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Discrimination of genotoxic and non-genotoxic hepatocarcinogens by statistical analysis based on gene expression profiling in the mouse liver as determined by quantitative real-time PCR☆

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ABSTRACT

The general aim of the present study is to discriminate between mouse genotoxic and non-genotoxic hepatocarcinogens via selected gene expression patterns in the liver as analyzed by quantitative real-time PCR (qPCR) and statistical analysis. qPCR was conducted on liver samples from groups of 5 male, 9-week-old B6C3F$_1$ mice, at 4 and 48 h following a single intraperitoneal administration of chemicals. We quantified 35 genes selected from our previous DNA microarray studies using 12 different chemicals: 8 genotoxic hepatocarcinogens (2-acetylaminofluorene, 2,4-diaminotoluene, diisopropanolnitrosamine, 4-dimethylaminoazobenzene, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, *N*-nitrosomorpholine, quinoline and urethane) and 4 non-genotoxic hepatocarcinogens (1,4-dichlorobenzene, dichlorodiphenyltrichloroethane, di(2-ethylhexyl)phthalate and furan). A considerable number of genes exhibited significant changes in their gene expression ratios (experimental group/control group) analyzed statistically by the Dunnett's test and Welch's t-test. Finally, we distinguished between the genotoxic and non-genotoxic hepatocarcinogens by statistical analysis using principal component analysis (PCA) of the gene expression profiles for 7 genes (*Btg2, Ccnf, Ccng1*, *Lpr1*, *Mbd1*, *Phlda3* and *Tubb2c*) at 4 h and for 12 genes (*Aen*, *Bax*, *Btg2*, *Ccnf*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Lrp1*, *Mbd1*, *Phlda3*, *Plk2* and *Tubb2c*) at 48 h. Seven major biological processes were extracted from the

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gene ontology analysis: apoptosis, the cell cycle, cell proliferation, DNA damage, DNA repair, oncogenes and tumor suppression. The major, biologically relevant gene pathway suggested was the DNA damage response pathway, resulting from signal transduction by a p53-class mediator leading to the induction of apoptosis. Eight genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Phlda3* and *Plk2*) that are directly associated with *Trp53* contributed to the PCA. The current findings demonstrate a successful discrimination between genotoxic and non-genotoxic hepatocarcinogens, using qPCR and PCA, on 12 genes associated with a *Trp53*-mediated signaling pathway for DNA damage response at 4 and 48 h after a single administration of chemicals.

1. Introduction

Based on their mechanisms of action, chemical carcinogens are classified as genotoxic or non-genotoxic carcinogens [1,2]. Genotoxic carcinogens induce positive genotoxic responses which can occur through any of a number of relevant processes, including direct DNA damage, delayed or inhibited repair, interferences with repair processing enzymes such as topoisomerase, and so forth [3]. Non-genotoxic carcinogens, however, do not induce positive genotoxic responses. According to Waters et al. although the number of presumed non-genotoxic rodent carcinogens has dramatically increased over the past two decades, the fact remains that ~90% of the known, probable and possible human carcinogens classified by the International Agency for Research on Cancer are detected in conventional short-term tests for genotoxicity and induce tumors at multiple sites in rodents [4].

Mathijs et al. hypothesized that genotoxic and non-genotoxic carcinogens induce distinct gene expression profiles, which consequently may be used for a mechanism-based classification of unknown compounds as either genotoxic carcinogens or non-genotoxic carcinogens [2]. The DNA microarray is a powerful technology for characterizing gene expression on a genomic scale [5], although issues of reliability, reproducibility and correlation of data produced across different DNA microarrays are still being addressed [6]. The combination of toxicogenomics data on chemical carcinogens coupled with DNA microarrays has gradually become more common and suggests their usefulness [3]. However, the published studies on in vivo rodent livers are limited.

Quantitative real-time PCR (qPCR) is generally considered the "gold-standard" assay for measuring gene expression and is often used to confirm DNA microarray data [7]. qPCR is the most sensitive technique for the detection and quantification of mRNA targets [8]. It has been suggested that qPCR may be a simpler, more reliable and more reproducible method than DNA microarray [9], although it requires more time for a large number of genes and samples; more recently though, a high-density qPCR technique has appeared [10]. There are only a few papers that have examined selected genes by qPCR in rodent livers in vivo.

