資料 2

研究機関等における動物実験等の実施に関する基本指針

文部科学省告示第七十一号
研究機関等における動物実験等の実施に関する基本指針を次のように定める。

平成十八年六月一日
文部科学大臣　小坂　憲次

研究機関等における動物実験等の実施に関する基本指針

前文

地球上の生物の生命活動を科学的に理解することは、人類の福祉、環境の保全と再生などの多くの課題の解決にとって極めて重要であり、動物実験等はそのために必要な、やむを得ない手段であるが、動物愛護の観点から、適正に行われなければならない。

このため、研究機関等においては、従前から「大学等における動物実験について（昭和 62 年 5 月 25 日文部省学術国際局長通知）」等に基づき、動物実験委員会を設けるなどして、動物実験指針の整備及びその適正な運用に努めてきたところであるが、今後も生命科学の進展、医療技術等の開発等に資するため、動物実験等が実施されていくものと考えられる。

一方、平成 17 年 6 月に動物の愛護及び管理に関する法律の一部を改正する法律（平成 17 年法律第 68 号）が公布され、動物実験等に関する理念であるいわゆる 3R のうち、Refinement（科学上の利用に必要な限度において、できる限り動物に苦痛を与えない方法によってしなければならないことをいう。）に関する規定に加え、Replacement（科学上の利用の目的を達することができる範囲において、できる限り動物を供する方法に代わり得るものを利用することをいう。）及び Reduction（科学上の利用の目的を達することができる範囲において、できる限りその利用に供される動物の数を少なくすることをいう。）に関する規定が盛り込まれた。

このような動物実験等を取り巻く環境の変化を受け、研究機関等においては、科学上の必要性のみならず、動物の愛護及び管理に関する法律（昭和 48 年法律第 105 号。以下「法」という。）及び実験動物の飼養及び保管並びに苦痛の軽減に関する基準（平成 18 年環境省告示第 88 号。以下「飼養保管基準」という。）の規定も踏まえ、科学的観点と動物の愛護の観点から、動物実験等を適正に実施することがより重要である。

このような現状を踏まえ、動物実験等の適正な実施に資するため、研究機関等における動物実験等の実施に関する基本指針（以下「基本指針」という。）を定める。

第 1　定義

この基本指針において、次の各号に掲げる用語の意義は、それぞれ当該各号に定めるところによる。

(1) 動物実験等　動物を教育、試験研究又は生物学的製剤の製造の用その他の科学上の利用に供することをいう。
(2) 実験動物　動物実験等のため、研究機関等における施設で飼養し、又は保管している哺乳類、鳥類及び爬虫類に属する動物をいう。
(3) 研究機関等　次に掲げる機関であって、科学技術に関する試験、研究若しくは開発又は学術研究を実施するものをいう。
  ① 大学
  ② 大学共同利用機関法人
  ③ 高等専門学校
  ④ 文部科学省の施設等機関
  ⑤ 独立行政法人（文部科学省が所管するものに限り、独立行政法人国立高等専門学校機構を除く。）
  ⑥ 民法（明治 29 年法律第 89 号）第 34 条の規定により設立された法人（文部科学省が所管するものに限る。）
(4) 動物実験計画　動物実験等の実施に関する計画をいう。
(5) 動物実験実施者　動物実験等を実施する者をいう。
(6) 動物実験責任者　動物実験実施者のうち、動物実験の実施に関する業務を統括する者をいう。

第 2　研究機関等の長の責務

1　研究機関等の長の責務

研究機関等の長は、研究機関等における動物実験等の実施に関する最終的な責任を有し、動物実験委員会の設置、2 に規定する機関内規程の策定、動物実験計画の承認、動物実験計画の実施の結果の把握その他動物実験等の適正な実施のために必要な措置を講じること。

2　機関内規程の策定
研究機関等の長は、法、飼養保管基準、基本方針その他の動物実験等に関する法令（告示を含む。以下同じ。）の規定を踏まえ、動物実験施設の整備及び管理の方法並びに動物実験等の具体的な実施方法等を定めた規程（以下「機関内規程」という。）を策定すること。

3　動物実験計画の承認
研究機関等の長は、動物実験等の開始前に動物実験責任者に動物実験計画を申請させ、その動物実験計画について動物実験委員会の審査を経てその申請を承認し、又は却下すること。

4　動物実験計画の実施の結果の把握
研究機関等の長は、動物実験等の終了の後、動物実験計画の実施の結果について報告を受け、必要に応じ適正な動物実験等の実施のための改善措置を講ずること。

第3　動物実験委員会
1　動物実験委員会の設置
研究機関等の長は、動物実験委員会を設置すること。

2　動物実験委員会の役割
動物実験委員会は、次に掲げる業務を実施すること。
① 研究機関等の長の諮問を受け、動物実験責任者が申請した動物実験計画が動物実験等に関する法令及び機関内規程に適合しているかどうかの審査を実施し、その結果を研究機関等の長に報告すること。
② 動物実験計画の実施の結果について、研究機関等の長より報告を受け、必要に応じ助言を行うこと。

3　動物実験委員会の構成
動物実験委員会は、研究機関等の長が次に掲げる者から任命した委員により構成することとし、その役割を十分に果たすのに適切なものとなるよう配慮すること。
① 動物実験等に関して優れた識見を有する者
② 実験動物に関して優れた識見を有する者
③ その他学識経験を有する者

第4　動物実験等の実施
1　科学的合理性の確保
動物実験責任者は、動物実験等により取得されるデータの信頼性を確保する等の観点から、次に掲げる事項を踏まえて動物実験計画を立案し、動物実験等を適正に実施すること。
(1) 適正な動物実験等の方法の選択
次に掲げる事項を踏まえ、適正な動物実験等の方法を選択して実施すること。
① 代替法の利用
動物実験等の実施に当たっては、科学上の利用の目的を達することができる範囲において、できる限り実験動物を供する方法に代わり得るものを利用すること等により実験動物を適切に利用することに配慮すること。
② 実験動物の選択
動物実験等の実施に当たっては、科学上の利用の目的を達することができる範囲において、できる限りその利用に供される実験動物の数を少なくすること等により実験動物を適切に利用することに配慮すること。この場合において、動物実験等の目的に適した実験動物種の選定、動物実験成績の精度及び再現性を左右する実験動物の数、遺伝学的及び微生物学的品質並びに飼養条件を考慮する必要があること。
③ 苦痛の軽減
動物実験等の実施に当たっては、法及び飼養保管基準を踏まえ、科学上の利用に必要な限度において、できる限りその実験動物に苦痛を与えない方法によってすること。
(2) 動物実験等の施設及び設備
適切に維持管理された施設及び設備を用いて実施すること。

2　安全管理に特に注意を払う必要がある動物実験等
研究機関等の長は、安全管理に特に注意を払う必要がある動物実験等を実施する際には、次に掲げる事項に配慮すること。
① 物理的、化学的な材料若しくは病原体を取り扱う動物実験等又は人の安全若しくは健康若しくは周辺環境に影響を及ぼす可能性のある動物実験等を実施する際には、研究機関等における施設及び設備の状況を踏まえつつ、動物実験実施者の安全の確保及び健康保持について特に注意を払うこと。
② 飼育環境の汚染により実験動物が傷害を受けることのないよう施設及び設備を保持するとともに、必要に応じ、検疫を実施するなどして、実験動物の健康保持に配慮すること。

③　遺伝子組換え動物を用いる動物実験等、生態系に影響を及ぼす可能性のある動物実験等を実施する際には、研究機関等における施設及び設備の状況を踏まえつつ、遺伝子組換え動物の逸走防止等に関して特に注意を払うこと。

第5　実験動物の飼養及び保管

動物実験等を実施する際の実験動物の飼養及び保管は、法及び飼養保管基準を踏まえ、科学的観点及び動物の愛護の観点から適切に実施すること。

第6　その他

1　教育訓練等の実施

研究機関等の長は、動物実験実施者及び実験動物の飼養又は保管に従事する者（以下「動物実験実施者等」という。）に対し、動物実験等の実施並びに実験動物の飼養及び保管を適切に実施するために必要な基礎知識の修得を目的とした教育訓練の実施その他動物実験実施者等の資質向上を図るために必要な措置を講じること。

2　基本指針への適合性に関する自己点検・評価及び検証

研究機関等の長は、動物実験等の実施に関する透明性を確保するため、定期的に、研究機関等における動物実験等の基本指針への適合性に関し、自ら点検及び評価を実施するとともに、当該点検及び評価の結果について、当該研究機関等以外の者による検証を実施することに努めること。

3　情報公開

研究機関等の長は、研究機関等における動物実験等に関する情報（例：機関内規程、動物実験等に関する点検及び評価、当該研究機関等以外の者による検証の結果、実験動物の飼養及び保管の状況等）を、毎年1回程度、インターネットの利用、年報の配付その他の適切な方法により公表すること。

附則

この基本指針は、平成18年6月1日から施行する。

(研究振興局ライフサイエンス課)

資料 3

動物実験に関する日本薬理学会指針

生命科学の急速な発展と社会に与える影響の著しい拡大により、一般社会にとっても、生命科学研究がより身近なものになっている。また、研究は多額の公的資金によって支えられており、薬理学を含む生命科学研究の推進において社会の支持が不可欠の要素となっている。

一方、動物を用いた研究は薬理学の発展に大きな役割を果たして来たし、今後もその意義が失われることはないと考える。しかし、動物実験については、社会に様々な考え方が存在することも事実である。薬理学研究が社会に受け入れられるためには、科学的・倫理的に適正な動物実験を行う環境を醸成し、実施することが不可欠である。

そこで、日本薬理学会では「動物実験ガイドラインの策定に関する勧告」(昭和 55 年 11 月 5 日　総学庶第 1513 号日本学術会議会長) および「大学等における動物実験について (通知)」(昭和 62 年 5 月 25 日　文学情第 141 号文部省学術国際局長) に定められている事項のほか、日本薬理学会員 (以下、会員という) が動物実験を計画、実施する際に、遵守すべき基本的事項を定め、平成 4 年と 13 年に「動物実験に関する日本薬理学会指針」を学会員に通知し、科学的、倫理的観点から適正な実験動物の飼養と動物実験の実施に努めてきた。一方、動物福祉への社会の関心が更に高まり、平成 17 年 6 月 15 日に「動物の愛護及び管理に関する法律 (動愛法)」が改正され、動物実験に関する 3R の原則*の尊重が盛り込まれた。また、平成 18 年 4 月 28 日に「実験動物の飼育及び保管並びに苦痛の軽減に関する基準 (環境省告示第 88 号)」が、平成 18 年 6 月 1 日に「研究機関等における動物実験等の実施に関する基本指針 (文部科学省告示　第 71 号)」、「厚生労働省の所管する動物実験等の実施に関する基本指針 (厚生労働省通知　科発 0601002 号)」、並びに日本学術会議から「動物実験の適正な実施に向けたガイドライン」が示されたことなど、わが国内外の動向も鑑み、指針を刷新することとした。

