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Research Paper

Discriminating between adaptive and carcinogenic liver hypertrophy in rat studies using logistic ridge regression analysis of toxicogenomic data: The mode of action and predictive models

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ABSTRACT

Chemical exposure often results in liver hypertrophy in animal tests, characterized by increased liver weight, hepatocellular hypertrophy, and/or cell proliferation. While most of these changes are considered adaptive responses, there is concern that they may be associated with carcinogenesis. In this study, we have employed a toxicogenomic approach using a logistic ridge regression model to identify genes responsible for liver hypertrophy and hypertrophic hepatocarcinogenesis and to develop a predictive model for assessing hypertrophy-inducing compounds. Logistic regression models have previously been used in the quantification of epidemiological risk factors. DNA microarray data from the Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System were used to identify hypertrophy-related genes that are expressed differently in hypertrophy induced by carcinogens and non-carcinogens. Data were collected for 134 chemicals (72 non-hypertrophy-inducing chemicals, 27 hypertrophy-inducing non-carcinogenic chemicals, and 15 hypertrophy-inducing carcinogenic compounds). After applying logistic ridge regression analysis, 35 genes for liver hypertrophy (*e.g.*, *Acot1* and *Abcc3*) and 13 genes for hypertrophic hepatocarcinogenesis (*e.g.*, *Asns* and *Gpx2*) were selected. The predictive models built using these genes were 94.8% and 82.7% accurate, respectively. Pathway analysis of the genes indicates that, aside from a xenobiotic metabolism-related pathway as an adaptive response for liver hypertrophy, amino acid biosynthesis and oxidative responses appear to be involved in hypertrophic hepatocarcinogenesis. Early detection and toxicogenomic characterization of liver hypertrophy using our models may be useful for predicting carcinogenesis. In addition, the identified genes provide novel insight into discrimination between adverse hypertrophy associated with carcinogenesis and adaptive hypertrophy in risk assessment.

1. Introduction

The evaluation of hepatotoxicity is particularly important in repeated-dose toxicity studies because the liver plays a central role in the clearance and metabolism of chemicals. Among the hepatotoxicity findings observed in toxicological studies, liver hypertrophy, which is characterized by an increase in liver weight, hepatocellular hypertrophy, and/or hepatocyte proliferation, is well known as one of the most frequent effects.

The toxicological significance of liver hypertrophy has been debated for more than 50 years, and this has become a critical issue in chemical safety assessment (Gilbert and Goldberg, 1965; Weil and McCollister, 1963). In the 2000s, the United States Environmental Protection Agency (U.S. EPA, 2002) and the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO, 2006) have stated that liver hypertrophy in the absence of histopathological and relevant clinical changes should not be considered an adverse effect. However, data suggest an association between liver hypertrophy and the development of liver tumors (Allen et al., 2004; Carmichael et al., 1997). The European Society of Toxicologic Pathology convened an expert panel to reach consensus on the role of liver hypertrophy in toxicology. The panel reached conclusions similar to those of the FAO/WHO and U.S. EPA, but they also mentioned that more scientific research, including a

Abbreviations: acc, accuracy; DEG, differentially expressed gene; f1, harmonic mean of precision and sensitivity; HC, hypertrophic compounds; HCC, hypertrophic carcinogenic compounds; HNCC, hypertrophic non-carcinogenic compounds; NHC, non-liver hypertrophic compounds; TG-GATEs, Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System.

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mode of action analysis, might be needed to elucidate the correlation between liver hypertrophy and hepatocarcinogenesis (Hall et al., 2012).