Previously, we examined differential gene expression using DNA microarrays upon the application of 13 different chemicals including 8 genotoxic hepatocarcinogens [*o*-aminoazotoluene, chrysene, dibenzo[*a*,*l*]pyrene, diethylnitrosamine (DEN), 7,12-dimethylbenz[*a*]anthracene, dimethylnitrosamine, dipropylnitrosamine and ethylnitrosourea (ENU)], 4 non-genotoxic hepatocarcinogens [carbon tetrachloride, di(2-ethylhexyl)phthalate (DEHP), phenobarbital and trichloroethylene] and a non-genotoxic non-hepatocarcinogen [ethanol]. DNA microarray analysis was conducted on 9-week-old male mouse liver samples at 4 h and up to 28 days following a single intraperitoneal administration. Many candidate genes were identified to discriminate the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogens; the results were reported in part [11] and registered to the GEO database (GEO accession GSE33248). Notably, the changes at 4 h were much greater than those at 20 h, 14 days and 28 days. Additionally, dose-dependent alterations in the gene expression were demonstrated in 31 out of 51 of the examined candidate genes at 4 h and 28 days after the administration of DEN (3, 9, 27 and 80 mg/kg bw, 1/40–1/2 of lethal dose 50% (LD50)) and ENU (6, 17, 50 and 150 mg/kg bw, 1/80–1/3 of LD50) as determined by qPCR [12].

In the present study, we evaluated the gene expression profiles of 12 genotoxic and non-genotoxic mouse hepatocarcinogens, using qPCR on 34 genes selected from our previous DNA microarray studies. The chemicals were intraperitoneally injected into 9-week-old male B6C3F$_1$ mice and analyzed at 4 and 48 h after administration. We speculated that the period at 4 h post-hepatocarcinogen administration in liver would be the time of DNA damage determined by in vivo unscheduled DNA synthesis test [13–15] and Comet assay [16] and that the period at 48 h would be the period of DNA replication after damage determined by replicative DNA synthesis test [13–15,17]. We examined genotoxic hepatocarcinogens, which are positive in the Ames test and in in vivo genotoxicity tests in the mouse liver (transgenic mouse mutation assay or micronucleus assay) and exhibit various chemical properties (summarized in Table 1), and non-genotoxic hepatocarcinogens, which are negative in the Ames test and in in vivo genotoxicity tests (micronucleus assay, unscheduled DNA synthesis assay or Comet assay) and exhibit various chemical properties (summarized in Table 1). Finally, we succeeded in discriminating the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogens by statistical analysis using PCA. We showed that the major biologically relevant gene pathway of PCA contributed genes is a *Trp53*-mediated signaling pathway for the DNA damage response resulting in the induction of apoptosis.

2. Materials and methods

2.1. Chemicals

All chemical names, abbreviations, genotoxic vs. non-genotoxic; CAS numbers; makers; doses; LD50; in vivo mouse genotoxic test and Ames test are summarized in Table 1. The solvents; olive oil (CAS 8001-25-0) and saline were obtained from Wako Pure Chemical Industries; Ltd.; Osaka; Japan.

2.2. Animal treatment

Male B6C3F$_1$ mice were obtained at 8 weeks of age from Charles River Japan, Inc. (Yokohama, Japan) and Japan SLC, Inc. (Shizuoka, Japan) and were kept in plastic cages with wood chip bedding and access to food (Oriental MF, Oriental Yeast Co., Tokyo) and water ad libitum in an air-conditioned room (12 h light, 12 h dark; 23 ± 2 °C; 55 ± 5% humidity) at the Biosafety Research Center, Foods, Drugs, and Pesticides in Shizuoka and the National Institute of Health Sciences in Tokyo. All animal experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee in the Biosafety Research Center, Foods, Drugs, and Pesticides and in the National Institute of Health Sciences. Groups of 5 mice at 9 weeks of age were injected i.p. with 8 genotoxic and 4 non-genotoxic mouse hepatocarcinogens. DIPN and URE were dissolved in saline, and the other chemicals were suspended in olive oil. The control animals received plain saline or olive oil. As shown in Table 1, the doses for the genotoxic hepatocarcinogens were similar to the positive doses used in previous in vivo mouse liver genotoxic studies (transgenic mouse studies (2AAF [18], DAT [19], DIPN; T. Suzuki unpublished data, NNM; T. Suzuki unpublished data, NNK [20], QN [21] and URE [22])) and in the micronucleus test (DAB [23]). Doses for the non-genotoxic hepatocarcinogens were 1/3–1/2 of the LD50 or similar to the doses used for previous in vivo genotoxic studies (DCB [24], DDT [25] and FUR [26]). The dose of DEHP (2000 mg/kg bw) constituted the highest recommended dose for