日本薬理学会は本指針に従った動物実験が行われることを期待するとともに、これに反する研究の成果は本会の刊行する学術雑誌から排除する所存である。

なお、遺伝子組換え動物に関しては、自然界への拡散を防止するため、「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律 (平成 15 年法律第 97 号)」ならびに「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律施行規則 (平成 15 年財務・文部科学・厚生労働・農林水産・経済産業・環境省令第 1 号)」が定められているが、これらの規制に関わる事項は対象外とした。

*：充分な倫理的配慮を行った上、科学上の利用の目的を達する事が出来る範囲において、動物に与える苦痛を最小限にし (refinement)、動物使用数を削減し (reduction)、また、動物を用いない代替法がある場合にはそれを利用すること (replacement)。

1.　目的

この指針は、大学およびその他の研究機関において行われる薬理学研究のための動物実験を計画し、実施する際に遵守すべき事項を示すことにより、科学的にはもとより、動物福祉の観点からも適正な動物実験の実施を促すことを目的とする。

2.　適用範囲

この指針は、会員によって行なわれる実験動物*を用いるすべての動物実験に適用されるものとする。

*：考慮の対象とする実験動物の範囲は基本的に生命を有する脊椎動物とその胚であるが、無脊椎動物が含まれることもある。また、これら以外も本指針を参考にする。

3.　基本原則

会員は「動物が命あるものであることにかんがみ、何人も動物をみだりに殺し、傷つけ、又は苦しめることのないようにするのみでなく、人と動物の共生に配慮しつつ、その習性を考慮して適正に取り扱うようにしなければならない」という動愛法第 2 条に示された基本原則を深く胸に刻み、ここに定められた事項を遵守するよう努めるとともに、動物実験に対する社会の動向や規制の移り変わりに留意し、常に適切な動物実験を実施するよう努めなくてはならない。

会員はヘルシンキ宣言のヒトを対象とする医学研究の倫理的原則 (2002 年追加) 第 12 項に示された「研究に使用される動物の健康を維持し、または生育を助けるためにも配慮」や動愛法に示された動物実験に関する 3R の原則を尊重しなくてはならない。

薬理学研究を行う研究機関の責任者は、動物が適正に飼養され、適正な動物実験が行われるよう、施設を整備するとともに、研究機関毎の動物実験指針を策定し、研究者を教育しなければならない。また、動物実験委員会を設置し、研究機関内で行われる動物実験の法令や機関の定めた指針への適合性や科学的・倫理的妥当性を審査させるとともに、動物実験の実施結果の報告を受け、必要に応じて適正な動物実験実施のための改善措置をとらなければならない。

本指針の目的を達成することは、会員のみの努力では困難である。研究機関の責任者や実験動物の専門家等の協力を得るよう努めなければならない。

4.　具体的な指針

1) 実験者

動物実験を行おうとする研究者は動物実験を行うに際しての法令や規制・基準、倫理、麻酔法、鎮痛法、動物

実験代替法についての教育、また、動物実験手技について訓練を受けていなければならない。

2）動物実験委員会

会員の属する研究機関においては、平成18年6月1日に示された文部科学省および厚生労働省の動物実験の実施に関する基本指針により、動物実験委員会を設置しなければならない。この委員会は動物実験が関係法令や機関の定めた指針に従い、科学的かつ倫理的に実施されるために動物実験計画を審査し、必要な助言を与え、また、適正な実施の監視を行う組織である。委員会は倫理的かつ科学的に妥当な動物実験を行う上で必要な知識と経験を有する実験動物の専門家、動物実験に関して優れた識見を有する者、その他必要と思われる者によって構成しなければならない。

3）動物実験の場所

動物実験は、動物実験委員会が承認した、適正に整備、管理された施設において、必要な設備のもとで行なわなければならない。

4）実験動物の飼育と管理

実験動物の入荷の際の検疫とその後の飼養については、そのための専門的な知識を有する動物管理責任者の協力を得て、適切な実験動物を確保すべきである。

動物実験の際の実験動物の取り扱いにあたっては、実験者自身も実験動物の生理、生態、習性ならびに飼育、管理方法に関する知識をもたなければならないが、それらの知識を十分にもつ専門家の助言を得ることも重要である。疾患モデル動物の作成や使用の場合においても同様である。

5）実験計画の立案

動物実験計画の立案にあたっては、動物を用いないで、その研究目的を達成できる代替法の有無を考慮しなければならない。動物を用いる場合は、適正な動物種や系統を選択し、使用動物数と動物に与える苦痛を必要最小限にとどめるよう、実験方法についての十分な配慮が必要である。また、適切な飼育環境（ケージの大きさ、収容動物数、温湿度、照明など）のもとに実験が実施できるよう実験計画を立案しなければならない。

なお、実験計画は研究機関内の動物実験委員会による審査と承認を受けなければならない。

6）実験実施上の配慮

動物実験は動物実験に熟達した者により、あるいはその指導のもとに行うべきである。また、動愛法および関連する規制・基準を遵守し、動物福祉の立場から、動物の不安や苦痛を、極力軽減するように努めなければならない。この際、国立大学法人動物実験施設協議会、NIH あるいは OECD の作成した安全性試験における人道的な指標に関するガイドラインが良い参考となる。

実験終了後の動物の取り扱いについては、「実験動物の飼養及び保管等に関する基準」（昭和55年3月27日 総理府告示第6号　平成14年5月28日一部改正）に従い、動物をすみやかに苦痛から解放するように努めなければならない。実験途中であっても、研究目的達成上不適切な強い苦痛が現れた場合には、動物をすみやかに苦痛から解放するように努めなければならない。

安楽死の方法については、国立大学法人動物実験施設協議会（2004）や日本獣医師会の解説（2000）を参照されたい。

動物実験および本指針遵守に関わる記録は適切に保管されなければならない。

7）安全管理上の配慮

物理的、化学的に注意を要する試料、または病原体を用いた動物実験を実施する場合には、施設管理者と協力し、一般留意事項、関係規則等を遵守して、安全の確保および環境汚染の防止のため十分な処置を講じなければならない。

5.　その他

この指針に示されていない必要事項については、会員の所属する研究機関における動物実験に関する諸規定、および「大学等における動物実験について（通知）」（昭和62年5月25日 文学情第141号 文部省学術国際局長）を遵守するものとする。

日本学術会議第7部は2004年に「動物実験・施設の第三者評価機構の設置について」の提言を行っており、動物の飼育や管理、また、動物実験が適正に行われていることについて、第三者による認証を得ることも考慮しておく必要がある。

なお、動愛法の改正に伴い、文部科学省において動物実験指針の検討が始まった。それが完成した場合においては、必要に応じて本指針も改正しなければならない。

6.　引用文献

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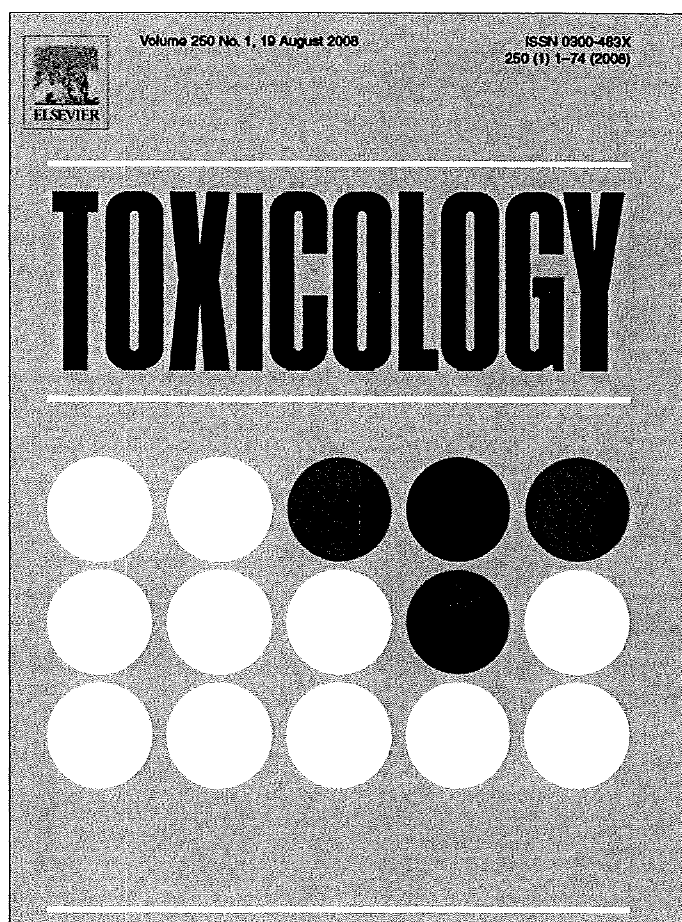

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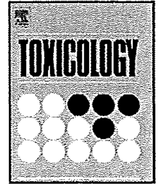

A toxicogenomics approach for early assessment of potential non-genotoxic hepatocarcinogenicity of chemicals in rats

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ABSTRACT

For assessing carcinogenicity in animals, it is difficult and costly, an alternative strategy has been desired. We explored the possibility of applying a toxicogenomics approach by using comprehensive gene expression data in rat liver treated with various compounds. As prototypic non-genotoxic hepatocarcinogens, thioacetamide (TAA) and methapyrilene (MP) were selected and 349 commonly changed genes were extracted by statistical analysis. Taking both compounds as positive with six compounds, acetaminophen, aspirin, phenylbutazone, rifampicin, alpha-naphthylisothiocyanate, and amiodarone as negative, prediction analysis of microarray (PAM) was performed. By training and 10-fold cross validation, a classifier containing 112 probe sets that gave an overall success rate of 95% was obtained. The validity of the present discriminator was checked for 30 chemicals. The PAM score showed characteristic time-dependent increases by treatment with several non-genotoxic hepatocarcinogens, including TAA, MP, coumarin, ethionine and WY-14643, while almost all of the non-carcinogenic samples were correctly predicted. Measurement of hepatic glutathione content suggested that MP and TAA cause glutathione depletion followed by a protective increase, but the protective response is exhausted during repeated administration. Therefore, the presently obtained PAM classifier could predict potential non-genotoxic hepatocarcinogenesis within 24 h after single dose and the inevitable pseudo-positives could be eliminated by checking data of repeated administrations up to 28 days. Tests for carcinogenicity using rats takes at least 2 years, while the present work suggests the possibility of lowering the time to 28 days with high precision, at least for a category of non-genotoxic hepatocarcinogens causing oxidative stress.