Hepatocellular hypertrophy that is accompanied by enzyme induction is generally regarded as an adaptive response (Maronpot et al., 2010; Thoolen et al., 2010), while liver hypertrophy with necrosis, inflammation, cell proliferation, or other significant changes is considered an adverse effect related to carcinogenesis (Hall et al., 2012). One route to elucidate the relationship between liver hypertrophy and carcinogenesis is to investigate the molecular mechanisms associated with hypertrophy. Previous studies on the mechanisms of liver hypertrophy have mostly focused on the induction of phase I and II drug-metabolizing enzymes by nuclear receptor activation, which is considered to trigger adaptive responses. However, adaptive hypertrophy with enzyme induction and adverse hypertrophy with a proliferative response have been found to share common upstream regulators such as aryl hydrocarbon receptor (AHR), constitutive androstane receptor (CAR), and peroxisome proliferator-activated receptor alpha (PPARα) (Budinsky et al., 2014; Corton et al., 2014; Elcombe et al., 2014). Nagata et al. employed a toxicogenomic approach using a DNA microarray to identify genes related to increases in liver weight, and reported several hypertrophy-related genes that are not drug-metabolizing enzymes (Nagata et al., 2014). Thus far, no studies have focused on identifying the molecular mechanisms of hypertrophic hepatocarcinogenesis.

Toxicogenomic analysis has been shown to be suitable for analyzing molecular mechanisms and predicting toxicity (Matsumoto et al., 2009; Suter et al., 2004; Watanabe et al., 2012). Computational techniques such as discriminant analysis (*e.g.*, support vector machines) are extensively used in toxicogenomics research (Rätsch et al., 2006). Machine learning makes it possible to build a high-performance predictive model, but sometimes leads to over-fitting. Furthermore, machine learning does little to interpret the mode of action, because it does not give added weight to each gene. For this reason, we have applied a logistic ridge regression model, which helps identify epidemiological risk factors, to identify genes important for liver hypertrophy and hypertrophic hepatocarcinogenesis and to develop a predictive model for discriminating between adaptive and carcinogenic liver hypertrophy.

2. Materials and methods

2.1. Data selection and compound classification

2.1.1. Data resources. DNA microarray data (Affymetrix Rat Genome 230 2.0 Arrays) for all compounds were gathered from the Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System (TG-GATEs), a toxicogenomic database developed in Japan. This database contains microarray data from *in vitro* and *in vivo* experiments as well as clinical and pathological examination results for rat single- and repeated-dose toxicity tests of 170 compounds (Igarashi et al., 2015). The repeated-dose toxicity test data consists of 4 time points (days 4, 8, 15, and 29) and 3 administration doses (low, middle, and high). This study used microarray data of 134 compounds that were obtained from 28-day, repeated-, high-dose toxicity tests.

2.1.2. Classification of selected TG-GATEs compounds. The compounds used in the present study are summarized in Table 1. Using data on liver weight and liver hypertrophy histopathological examination from TG-GATEs and its public database Open TG-GATEs (http://toxico.nibiohn.go.jp/english/index.html), the compounds were categorized as liver hypertrophic if they met either of the following criteria: (1) the increase in the weight of the liver compared to the total body weight was >1.2-fold for at least one dose at one time point, with a p-value of <0.05 using Student's t-test, and (2) centrilobular hypertrophy or hepatocyte hypertrophy was observed in the liver histopathology in ≧3 animals at the same time point and dose. The 62 compounds that satisfied either of these criteria were classified as liver hypertrophic compounds (HC), and the remaining 72 compounds were classified as non-liver hypertrophic compounds (NHC). Compounds known to be liver hypertrophic, such as omeprazole, phenobarbital, and clofibrate were correctly classified using this method.

To investigate the mechanism of hypertrophic hepatocarcinogenesis, HC were further classified according to their carcinogenicity in rodent livers and their mutagenicity status, based on data from the Carcinogenic

Table 1
Compounds used in this study.

