each other. The bias evidenced here does not allow a direct comparison between microdissected samples and whole liver samples.

The staining protocol chosen needs to satisfy several criteria: it has to preserve tissue morphology while allowing the CLO-induced preneoplastic foci to be distinguished. In addition, it has to enable the PixCell II instrument to capture cells efficiently and also to preserve the integrity of RNA from the captured cells. PP-induced lesions do not express common markers that could facilitate detection of early pre-neoplastic change^{15–21} such as γ -glutamyltransferase or glutathione S-transferase placental form. They are mainly basophilic foci and can be distinguished by a H&E staining.²² It is important to note

Figure 5. Heat map of genes representative of CLO exposure after each step of the LCM procedure, grouped by biochemical categories. The complete data set can be accessed at the website <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE691>. Genes were selected if their fold change was statistically significant (P value < 0.05) in the 0.29% CLO *versus* treatment control group for whole liver sample. The 98 most significant genes obtained were manually classified into biochemical functions. The 0.29% CLO gene list was then mapped to the 0.54% CLO fold-changes. The fold-changes between CLO-treated and treatment controls were color-coded. The **green** color indicates a transcriptional down-regulation of the gene by CLO treatment and the **red** an up-regulation, whereas the **black** color represents no modulation. The color intensity represents the magnitude of the change in gene expression.³⁹ These fold-changes have been calculated for three stages of the process: whole liver samples, stained, and microdissected/LCM time reference samples (considered as replicates).

that, as expected, no foci were detected in the liver of rats treated for 1 month with CLO. However, evaluation of the potential of H&E staining to affect RNA quality was a key part of this study since this staining will be used to detect foci expected in later time points of an ongoing long-term hepatocarcinogenesis study. Hematoxylin and eosin stain by interfering with biomolecules. Hematoxylin is a basic dye, which stains the nucleus via interactions with nucleotides. In addition, basic pH is known to degrade oligonucleotides.²³ Taken together, and in agreement with Burton et al and Uneyama et al,^{24,25} this would suggest that the H&E staining process could be partly responsible for the observed degradation of total RNA samples. To check the influence of the H&E staining step, we compared dehydrated and stained samples, differing only by the addition of an H&E staining step. Analysis of total RNA profiles as well as the cRNA electrophoregram showed no differences between these two samples, demonstrating that the eosin and hematoxylin dyes are not responsible for RNA degradation. Banaschak et al and Ehrig et al^{26,27} showed no effect of H&E on DNA amplification. We demonstrated here that the dyes have no effect on RNA quality. Thus, our data show that the staining protocol satisfies the criteria described above.

Similarly, comparison of total RNA profiles and cRNA electrophoregram from LCM time reference and microdissected samples led to the conclusion that the LCM laser induces no additional changes. Taken together, our results suggest that no specific mechanical step of the LCM procedure is responsible for the decrease in RNA quality during sample preparation. However, to avoid adding a step of amplification in our protocol, a large number of cells were microdissected. Though this procedure was limited to 20 minutes per slide, it increased the time of the overall process. So, the time needed for processing the LCM sample could be the main factor for the observed RNA degradation. To improve the quality of the RNA from LCM processed samples, it would be useful to microdissect less material, thus decreasing the duration. However, RNA will have to be amplified to get the required amount of cRNA needed for Affymetrix gene profiling. Protocols of amplification are currently under evaluation in several laboratories.²⁸ For example, the protocol from Baugh et al²⁸ is designed to reduce the required starting material down to 2 ng of total RNA. This amount of total RNA would only require the microdissection of around one hundred cells. In the near future, properly validated amplification protocols will be available to obtain RNA from microdissected cells that should be of better quality. This will facilitate the evaluation of gene expression modulation in small lesions of a few tens of cells, such as preneoplastic foci.

Concerning the results of the CLO study itself, the histological changes we noted are similar to those previously described for this class of compound in rodent livers in association with proliferation of peroxisomes.²⁹ Looking at gene expression levels in whole liver samples, the molecular changes and pathway relationships noted were in agreement with previously described changes in response to PP exposure such as triglyceride hydrolysis, fatty acid uptake and β -oxidation stimulation (Figure 5),

also corroborating previous microarrays data.^{30–32} Up-regulation of the fatty acid binding protein by CLO has been widely described as a pharmacological effect of the PPs, as described by Fujishiro et al.³³ $\delta 3, \delta 2$ -enoyl-coA-isomerase, a gene involved in fatty acid metabolism was up-regulated in microarray analysis performed by Cherkaoui-Malki et al.³¹ Nakamura and Nara showed the up-regulation of sterol-regulatory element binding protein-1, a regulator of polyunsaturated fatty acid metabolism together with PPAR α .³⁴ Up-regulation of cytochrome CYP4A1/P452 has been reported in another microarray studies^{16,31} and by immunohistochemistry.¹⁶ Up-regulation of CYP 4A10 was also observed by Yamakazi et al.³⁰ In addition, CLO-molecular signature was not significantly affected by the key stages of the microdissection process for medium to highly modulated genes and the shape of the gene profiles was conserved. Only small expression variations due to CLO-treatment were lost during the LCM process.

In line with the non-genotoxic hepatocarcinogenesis potential of CLO in rodent, several candidate markers can be highlighted. Kallistatin, which is down-regulated in most of our CLO-treated samples, was recently shown to be an inhibitor of angiogenesis and tumor growth.³⁵ Prohibitin, up-regulated in our study, was described as an antiproliferative protein.³⁶ Similarly, regucalcin also named senescence marker protein 30 was down-regulated. It is regulated during apoptosis and was recently shown to have a suppressive effect on the enhancement of RNA synthesis during liver regeneration.³⁷ These last two proteins were found similarly regulated in proteomics studies performed in our laboratory (Leonard JF, Boitier E, Courcol M, Saulnier C, Duchesne M, Parker F, Roberts RA, and Gautier JC, manuscript in preparation), validating the gene expression variation observed.

In summary, LCM can be combined with gene profiling to accurately study a compound-specific molecular signature. The quality of total RNA obtained from LCM samples is sufficient to perform reliable gene expression profiles: the modulation of gene expression obtained by comparing CLO-treated and treatment control samples is representative for CLO treatment, independently of sample processing. Moreover, this modulation in gene expression is reproducible between whole liver and microdissected samples. In the future, gene expression profiling of microdissected preneoplastic foci (or cells at other stages of the hepatocarcinogenic process), followed by a RNA amplification step, will help in understanding the molecular mechanisms underlying CLO-induced non-genotoxic hepatocarcinogenesis in the rat. In addition, this combined LCM-gene expression profiling approach could be successfully applied for molecular analysis at any cellular level.

Acknowledgments

We thank J-M. Monichon's team for the histopathological preparations; A. Benevaut and the General Toxicology team for animal care; J. Theilhaber and J-P. Marchandeau for assistance in data analysis; V. Thiers, J-B. Tabut

and A. Dos Santos, INSERM U370, for the discussions on microdissection; J-F. Leonard and M. Courcol for corrections, and we thank P. Defrenaux, Arcturus France, for technical and scientific support on LCM.

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