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1. Introduction

Chemical carcinogenesis is a multistage process, *i.e.*, initiation, promotion and progression (Dragan et al., 1993; Miller and Miller, 1981; Scott et al., 1984). Based on this mechanism of action, chemical carcinogens are classified as genotoxic (mutagenic) and non-genotoxic (non-mutagenic) agents (Hayashi, 1992; Melnick et al., 1996). Genotoxic agents covalently react with DNA to form DNA adducts within the cells of the target organ, contributing to the initiation process. Such chemicals could be assessed by several short-term *in vitro* and *in vivo* assays that measure DNA damage, mutagenic effects, and chromosomal aberrations (Weisburger and Williams, 2000). In the case of non-genotoxic agents, the mechanism is much more complicated. Non-genotoxic carcinogens lack chemical reactivity with DNA and hence do not form DNA adducts, but rather induce effects that indirectly lead to neoplastic transformation or enhance the development of tumors from pre-initiated cells. Although the mechanism of action of such non-genotoxic carcinogens is not fully understood, several possibilities have been postulated in liver, such as oxidative stress, modulation of metabolizing enzymes, induction of peroxisome proliferation, alteration of intercellular communication, and disruption of the balance between proliferation and apoptosis (Butterworth and Bogdanffy, 1999; Cohen and Ellwein, 1990; Klaunig et al., 1998; Klaunig and Kamendulis, 2004; Nguyen-Ba and Vasseur, 1999; Silva Lima and Van der Laan, 2000; Williams et al., 1996). Even more complicated is the fact that many non-genotoxic carcinogens frequently cause several of these effects at once. The effects of non-genotoxic

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carcinogens in rodents are only manifested after *in vivo* exposure at high dosage levels over long periods (*e.g.*, 2-year rodent carcinogenicity assays). Consequently, the current strategy for evaluating non-genotoxic carcinogens is not satisfactory because the test is time consuming and expensive, and it requires the use of many animals and large amounts of chemicals.

The present report is focused on the application of toxicogenomics for early assessment of potential non-genotoxic hepatocarcinogenicity of chemicals. Non-genotoxic hepatocarcinogenesis has been studied extensively, and postulated to act via a number of mechanisms: oxidative stress, increased mitogenesis, decreased apoptosis, interference with gap junction intercellular communication, and interference with tubulin polymerization (Combes, 2000; Klaunig et al., 1998). Several recent publications have described applications of microarrays and expression profiling for non-genotoxic carcinogenesis in liver (Ellinger-Ziegelbauer et al., 2005, 2008; Fielden et al., 2007; Nie et al., 2006). They attempted to extract common gene sets coordinately deregulated by several different classes of genotoxic and/or non-genotoxic hepatocarcinogenesis. It was then revealed that the modulation of extracted genes was dependent upon the class of the carcinogenesis. This strongly suggests that mechanism-based strategy should be employed in order to obtain useful biomarker gene sets for carcinogenesis. The specific aim of the present study was to develop identifiers for early assessment of non-genotoxic hepatocarcinogenicity in specific class of chemical based on gene expression profiles in reference to our large-scale database named as TG-GATEs (genomics assisted toxicity evaluation system developed by Toxicogenomics Project, Japan) (Urushidani, 2007). Our strategy was to focus on common gene expression changes in livers treated with two well-known oxidative stressors, methapyrilene (MP) (Lijinsky et al., 1980; National Toxicology Program, 2000; Ohshima et al., 1984; Ratra et al., 1998) and thioacetamide (TAA) (Becker, 1983; Diez-Fernandez et al., 1998; Duivenvoorden and Maier, 1994; Ohtsuka et al., 1998; Sanz et al., 1995) to identify a characteristic set of genes reflecting the early stage of oxidative stress-mediated non-genotoxic hepatocarcinogenesis.

2. Materials and methods

2.1. Animals and experimental design

Five-week-old male Sprague–Dawley rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan). After a 7-day quarantine and acclimatization period, the animals (6-week old) were assigned to dosage groups (five rats per group) using a computerized stratified random grouping method based on individual body weight. The animals were individually housed in stainless-steel cages in an animal room that was lighted for 12 h (7:00–19:00) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21–25 °C with a relative humidity of 40–70%. Each animal was allowed free access to water and pellet diet (CRF-1, sterilized by radiation, Oriental Yeast Co., Ltd., Tokyo, Japan).

Table 1 lists the overview of the compounds used in this study. A total of 30 compounds (10 non-genotoxic hepatocarcinogens and 20 non-hepatocarcinogens) were available in the database when the present analysis was performed. They were subdivided in a training set, consisting of 2 non-genotoxic carcinogens (positive training set) and 6 non-hepatocarcinogens (negative training set) with the test set for additional validation consisting of 8 non-genotoxic carcinogens and 14 non-hepatocarcinogens.

According to the standard protocol in our project (Takashima et al., 2006), five rats per group were orally administered at three doses with these compounds suspended or dissolved either in 0.5% methylcellulose (MC) solution or corn oil according to their dispersibility. Traditionally, carcinogenicity studies for chemical agents have relied upon the maximally tolerated dose (MTD) as the standard method for high dose selection. In the present study, the MTD was chosen based on data derived from preliminary toxicity studies of 7 days duration.

For single-dose studies, rats were sacrificed at 3, 6, 9 and 24 h after dosing (3H, 6H, 9H and 24H, respectively). For repeated dose studies, the animals were treated daily for 3, 7, 14 and 28 days, and sacrificed 24 h after the last dosing [day 4 (4D), 8 (8D), 15 (15D) and 29 (29D), respectively]. The animals were euthanized by exsanguination from the abdominal aorta under ether anesthesia, and the liver samples were obtained from the left lateral lobe of the liver in each animal immediately after sacrifice for examination.

The experimental protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation of National Institute of Health Sciences.

2.2. Histopathology of livers treated with MP or TAA

For light microscopical examination, the liver sample of each animal was fixed in 10% neutral buffered formalin, dehydrated in alcohol and embedded in paraffin. Paraffin sections were prepared and stained by a routine method with hematoxylin and eosin (H&E).

2.3. Microarray analysis

An aliquot of the sample (about 30 mg) for microarray analysis was obtained from the left lateral lobe of the liver in each animal immediately after sacrifice, kept in RNAlater® (Ambion, Austin, TX, USA) overnight at 4 °C, and then frozen at −80 °C until use. Liver samples were homogenized with the buffer RLT supplied in RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and total RNA was isolated according to the manufacturer's instructions. Microarray analysis was conducted on three out of five samples for each group by using GeneChip® RAE230A probe arrays (Affymetrix, Santa Clara, CA, USA). The procedure was basically conducted according to the manufacture's instructions as previously reported (Uehara et al., 2008a,b). Microarray Analysis Suite 5.0 (MAS; Affymetrix) was used to quantify microarray signals and the intensities were normalized for each chip by setting the mean intensity to 500 (per chip normalization).

2.4. Selection of persistently up/down-regulated genes in common with MP and TAA

By using statistical and clustering tools, persistently up/down-regulated genes in common with MP and TAA throughout the study periods were extracted. First, data were imported into GeneSpring 6.0 software (Silicon Genetics, Redwood City, CA), and comparisons among time-matched groups from each study of MP and TAA were performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test for post hoc comparisons when significance was determined by ANOVA with a false discovery rate ($p < 0.05$). Probe sets exhibiting significant changes in expression by Tukey's multiple comparison test in both high- and middle-dose groups for one or more time points in each study were selected. In the next step, significant selected probe sets (452 probe sets) were divided into subsets with distinct expression profiles by K-means clustering using Tigr Mev 3.1 software (http://www.tm4.org/mev.html) (current metric: Euclidean distance; divided into nine clusters) based on logarithm (log 2) of the ratio to control for individual gene expression. Genes not categorized in the clusters showing clear time- and dose-independent expression pattern were excluded from further analysis. Finally, a subset of 349 probe sets containing 276 up-regulated and 73 down-regulated probe sets was selected for common intersection to single and repeated studies of MP and TAA (for more information, see supplemental figures).

2.5. Class discrimination by prediction analysis of microarray (PAM)

Prediction of potential carcinogenesis was performed by an approach using PAM for R package (http://www-stat.stanford.edu/~tibs/PAM). PAM makes sample classification using the nearest shrunken centroid method with an automated gene selection step integrated into the algorithm (Tibshirani et al., 2002). It employs a parameter threshold Δ to select genes for class discrimination. PAM training is performed by comparing 2 positive compounds as non-genotoxic carcinogenesis (MP and TAA, high dose group only) with 6 negative compounds, *i.e.*, APAP (Iida et al., 2005; National Toxicology Program, 1993), ASA (Giri, 1993), PhB (Meakawa et al., 1987; National Toxicology Program, 1990), RIF (Sodhi et al., 1997), ANIT (Jean and Roth, 1995; Leonard et al., 1981) and AM (Agoston et al., 2003; Delaney et al., 2004) for the ratio of expression levels of the selected 349 probe sets at various time points (a total of 64 training samples).