Liver hypertrophic compounds[a] (HC) (62)	
Carcinogens (18)	
Genotoxic carcinogens	Coumarin (CMA), Isoniazid (INAH), Methylene Dianiline (DAPM)
Non-genotoxic carcinogens[a] (HCC) (15)	Acetaminophen (APAP), Carbamazepine (CBZ), Carbon Tetrachloride (CCL4), Clofibrate (CFB), Ethinylestradiol (EE), Fenofibrate (FFB), Gemfibrozil (GFZ), Hexachlorobenzene (HCB), Methapyrilene (MP), Omeprazole (OPZ), Phenobarbital (PB), Rifampicin (RIF), Simvastatin (SST), Thioacetamide (TAA), Wy-14643 (WY)
Non-carcinogens[a] (HNCC) (27)	Amiodarone (AM), Aspirin (ASA), Bendazac (BDZ), Benzbromarone (BBr), Benziodarone (BZD), Bromoethylamine (BEA), Butylated Hydroxyanisole (BHA), Caffeine (CAF), Chloramphenicol (CMP), Chlorpropamide (CPP), Danazol (DNZ), Diazepam (DZP), Diltiazem (DIL), Disulfiram (DSF), Erythromycin Ethylsuccinate (EME), Ethambutol (EBU), Ketoconazole (KC), Methimazole (MTZ), Methyltestosterone (MTS), Mexiletine (MEX), Phenacetin (PCT), Phenylbutazone (PhB), Phenytoin (PHE), Propylthiouracil (PTU), Quinidine (QND), Tolbutamide (TLB), Trimethadione (TMD), Methimazole (MTZ), Methyltestosterone (MTS), Mexiletine (MEX), Phenacetin (PCT), Phenylbutazone (PhB), Phenytoin (PHE), Propylthiouracil (PTU), Quinidine (QND), Tolbutamide (TLB), Trimethadione (TMD)
Unknown (17)	1% Cholesterol + 0.25% Sodium Cholate (CH + DS-Na)[b], 2,4-Dinitrophenol (DNP), Amitriptyline (AMT), Bromobenzene (BBZ), Chlormezanone (CMN), Chlorpheniramine (CHL), Dantrolene (DTL), Fluoxetine Hydrochloride (FLX), Flutamide (FT), Hydroxyzine (HYZ), Imipramine (IMI), Nimesulide (NIM), Papaverine (PAP), Promethazine (PMZ), Tamoxifen (TMX), Terbinafine (TBF), Ticlopidine (TCP)
Non-liver hypertrophic compounds[a] (NHC) (72)	
Carcinogens (5)	
Genotoxic carcinogens	Acetamidofluorene (AAF), Lomustine (LS)
Non-genotoxic carcinogens	Acetamide (AAA), Ethanol (ETN), Ethionine (ET)
Non-carcinogens (57)	Acarbose (ACA), Acetazolamide (ACZ), Allopurinol (APL), Allyl Alcohol (AA), Azathioprine (AZP), Captopril (CAP), Cephalothin (CLT), Chlormadinone (CLM), Chlorpromazine (CPZ), Cimetidine (CIM), Ciprofloxacin (CPX), Cisplatin (CSP), Clomipramine (CPM), Colchicine (COL), Cyclophosphamide (CPA), Cyclosporine A (CSA), Diclofenac (DFNa), Disopyramide (DIS), Doxorubicin (DOX), Enalapril (ENA), Famotidine (FAM), Fluphenazine (FP), Furosemide (FUR), Gentamicin (GMC), Glibenclamide (GBC), Griseofulvin (GF), Haloperidol (HPL), Ibuprofen (IBU), Iproniazid (IPA), Mefenamic Acid (MEF), Metformin (MFM), Methyldopa (MDP), Moxisylyte (MXS), Naphthyl Isothiocyanate (ANIT), Nicotinic Acid (NIC), Nifedipine (NIF), Nitrofurantoin (NFT), Nitrofurazone (NFZ), Pemoline (PML), Penicillamine (PEN), Perhexiline (PH), Phenylanthranilic Acid (NPAA), Propranolol (PPL), Ranitidine (RAN), Rosiglitazone Maleate (RGZ), Rotenone (ROT), Sulfasalazine (SS), Sulindac (SUL), Sulpiride (SLP),Tannic Acid (TAN), Tetracycline (TC), Theophylline (TEO), Thioridazine (TRZ), Tiopronin (TIO), Triamterene (TRI), Valproic Acid (VPA), Vitamin A (VA)
Unknown (10)	Adapin (ADP), Ajmaline (AJM), Amphotericin B (AMB), Bucetin (BCT), Carboplatin (CBP), Desmopressin Acetate (DDAVP), Etoposide (ETP), Labetalol (LBT), Tacrine (TAC), Triazolam (TZM)

Data obatained from Japan's Toxicogenomics Project-Genomics Assisted Toxicity Evalauation System database.

[a] The microarray data of HC, NHC, HNCC, and HCC were analyzed in the present study.

[b] This mixture is treated as a chemical in the present study.