Ten-fold cross validation was performed to find out the optimal classifier performance, which minimized classification errors for training sets. During the validation, a threshold Δ was varied in search of the optimal classifier performance. The Δ value that settled at the lowest classification error with the fewest genes was favored as the optimal. For validation of the classifier, the optimized threshold value obtained from training was subsequently used for prediction of potential carcinogenicity for the total of 30 compounds, including training sets. PAM prediction results were expressed as a logarithm transformed score (PAM prediction score) of the ratio of positive class probability relative to negative class probability associated with the classification of each sample, *i.e.*,

$$\text{PAM prediction score} = \log_{10}\frac{\text{class probability}: \text{positive}}{\text{class probability}: \text{negative}}$$

Table 1
Overview of the compounds used for prediction analysis of microarrays training and/or test

Compound	Abbreviation	CAS-number	Mode of action	Supplier	Vehicle	Dose (mg/kg)	PAM training/test
Non-genotoxic hepatocarcinogens[a,b]							
Methapyrilene	MP	135-23-9	Oxidative stress induction	Sigma	0.5%MC	10, 30, 100	Positive training/test set
Thioacetamide	TAA	62-55-5	Oxidative stress induction	Sigma	0.5%MC	4.5, 15, 45	Positive training/test set
Coumarin	CMA	91-64-5	Oxidative stress induction	Tokyo Chemical Industry	Corn oil	150	Test set
Ethionine	ET	67-21-0	Oxidative stress induction	Tokyo Chemical Industry	0.5%MC	250	Test set
Carbon tetrachloride	CCL4	56-23-5	Oxidative stress induction	Wako Pure Chemical Industries	Corn oil	300	Test set
Phenobarbital	PB	57-30-7	Hepatic enzyme induction	Sigma	0.5%MC	100	Test set
Hexachlorobenzene	HCB	118-74-1	Hepatic enzyme induction	Tokyo Chemical Industry	Corn oil	300	Test set
Clofibrate	CFB	637-07-0	Peroxisome proliferation	Wako Pure Chemical Industries	Corn oil	300	Test set
Gemfibrozil	GFZ	25812-30-0	Peroxisome proliferation	Sigma	Corn oil	300	Test set
Wy-14,643	WY	50892-23-4	Peroxisome proliferation	Tokyo Chemical Industry	Corn oil	100	Test set
Non-hepatocarcinogens[a,b]							
Acetaminophen	APAP	103-90-2	–	Sigma	0.5%MC	600	Negative training set
Aspirin	ASA	50-78-2	–	Wako Pure Chemical Industries	0.5%MC	450	Negative training set
Phenylbutazone	PhB	50-33-9	–	Sigma	0.5%MC	200	Negative training set
Rifampicin	RIF	13292-46-1	–	Wako Pure Chemical Industries	0.5%MC	200	Negative training set
Alpha-naphthylisothiocyanate	ANIT	551-06-4	–	Tokyo Chemical Industry	Corn oil	15	Negative training set
Amiodarone hydrochloride	AM	1951-25-3	–	Sigma	0.5%MC	200	Negative training set
Allopurinol	APL	315-30-0	–	Sigma	0.5%MC	150	Negative test set
Allyl alcohol	AA	107-18-6	–	Tokyo Chemical Industry	Corn oil	30	Negative test set
Benzbromarone	BBr	3562-84-3	–	Sigma	0.5%MC	200	Negative test set
Bromobenzene	BBZ	108-86-1	–	Tokyo Chemical Industry	Corn oil	300	Negative test set
Carbamazepine	CBZ	298-46-4	–	Sigma	0.5%MC	300	Negative test set
Chlorpromazine	CPZ	69-09-0	–	Wako Pure Chemical Industries	0.5%MC	45	Negative test set
Diclofenac sodium	DFNa	15307-79-6	–	Cayman Chemical Company	0.5%MC	10	Negative test set
Diazepam	DZP	439-14-5	–	Wako Pure Chemical Industries	0.5%MC	250	Negative test set
Isoniazid	INAH	54-85-3	–	Sigma	0.5%MC	200	Negative test set
Nitrofurantoin	NFT	67-20-9	–	ICN Biomedicals	0.5%MC	100	Negative test set
Phenytoin	PHE	57-41-0	–	Tokyo Chemical Industry	0.5%MC	600	Negative test set
Propylthiouracil	PTU	51-52-5	–	Tokyo Chemical Industry	0.5%MC	100	Negative test set
Sulfasalazine	SS	599-79-1	–	Sigma	0.5%MC	1000	Negative test set
Valproate sodium	VPA	1069-66-5	–	Sigma	0.5%MC	450	Negative test set

[a] Genotoxicity is based on *in vitro* genotoxicity tests (Salmonella and mammalian gene mutation tests) as reviewed in NTP (http://ntp-server.niehs.nih.gov/), IARC (http://monographs.iarc.fr.) and several published papers.
[b] Carcinogenicity is based on reviews by NTP (http://ntp-server.niehs.nih.gov/), IARC (http://monographs.iarc.fr.) and several published papers.

2.6. Gene ontology (GO) analysis of PAM classifier

The identified probe sets were subjected to GO analysis by DAVID (database for annotation, visualization, and integrated discovery; http://apps1.niaid.nih.gov/david/) using Fisher's exact test. Level 3 analysis was adopted.

2.7. Measurement for hepatic total glutathione contents

Hepatic total glutathione was measured in the liver of rats receiving a high dose of MP, TAA or BBZ, and their corresponding controls. Measurements were performed for three rats (gene expression was measured) per group using Glutathione Quantification Kit (Dojindo Mol. Tech, Inc., Kumamoto, Japan). In brief, the liver tissue was homogenized in 5% 5-sulfosalicylic acid and the particulate cellular debris was removed by centrifugation ($8000 \times g$) for 10 min. The internal standards consist of serial dilutions of glutathione (1000, 750, 500, 250, 100, 50 and 0 μM). The change in absorbance at 405 nm was measured and total glutathione was calculated according to the glutathione standard curve. The results were analyzed with the use of an unpaired two-tailed Student's t-test or Welch's t-test as appropriate, and a p-value of <0.05 was considered statistically significant.

3. Results

3.1. Histopathology

Except for the death of one animal in the high dose group of MP on 20D, there were no other deaths in these studies of MP and TAA.

MP- or TAA-treated rats revealed typical liver damage throughout the study periods. Although the extent of the liver damage differed slightly among the animals, a similar pattern was obtained for those in the same dose group.

At high dosage of MP in the single dose study, periportal hepatocytes exhibited hypertrophy characterized by granular eosinophilic cytoplasm and enlarged nuclei with variable anisonucleosis at each time point. More striking abnormalities include mononuclear cell infiltration and hepatocellular single cell necrosis containing shrunken cells with pyknotic nuclei randomly scattered throughout the periportal region of the hepatic lobule. Associated with these lesions, increased numbers of hepatocellular mitotic figures and bile duct hyperplasia were present at each time point in the repeated dose study. At 29D, hepatocellular hyperplasia became evident, and some affected portal regions contained an increased number of oval cells arranged in clusters without a distinct lumen (Fig. 1a). In addition, for the same dose group, a pre-neoplastic altered hepatocellular focus was also observed (Fig. 1b). Middle-dose MP treatment resulted in minimal hepatocellular hypertrophy, single cell necrosis of hepatocytes, and mononuclear cell infiltration in the periportal region at 15D and 29D. Moreover, no significant histopathological alterations were observed at early time points except hepatocellular hypertrophy. In the low-dose MP-treated groups, no significant changes were observed throughout the study periods except for minimal hypertrophy of hepatocytes, observed in one animal each at 8D and 29D.

At high- and middle-dose of TAA, centrilobular hepatocytes exhibited hypertrophy with large, atypical nuclei in single and repeated dose studies (Fig. 1c). Moreover inflammatory cell infiltration and hepatocellular single cell necrosis were also observed at the centrilobular region. The degree of these lesions increased in a dose and time-dependent manner. At 15D and 29D, bile duct hyperplasia and oval cell proliferation at the periportal region became evident, and a pre-neoplastic altered hepatocellular focus was also observed (Fig. 1d). No significant histopathological alterations were observed in the low-dose groups throughout the study periods except degeneration of hepatocytes with granular and eosinophilic cytoplasm, observed in two animals at 29D.

3.2. Class discrimination by PAM in the training set

PAM training was performed using the training set to identify a minimal subset of genes expected to best characterize the early stage of non-genotoxic hepatocarcinogenesis-specific responses. Fig. 2 shows the training and cross-validation errors for different threshold values. Both the training and cross-validated errors were minimized near the threshold = 4.00, where 112 genes were selected. At this threshold, both classes of the training samples were clearly separated based on the expression pattern of these 112 genes with an overall success rate of 95%. Namely, 13 of the 16 positive sets (81%) and all of the negative sets (100%) were correctly classified (Fig. 3a). However, three positive sets (MP-3H, -4D and TAA-3H) were classified as negative, together with all of the negative sets (Fig. 3b).

The list of the genes involved in the PAM classifier is shown in Table 2 (for more information, see supplemental data). Genes were sorted according to the best prediction between the two classes. The top three important discriminators identified by PAM were "nuclear RNA helicase, DECD variant of DEAD box family (Ddx39)", "interferon-related developmental regulator 1 (Ifrd1)", and "mdm2, transformed mouse 3T3 cell double minute 2 (Mdm2)", which were highly up-regulated by MP and TAA. In the extracted 112 probe sets, 111 were prominently up-regulated in the positive training set and the remaining 1 gene (cytochrome P450 4F4) was down-regulated. Based on gene ontology, the contents of genes related to cellular metabolism including several anti-oxidative metabolism, cell proliferation, cell cycle, response to DNA damage stimulus were significantly high (Table 3). These features might reflect the cellular changes related to sustained oxidative stress in association with non-genotoxic hepatocarcinogenesis by MP and TAA.

3.3. Validation of usefulness of the PAM classifier

The 112-gene classifier generated on the training set was next applied to class discrimination for the 30 total compounds as a validation test. The classifier predicted the following samples as positive: high dose MP-6H, 9H, 24H, 8D, 15D and 29D; middle-dose TAA-29D; high dose TAA-6H, 9H, 24H, 4D, 8D, 15D and 29D; CMA-3H, 6H and 9H; ET-24H, 4D, 8D, 15D and 29D; WY-15D and 29D; BBZ-24H. All of other samples (including enzyme inducers, PB and HCB; peroxisome proliferators other than WY, such as CFB and GFZ; and other compounds) were predicted as negative.

In the present study, these prediction results were visualized as a numerical score reflecting the probabilities of class discrimination between the two classes, namely the PAM prediction score. The PAM score showed characteristic time-dependent changes by treatment with several non-genotoxic hepatocarcinogens. In the MP- or TAA-treated group, the score increased dose-dependently with a peak value at 6H for MP, 9H and 24H for TAA after single dosing, and then it markedly increased with repeated administrations (Fig. 4b, c, e, f). CMA, ET or WY treatment also resulted in an increase in the score with a peak value at 6H for CMA, 24H for ET and WY, and also showed an increase or tendency to increase with repeated dosing (Fig. 4g, h, j). Although all of the CCL4-treated groups were predicted as negative, the score showed a tendency to increase with repeated dosing (Fig. 4i). On the other hand, all of the low dose MP- or TAA-treated groups were predicted as negative without any tendency to increase in the score with repeated dosing (Fig. 4a and d). As for the enzyme inducers with carcinogenic activity, PB and HCB (Fig. 4l and m), and peroxisome proliferators other than WY, *i.e.*, CFB (Fig. 4n) and GFZ (within Fig. 4r), showed negative scores throughout the time points. Of the non-carcinogenic samples, BBZ showed a transient increase in the score at 24H but returned to negative during repeated dosing (Fig. 4k). Other non-carcinogenic

Table 2
The list of the genes involved in the PAM classifier

Probe ID	Accession number	Gene title	Gene symbol
1387048_at	NM_053563	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	Ddx39
1367795_at	NM_019242	Interferon-related developmental regulator 1	Ifrd1
1384427_at	XM_001080981	Transformed mouse 3T3 cell double minute 2 homolog (mouse) (predicted)	Mdm2_predicted
1388986_at	–	EST	–
1369921_at	NM_020540	Glutathione S-transferase M4	Gstm4
1368072_at	NM_019290	B-cell translocation gene 3	Btg3
1387060_at	NM_031642	Kruppel-like factor 6	Klf6
1376098_a_at	XM_001069724	Myosin IG	Myo1g
1368173_at	NM_021754	Nucleolar protein 5	Nol5
1373200_at	XM_001063564	Eukaryotic translation elongation factor 1 epsilon 1 (predicted)	Eef1e1_predicted
1388560_at	NM_001008771	WD repeat domain 77	Wdr77
1374945_at	NM_001007706	GCD14/PCMT domain containing protein RGD1359191	RGD1359191
1376737_at	XM_001073157	EST	LOC686259
1388397_at	NM_001008721	EBNA1 binding protein 2	Ebna1bp2
1371785_at	NM_181086	Tumor necrosis factor receptor superfamily, member 12a	Tnfrsf12a
1375895_at	–	EST	–
1367764_at	NM_012923	Cyclin G1	Ccng1
1388674_at	NM_080782	Cyclin-dependent kinase inhibitor 1A	Cdkn1a
1373499_at	NR_002704	Growth arrest specific 5	Gas5
1386897_at	NM_024363	Heterogeneous nuclear ribonucleoproteins methyltransferase-like 2 (*S. cerevisiae*)	Hrmt1l2
1372211_at	NM_145673	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein K (avian)	Mafk
1386995_at	NM_017259	B-cell translocation gene 2, anti-proliferative	Btg2
1372510_at	NM_001047858	Sulfiredoxin 1 homolog (*S. cerevisiae*)	Srxn1
1388900_at	XM_001076548	RGD1566118 (predicted)	RGD1566118_predicted
1370583_s_at	NM_012623	ATP-binding cassette, sub-family B (MDR/TAP), member 1A/1B	Abcb1a/Abcb1b
1398756_at	NM_012992	Nucleophosmin 1	Npm1
1375224_at	NM_001012206	Pleckstrin homology-like domain, family A, member 3	Phlda3
1388155_at	NM_053976	Keratin complex 1, acidic, gene 18	Krt1-18
1368032_at	NM_022869	Nucleolar and coiled-body phosphoprotein 1	Nolc1
1388629_at	NM_199099	Inosine 5-monophosphate dehydrogenase 2	Impdh2
1371936_at	NM_199372	Eukaryotic translation initiation factor 4A1	Eif4a1
1377387_a_at	–	EST	–
1374326_at	NM_001011980	Peter pan homolog (Drosophila)	Ppan
1367617_at	NM_012495	Aldolase A	Aldoa
1376001_at	XM_001065234	Polymerase (RNA) I associated factor 1 (predicted)	Praf1_predicted
1398832_at	NM_012749	Nucleolin	Ncl
1368121_at	NM_013215	Aldo–keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	Akr7a3
1370174_at	NM_133546	Myeloid differentiation primary response gene 116	Myd116
1398771_at	NM_019283	Solute carrier family 3, member 2	Slc3a2
1389450_at	XM_001071583	EST	LOC360830
1371530_at	NM_199370	Keratin complex 2, basic, gene 8	Krt2-8
1367834_at	NM_053464	Spermidine synthase	Srm
1387282_at	NM_053612	Heat shock 22 kDa protein 8	Hspb8
1372043_at	XM_001071573	EST	RGD1311709_predicted
1372150_at	NM_001034146	Ubiquitin-specific protease 10	Usp10
1389569_at	NM_001029915	Brix domain containing 2	Bxdc2
1371498_at	NM_001037348	JTV1	MGC125271
1389815_at	NM_172045	Protein phosphatase 1, regulatory (inhibitor) subunit 14B	Ppp1r14b
1370314_at	NM_031148	Solute carrier family 20, member 1	Slc20a1
1372218_at	NM_199410	WD repeat domain 12	Wdr12
1372354_at	–	EST	–
1367654_at	NM_031819	Fat tumor suppressor homolog (Drosophila)	Fath
1388107_at	NM_144746	Protein phosphatase 2, regulatory subunit B, delta isoform	Ppp2r2d
1372028_at	NM_001047095	EST	RGD1305727_predicted
1373767_at	NM_001008363	Zinc finger, AN1-type domain 2A	Zfand2a
1390579_at	XM_001073162	EST	RGD1305222_predicted
1388588_at	NM_001015013	Mammary tumor virus receptor 2	Mtvr2
1370309_a_at	NM_031330	Heterogeneous nuclear ribonucleoprotein A/B	Hnrpab
1367732_at	NM_030987	Guanine nucleotide binding protein, beta 1	Gnb1
1399158_a_at	NM_012992	Nucleophosmin 1	Npm1
1389577_at	NM_001009640	Cirrhosis, autosomal recessive 1A (human)	Cirh1a
1398757_at	NM_012992	Nucleophosmin 1	Npm1
1370947_at	XM_001070821	EST	Rda279
1373677_at	XM_001061829	Solute carrier family 39 (zinc transporter), member 10 (predicted)	Slc39a10_predicted
1388244_s_at	NM_017138	Ribosomal protein SA	Rpsa
1388150_at	NM_053490	Exportin 1, CRM1 homolog (yeast)	Xpo1
1388666_at	NM_001003401	Ectodermal–neural cortex 1	Enc1
1367713_at	NM_019356	Eukaryotic translation initiation factor 2, subunit 1 alpha	Eif2s1
1386910_a_at	NM_024148	Apurinic/apyrimidinic endonuclease 1	Apex1
1372019_at	XM_001062474	EST	RGD1310128_predicted
1373647_at	NM_001009652	Zinc finger protein 622	Zfp622
1387072_at	NM_053794	Protein kinase, lysine deficient 1	Prkwnk1
1388754_at	–	EST	–
1367870_at	NM_032614	Thioredoxin-like 2	Txnl2

Table 2 (*Continued*)

Probe ID	Accession number	Gene title	Gene symbol
1387950_at	NM_138847	Nuclear import 7 homolog (*S. cerevisiae*)	Nip7
1387807_at	NM_031763	Platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45 kDa	Pafah1b1
1371378_at	XM_001053247	EST	LOC678808
1371735_at	-	EST	-
1398791_at	NM_031614	Thioredoxin reductase 1	Txnrd1
1386958_at	NM_031614	Thioredoxin reductase 1	Txnrd1
1385616_a_at	XM_001059946	ASF1 anti-silencing function 1 homolog A (*S. cerevisiae*) (predicted)	Asf1a_predicted
1388990_at	NM_139186	Mki67 (FHA domain) interacting nucleolar phosphoprotein	Mki67ip
1388449_at	XM_001071102	Eukaryotic translation elongation factor 1 beta 2 (predicted)	Eef1b2_predicted
1373850_at	NM_001025737	Sphingomyelin phosphodiesterase, acid-like 3B	Smpdl3b
1371539_at	XM_001071992	Nucleolar protein family A, member 2 (predicted)	Nola2_predicted
1387774_at	NM_013011	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Ywhaz
1371980_at	NM_001034922	ATPase family, AAA domain containing 3A	Atad3a
1373075_at	XM_001061556	EST	RGD1560888_predicted
1367693_at	NM_013052	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	Ywhah
1387973_at	NM_173123	Cytochrome P450, family 4, subfamily f, polypeptide 4	Cyp4f4
1390317_at	-	EST	-
1371377_at	NM_001037346	Ribosomal protein S19	Rps19
1373380_at	NM_001010963	Brain zinc finger protein	LOC362154
1367590_at	NM_053439	RAN, member RAS oncogene family	Ran
1370295_at	NM_138548	Expressed in non-metastatic cells 1	Nme1
1374632_at	NM_001012143	Phosphatidylserine receptor	Ptdsr
1388381_at	NM_001013095	Eukaryotic translation initiation factor 3, subunit 4 (delta)	Eif3s4
1370785_s_at	NM_152935	Translocase of outer mitochondrial membrane 20 homolog (yeast)	Tomm20
1398801_at	NM_134415	CDK105 protein	Cdk105
1374764_at	XM_001058941	EST	RGD1305605_predicted
1374793_at	XM_001065786	WD repeat domain 3 (predicted)	Wdr3_predicted
1368106_at	NM_031821	polo-like kinase 2 (Drosophila)	Plk2
1372116_at	XM_001079091	Mitochondrial ribosomal protein S2 (predicted)	Mrps2_predicted
1388507_at	NM_001037352	Integrin beta 4 binding protein	Itgb4bp
1389200_at	NM_182674	Bystin-like	Bysl
1372558_at	XM_001053949	NMDA receptor-regulated gene 1 (predicted)	Narg1_predicted
1371809_at	NM_212534	Mitochondrial ribosomal protein S18B	Mrps18b
1387911_at	NM_138708	RAB geranylgeranyl transferase, b subunit	Rabggtb
1372243_at	XM_001063411	Calcium binding protein 39 (predicted)	Cab39_predicted
1372255_at	XM_001065238	Arginyl-tRNA synthetase (predicted)	Rars_predicted
1370184_at	NM_017147	Cofilin 1, non-muscle	Cfl1
1372461_at	NM_001012504	EST	Set_predicted

compounds including APL, AA, and BBr (Fig. 4o–q), and remaining 16 (Fig. 4r) were correctly predicted as negative.

3.4. Additional biological validation

In order to support the class discrimination results by PAM, hepatic total glutathione was quantified for the following selected samples: high dose MP- and TAA-treated groups, and BBZ-treated groups.

Hepatic glutathione contents transiently reduced with peak values at 3H for MP, 6H for TAA and 9H for BBZ after single dosing, and rapidly recovered 24H after the treatment (Fig. 5). Although hepatic glutathione content was kept at normal or higher in the BBZ-treated group at all time points of repeated dose study, in the MP- and TAA-treated groups it reduced with repeated dosing (Fig. 5). These time course changes of the glutathione contents are clearly correlated with the change of the PAM score.

Of the PPARα agonists, only WY, but not CFB and GFZ, showed positive scores at 15D and 29D. If the PAM classifier detects carcinogenesis via the activation of PPARα and these three agonists stimulated the receptor to the same extent, all of three agonists should have been classified as positive. The dose of each compound had been determined based on a 7-day repeated preliminary study and thus the doses would not be proportional to their potency to the receptor. To assess the biological potency of each agonist *in vivo*, we compared the induction of acyl-coenzyme A oxidase 1, a gene directly regulated by PPARα. As shown in Fig. 6, the dose of WY appeared to be too high, since enzyme induction reached its maximum by the low dose of WY. During repeated administrations, however, the extent of the induction was almost the same as in the high dose of these three agonists. If the positive score of WY was due to its PPARα activation, not only the high dose but also the middle and low dose should be classified as positive. We then performed PAM using the present classifier for the three doses of these three agonists, but no positive scores were obtained other than the high dose of WY at 15D and 29D (data not shown).

4. Discussion

The goal of the present study was to develop a classifier for early assessment of potential non-genotoxic hepatocarcinogenic-

Table 3
GO analysis of the PAM classifier

Term	Count	Percentage	*p*-Value
Cellular metabolism	41	34.75	5.07E−03
Primary metabolism	38	32.20	1.80E−02
Macromolecule metabolism	31	26.27	8.64E−04
Cell organization and biogenesis	22	18.64	3.84E−05
Biosynthesis	14	11.86	8.19E−03
Cellular localization	12	10.17	3.03E−04
Cell proliferation	10	8.47	6.26E−03
Negative regulation of physiological process	10	8.47	1.66E−02
Negative regulation of cellular process	10	8.47	2.75E−02
Protein localization	9	7.63	7.12E−03
Cell cycle	9	7.63	1.10E−02
Cell death	8	6.78	4.27E−02
Cellular morphogenesis	7	5.93	2.60E−02
Response to DNA damage stimulus	5	4.24	2.58E−02
Regulation of response to stimulus	2	1.69	2.33E−02

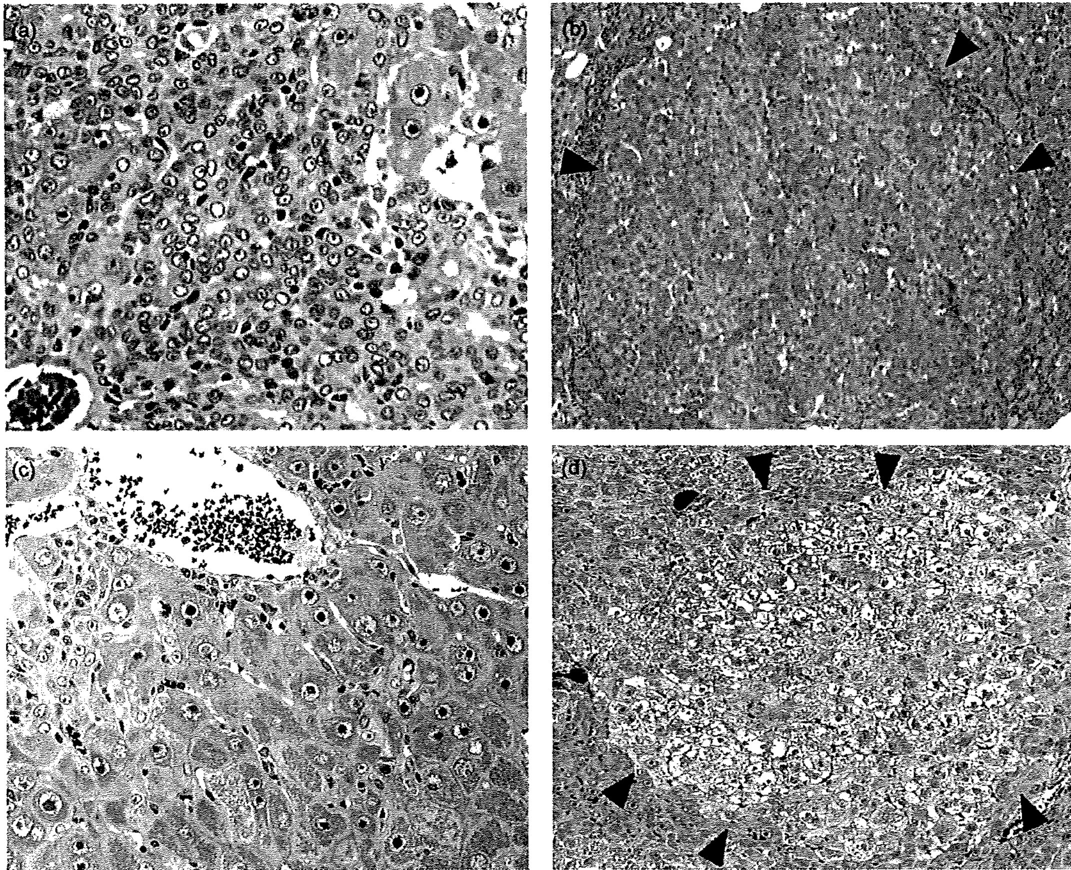

Fig. 1. Histopathology of rat liver treated with MP or TAA for 28 days. Repeated administrations of high dose of MP (100 mg/kg) for 28 days caused hepatocellular hyperplasia and some affected portal regions contained increased numbers of oval cells arranged in clusters without a distinct lumen (a), and in some cases, a pre-neoplastic altered hepatocellular focus was seen (b; arrowheads). In the centrilobular region of rat liver treated with repeated administrations of high dose of TAA (45 mg/kg) for 28 days, hepatocytes exhibited hypertrophy with large, atypical nuclei (c). As in methapyrilene, a pre-neoplastic altered hepatocellular focus was also observed (d; arrowheads).

ity of chemicals based on gene expression changes stored in our database, TG-GATEs. In order to utilize the classifier for practical drug development, we did not attempt to explore an original algorism but to use a well-established one, *i.e.*, PAM in the present case. Our advantage over the previous similar works was the quality of the database, *i.e.*, the quantitative gene expression data obtained in the single platform employing standardized and enriched protocol with three dose levels and eight time points (four for single and four for repeated). The enrichment of time and dose in the data has been shown to be quite powerful in toxicological analysis in various

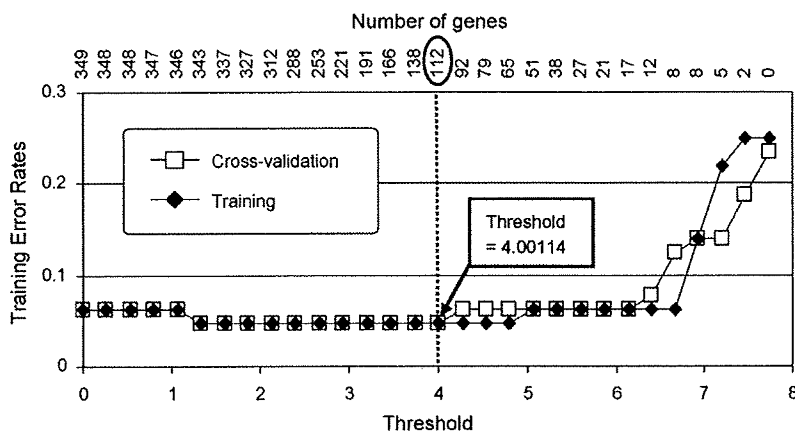

Fig. 2. PAM training and cross validation. PAM training was performed by comparing 2 positive compounds (MP and TAA, high dose group only) with 6 negative compounds (APAP, ASA, PhB, RIF, ANIT and AM) on the ratio of expression levels of the selected 349 probe sets for various time points (total of 64 training samples). Ten-fold cross validation was performed to find out the optimal classifier performance, which minimized classification errors for training sets. Both the training (black symbol) and cross-validated errors (white symbol) were minimized near the threshold = 4.00, where 112 genes (circled) were selected.

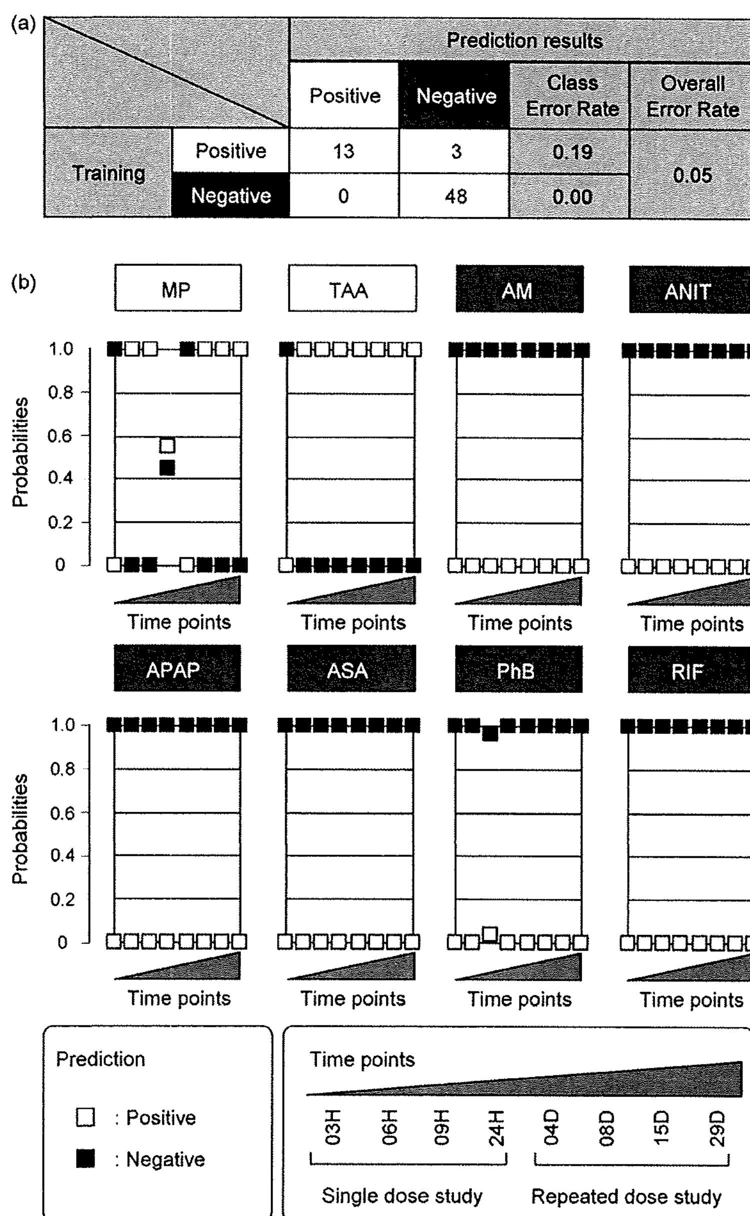

Fig. 3. Class discrimination by PAM. PAM prediction results for the condition determined in Fig. 2 are shown. (a) Prediction results of the training sets (13 positives and 48 negatives) are shown. Note that the overall success rate was 95%, *i.e.*, 13 of the 16 positive sets (81%) and all of the negative sets (100%) were correctly classified. (b) Prediction result of individual sample. For each chemical, the samples are aligned with time as shown on the bottom. The samples predicted as positive are depicted with white and negative with black. Note that two out of three errors occurred at 3 h after single dosing.

ways (Urushidani, 2007). In the present study, genes showing clear dose- and time-dependent changes were successfully extracted by K-means clustering, and we could detect the changes of the score transient after single administration which then turned to be sustained after repeated administration. These also helped us consider the toxicological mechanism.

After PAM training, we produced a discriminator consisting of 112 of the mobilized probe sets that could discriminate between both classes with a high probability, >95%. In the training procedure, MP-3H, 4D, and TAA-3H were judged as false negatives. However, these results were considered to be reasonable because 3H of both compounds was too early for development of hepatotoxicity and 4D of MP treatment was the period when homeostatic recovery of the hepatic glutathione contents occurred.

In the present experiments, MP and TAA showed similar early morphological changes in rat liver, characterized as hepatocellular single cell necrosis with inflammatory response and hypertrophy with granular eosinophilic changes. This was confirmed by electron microscopy as proliferation and swelling of mitochondria (unpublished observations). In addition, hepatocellular altered foci were observed at 15D and/or 29D in the MP and TAA-treated groups. It is well known that this type of lesion is a pre-neoplastic transformation of the cells and is induced in the early stage of non-genotoxic hepatocarcinogenesis in the liver (Bannasch, 1976; Fischer et al., 1983). Therefore, early gene expression profiling in liver treated with these two compounds is considered to be closely related to future carcinogenesis.

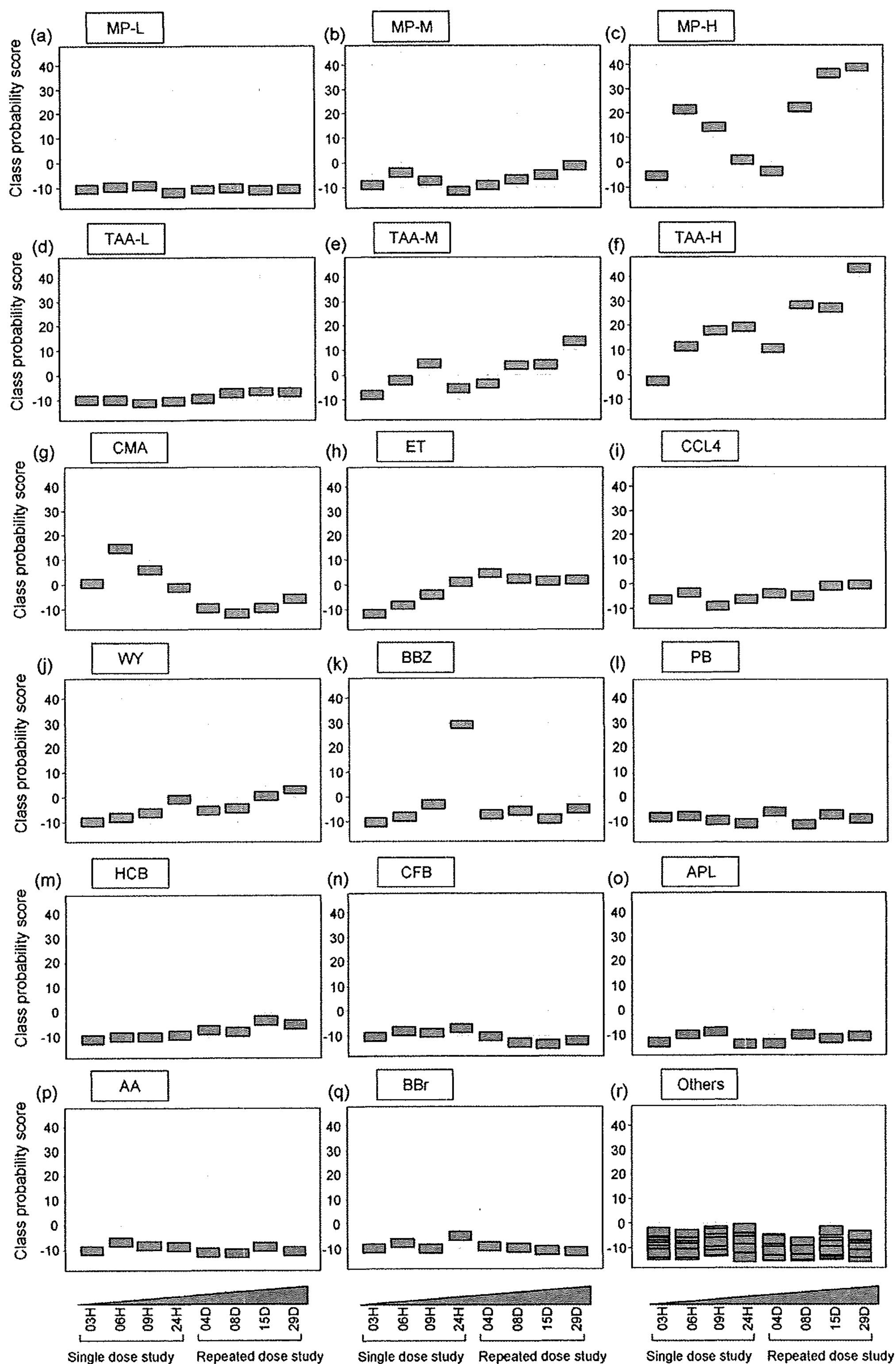

Fig. 4. PAM prediction score. The PAM class probability was converted to a score as described in Section 2 in order to enable quantitative comparison. The score is shown for MP ((a) 10 mg/kg, L; (b) 30 mg/kg, M; (c) 100 mg/kg, H), TAA ((d) 4.5 mg/kg, L; (e) 15 mg/kg, M; (f) 45 mg/kg, H), CMA ((g) 150 mg/kg), ET ((h) 250 mg/kg), CCL4 ((i) 300 mg/kg), WY ((j) 100 mg/kg), BBZ ((k) 300 mg/kg), PB ((l) 100 mg/kg), HCB ((m) 300 mg/kg), CFB ((n) 300 mg/kg), APL ((o) 150 mg/kg), AA ((p) 30 mg/kg), BBr ((q) 200 mg/kg), and (r) the other 17 chemicals. For abbreviation of the compounds, see Table 1.

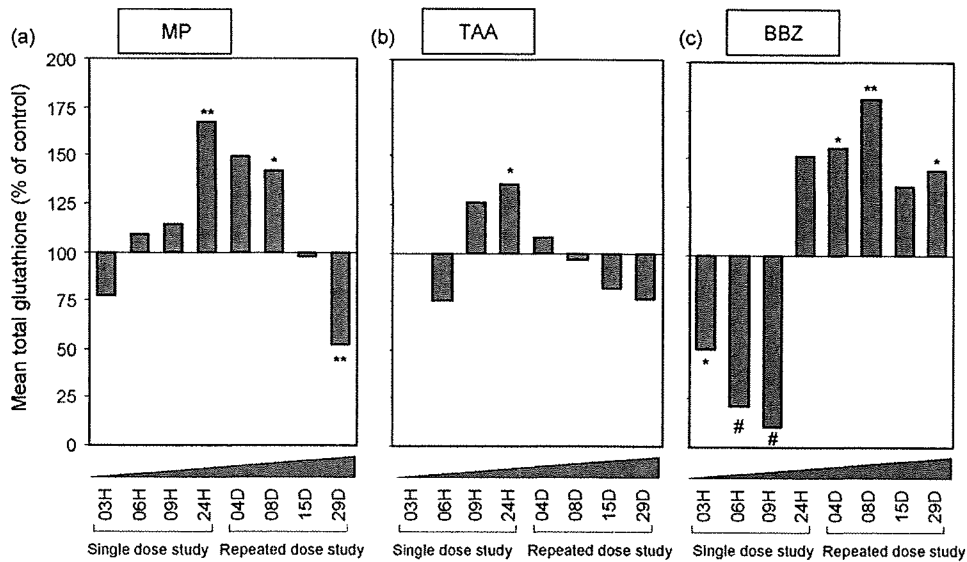

Fig. 5. Effects of MP, TAA or BBZ on glutathione contents in rat liver. Hepatic total glutathione was measured in the liver of rats receiving a high dose of MP (a), TAA (b) or BBZ (c), and their corresponding controls. Measurements were performed for five rats per group using Glutathione Quantification Kit. The results were expressed as percent of control at each time point. Statistical analysis was done by an unpaired two-tailed Student's t-test or Welch's t-test as appropriate. *$p < 0.05$, **$p < 0.01$, by Student's t-test, #$p < 0.05$ by Welch's t-test.

The gene list selected as a marker for predicting hepatic carcinogenicity contained oxidative stress-, oxidative DNA damage-, and cell cycle regulation-related genes, which were changed in the early stage of administration. The oxidative stress is due to the production of reactive oxygen species more than the anti-oxidant capability of the target cells. Unregulated or prolonged production of cellular oxidants has been thought to lead to mutation as a result of oxidant-induced DNA damage, thought to participate in non-genotoxic carcinogenesis (Klaunig et al., 1998; Klaunig and Kamendulis, 2004). The observed expression changes in these genes is in accordance with previous reports that the repetitive cycle of DNA damage (initiation) and reproduction (promotion) caused by sustained oxidative stress is closely related to the carcinogenic process of non-genotoxic carcinogens. This does not mean that the classifier detects any compounds causing oxidative stress. Of the compounds used as negative sets, APAP is known as a prototypic oxidative stressor, which induces glutathione depletion in liver when overdosed (James et al., 2003; Kiyosawa et al., 2004). ASA was reported to induce some antioxidant enzymes and components (Cai et al., 1995), and stimulates some beta-oxidation enzymes, bringing about an overproduction of H_2O_2 (Rivero et al., 1994). PhB was reported to accelerate glutathione oxidation and it induces lipid peroxidation of microsomes (Miura et al., 2002). All of these were successfully classified as negative, suggesting that the classifier discriminates non-carcinogens causing oxidative stress.

The validity of the presently developed discriminator for carcinogenesis was examined on our large-scale database, and all of the 20 chemicals except BBZ (selected as a non-carcinogen) were judged as negative at any time points. Of the eight chemicals classified as non-genotoxic carcinogens, CMA, ET, CCl4 and WY showed positive prediction and increase in the PAM prediction scores in repeated administrations, whereas enzyme inducers such as PB and HCB, and other peroxisome proliferators were all judged as negative.

For CMA (Lake et al., 2002; National Toxicol Program, 1993), ET (Ogiso et al., 1990; Svardal et al., 1988), and CCl4 (Castro et al., 1989; Natarajan et al., 2006), oxidative stress was reported as being involved in their hepatotoxicity and carcinogenesis. It could be con-

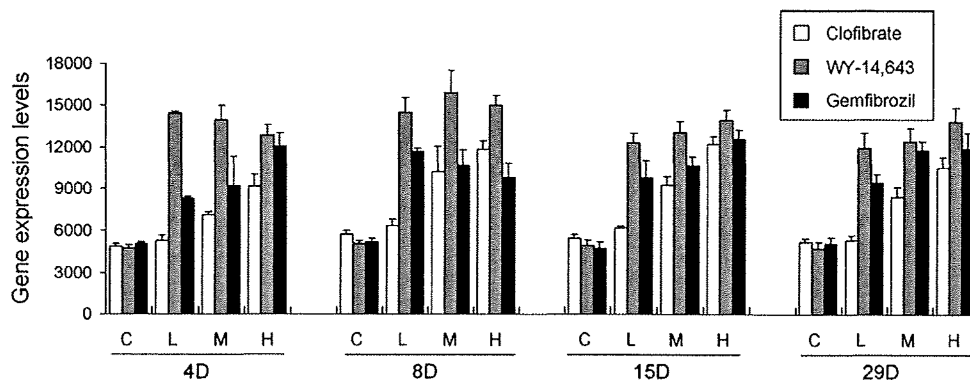

Fig. 6. Effects of repeated administration of CFB, WY or GFZ on expression of acyl-CoA oxidase-1. Expression of acyl-CoA oxidase-1, a gene directly regulated by PPARα, was measured by GeneChip, and the intensities were normalized for each chip by setting the mean intensity to 500 (per chip normalization). The results were expressed as mean ± S.D. ($n = 3$). For each panel, C: control, L: low dose, M: middle dose, H: high dose, for CFB: 30, 100, 300 mg/kg; WY: 10, 30, 100 mg/kg; GFZ: 30, 100, 300 mg/kg, respectively.

cluded that sustained oxidative stress plays an important role in their carcinogenesis, as in MP and TAA.

The induction of PPARα in rodents treated with peroxisome proliferators was considered to be related to hepatocarcinogenesis (Holden and Tugwood, 1999). Moreover, increased levels of H_2O_2 generation, hydroxyl free-radical formation and lipid peroxidation were found in the liver of rats following long-term treatment with peroxisome proliferators. It was also reported that 8-hydroxydeoxyguanosine was found in the liver DNA of rats chronically treated with a PPARα (Reddy and Lalwai, 1983; Reddy and Rao, 1989). In the present study, our discriminator designated WY as positive among the PPARα agonists, CFB, GFZ, and WY. This result suggests that either the discriminator could predict the carcinogenesis of PPARα agonists (although its sensitivity is relatively low) or that WY had an additional carcinogenicity differing from other PPARα agonists. The latter would be more likely since the low and middle doses of WY (by which the induction of acyl-CoA oxidase 1 reached a maximum) did not classify as positive and since the highest doses of CFB and GFZ induce acyl-CoA oxidase 1 to almost the same extent as WY. It was also suggested that WY might share a carcinogenic mechanism with MP and TAA apart from its PPARα agonist's activity.

The P450 enzymes generate oxygen free radicals in the process of metabolizing xenobiotic chemicals (Parke and Ioannides, 1990), including PB (Utley and Mehendale, 1991) and HCB (Smith and De Matteis, 1990). Kinoshita et al. (2002) reported that PB-induced reversible alteration to nuclear 8-hydroxydeoxyguanosine by oxidative stress in rat liver after several days of continuous application. Furthermore, Elrick et al. (2005) provided evidence for the relationship between oxidative stress and PB-induced non-genotoxic hepatic carcinogenesis. On the other hand, HCB exposure induces long-term alterations in intercellular communication via gap junction in rat liver. This effect is thought to be a critical mechanism of HCB-induced non-genotoxic hepatocarcinogenesis and tumor promotion (Plante et al., 2002). However, these chemicals were classified as non-carcinogens based on gene expression profiling. There are likely to be numerous mechanisms involved in non-genotoxic rodent hepatic carcinogenesis. Therefore, it is thought that these chemicals induce non-genotoxic hepatocarcinogenesis through chemical-specific mechanisms.

For the evaluation of these results of prediction, we developed a PAM prediction score based on the positive/negative class probability. In the present study, we compared the score with the hepatic glutathione contents in order to examine the validity of the prediction. In association with the largest decrease of hepatic glutathione contents at 3H (MP), 6H (TAA) and 9H (BBZ), the PAM prediction score increased with the peak at 6H (MP), 9H (TAA) and 24H (BBZ). This could be explained as follows: hepatic glutathione was rapidly consumed to detoxify the oxidants produced by these toxicants, and in the subsequent glutathione-depleted state the expression of these marker genes was up-regulated. The excess production of glutathione for homeostasis tended to decrease in MP or TAA, whereas its high value was maintained in BBZ during their repeated administrations. It is known that some reactive intermediates are conjugated with glutathione to be excreted from the cell. The hepatotoxicity of the acute dose of BBZ was significantly reduced by prior sub-chronic exposure to BBZ. Therefore, the enhanced BBZ excretion by glutathione conjugation could partly explain such potential tolerance against its acute hepatotoxicity (Chakrabarti and Brodeur, 1984). It would be reasonable to speculate that BBZ, which causes transient hepatic and DNA damage by oxidative stress at the early stage of dosing, does not result in hepatic cancer since metabolic protection against oxidative stress does not allow the sustained stressful condition up to 28 days of administration, whereas a breakdown of protection occurs in the case of MP and TAA suggested by the glutathione contents. There was a close correlation between the pattern of change in glutathione and PAM scores, supporting the usefulness of the present marker genes. The present scoring system also enables us to make a prediction based on important toxicological points, *e.g.*, dose- and time-dependency and it would be a quite convenient way for evaluation of the results of discriminant analysis.

In summary, we showed that the expression profile of 112 genes selected by the PAM method could make a prediction of oxidative stress-related hepatocarcinogenicity with high precision at the early stage of administration. The possibility of non-genotoxic carcinogenicity is suggested as early as 24 h after the single dosing. Although pseudo-positives are included in the chemicals selected by the single dose experiments, these can be discriminated by the prediction based on repeated administration up to 28 days. At present, tests for carcinogenicity using rats takes at least 2 years. The present study has suggested a possibility to enable it to take as short as 28 days with high precision. Although neither a single gene nor a single pathway is sufficient to predict non-genotoxic hepatocarcinogens at present, it is evident that combinations of biomarker gene sets appeared to be useful for prediction of carcinogenesis. Further study is clearly necessary to clarify the pathophysiological roles of the genes included in the marker gene list for the process of carcinogenesis.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2008.05.013.

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キーワード解説

Toxicogenomics
トキシコゲノミクス

漆谷 徹郎

1. トキシコゲノミクスとは

最近生命科学の分野に大きなインパクトを与えた技術に，オミクステクノロジーomics technologyがある．Omicsとは，「全体」を表すラテン語omeに「学問」を表す接尾語icsをつけた造語で，「すべて」を扱う学問であり，遺伝子全体genomeを扱うゲノミクスgenomicsに始まる．遺伝子を扱う学問であるジェネティクスgeneticsがゲノミクスと呼びうるものに進化しえたのは，ヒトゲノムプロジェクト，および全遺伝子の発現量を一枚のチップで一挙に測定することを可能としたマイクロアレイの開発による．

生体成分の分離・検出・定量法の発達は，タンパク質全体proteomeや代謝物全体metabolomeを取り扱うことを可能にし，プロテオミクスproteomics，メタボロミクスmetabolomics（metabonomics）という領域をも生み出した．タンパク質の網羅的解析には，2次元電気泳動法，代謝物の網羅的解析にはHPLCやNMRの飛躍的な改良が寄与した．また，分離された各成分を同定するに当たって，質量分析装置の改良がブレークスルーとなったことから，島津の田中耕一研究員のノーベル賞は記憶に新しい．なお，オミクステクノロジーの測定原理に関しては別稿を参照されたい(1)．

ゲノミクステクノロジーを毒性学toxicologyや薬理学pharmacologyに応用すれば，トキシコゲノミクスtoxicogenomics，ファーマコゲノミクスpharmacogenomicsとなる．ただしこれらの用語は上記の定義よりも限定的に使用される場合が多い．ファーマコゲノミクスは通常，特定疾患群の患者に共通な遺伝的特徴を把握して最適な薬剤の開発を目指す「ゲノム創薬」，および患者個々の遺伝的特徴に最適な薬剤を投与する「テーラーメード医療」への応用を意味し，トキシコゲノミクスは，非臨床毒性試験において網羅的遺伝子発現解析によって毒性予測を行おうとする戦略を指す場合が多い．それは，実際の応用例がその分野に集中しているからである．

旧来の薬理学では病態モデル動物で薬効評価を行っていたが，ヒトの疾患関連遺伝子を同定することが可能となった今，「ゲノム創薬」の効率が良いのは明らかである．また，臨床における薬効の個体差の多くが，薬物代謝酵素やトランスポーターの一塩基多型で説明できるようになり，実質的な成果が得られているため，ファーマコゲノミクス＝SNPs解析，という印象が強い．毒性が薬効の延長である場合には，そのままトキシコゲノミクスにつながるはずであるが，現在トキシコゲノミクスに期待されているのは「新薬が臨床展開して初めて明らかになるような予想外の有害作用を前臨床の段階で予測すること」である．例えば糖尿病治療薬troglitazoneが市販後重篤な肝障害の発生により回収されたことは記憶に新しいが，現在の技術ではこれを前臨床の段階で予測することは原理的に不可能である．これを可能とするものとしてトキシコゲノミクスに期待が集まっている．この場合には，動物実験で，薬物に対する応答をすべての遺伝子の発現変化として観察する，という戦略をとる．転写産物transcriptの全体を扱うという意味でトランスクリプトミクスtranscriptomicsと呼ぶべきであるが，これも通常ゲノミクスと称される．

2. 網羅的遺伝子発現解析による毒性予測とトキシコゲノミクスプロジェクト

オミクステクノロジーが目新しかった頃，きっかけとなるデータをマイクロアレイから得た場合，研究者としての主体性がない，と軽蔑される傾向にあった．現在でも「何が重要か良く分からないから，とりあえず全部測定してみよう」などという学生が研究室に溢れたら，教授の血圧は上がりっぱなしであろう．教育的効果はさておき，毒性学領域では，「全部測定してみよう」という姿勢は必須である．troglitazoneの例で言えば，PPARγ刺激作用を詳しく調べてもその肝

キーワード：オミクステクノロジー，多変量解析，判別分析
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