



Workshop 5.12

Toxicogenomics – potential, validation, and case studies

Lecture

A novel computational approach for the prediction of networked transcription factors of aryl hydrocarbon-receptor-regulated genes

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A novel computational method based on a genetic algorithm was developed to study composite structure of promoters of co-expressed genes. Our method enabled an identification of combinations of multiple transcription factor binding sites regulating the concerted expression of genes. In my presentation, I report the study of genes whose expression is regulated by the aryl hydrocarbon receptor (AhR), that mediates responses to a variety of toxins. AhR-mediated change in expression of AhR target genes was measured by oligonucleotide microarrays and by reverse transcription-polymerase chain reaction in human and rat hepatocytes. Promoters and long-distance regulatory regions

(>10 kb) of AhR-responsive genes were analysed by the genetic algorithm and a variety of other computational methods. Rules were established on the local oligonucleotide context in the flanks of the AhR binding sites, on the occurrence of clusters of AhR recognition elements, and on the presence in the promoters of specific combinations of multiple binding sites for the transcription factors cooperating in the AhR regulatory network. Our rules were applied to search for yet unknown Ah-receptor target genes. Experimental evidence is presented to demonstrate high fidelity of this novel *in silico* approach.



Lecture

Validation of toxicogenomics-based tests, a new generation of alternatives

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Toxicogenomics-based methods are being widely applied in toxicology and biomedical research. Since data are already being generated using these technologies, it is both timely and important to address the critical validation issues now with the aim of establishing a foundation that will facilitate future regulatory acceptance of scientifically valid toxicogenomics-based test methods. Addressing such issues early on, will also facilitate early buy-in and confidence in the technologies by the regulatory arena in its quest for new and improved methods by which to help ensure human health, protect the environment, and demonstrate responsiveness to animal welfare issues.

For that reason, the European Centre for the Validation of Alternative Methods (ECVAM), the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the National Toxicology Program

(NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) have started to investigate the specific considerations necessary for adequate validation of toxicogenomics-based test methods. Experience in validation of conventional alternative test methods has led to an understanding that the validation approach will have to be adapted to the evaluation of methods based on toxicogenomics. The toxicogenomics field is rapidly evolving; therefore the validation process should accommodate the anticipated changes in the technology and must not be at the expenses of innovation. Moreover, other international Organisation as the OECD and the WHO/IPCS are currently drafting activity programs related to the possible use of toxicogenomics-based test methods for hazard and risk assessment purposes.

Lecture

Achieving the potential of toxicogenomics

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Microarray technology is gaining widespread attention in toxicology studies. This technology allows the global assessment of gene expression profiles in tissues and purified cell subtypes, as well as tissue cultures. Thus, the effect of a test substance on the activity of nearly every gene can be determined in a single experiment. Although presently not a high throughput technology, the high information content of the assay may allow the elucidation of possible toxicity, as well as mechanisms of action. Microarrays, combined with other high information content technologies, such as metabolomics and proteomics, will provide a global picture of the effect of test substances on a biological system. With the understanding of underlying molecular events caused by a test substance, extrapolation between surrogate test systems and humans can be rationally performed. In

order to fulfill the promise of such toxicogenomics approaches, standard reference materials are needed for quality control, standardisation, and performance proficiency. The Microarray Quality Control Project (MAQC) was initiated to provide such standards for the microarray community. Large reference datasets will be produced by different microarray platforms using common RNA samples that will be available to the microarray community. The derived QC metrics/thresholds will aid individual laboratories in better assessing performance and to avoid procedural failures. The recently issued U.S. FDA Guidance for Industry on Pharmacogenomic Data Submissions will help facilitate the use of this type of data in pharmaceutical drug development.



Lecture

“Percellome” and “Mille-Feuille data” system for toxicogenomics

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A systematic approach is proposed to normalise the mRNA expression values from DNA microarrays and quantitative-PCR in a “per one cell” basis, designated as “Percellome”. This Percellome system will enable us to plot all data on to one common linear scale. Transcriptome data between experimental groups, between studies, and even between different organs can be directly compared without further normalisation. This system, developed primarily for Affymetrix Gene Chips, can be expanded to other platforms and quantitative-PCR as long as they meet a few requirements. Direct data comparison between different laboratories would be possible in this respect.

Our Percellome toxicogenomics projects monitors, for example, the time- and dose-dependent alteration of gene expression induced by various chemicals in mouse (4 time points and 4 dose levels, total of 16 groups, 3 mice each). The

percellome data can be visualised as a three-dimensional graph (X=time, Y=dose, Z=expression per one cell) containing 45,000 layers of surface corresponding to each of the probe sets in the Affymetrix MOE430v2 Gene Chip (“MilleFeuille” data). X and Y axes can be any experimental parameters. This MF data is biologist-friendly that it is helpful to extract biologically plausible alterations and to develop further methods for drawing the gene cascades.

The aim of this Percellome toxicogenomics project is to develop the predictive toxicology through the development of the gene cascade data based on the high-precision transcriptome database/informatics. We believe that this approach should lead to our ultimate goal of generating “virtual mouse” *in silico* in the future.

Poster

Toxicogenomic analysis of rat liver carcinogenesis: Mechanistic insights and implications for risk assessment

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Genomic and proteomic approaches are widely explored to evaluate their usefulness for detecting early toxicological endpoints and mechanisms. In order to relate changes of transcriptional and translational profiles to conventional endpoints of toxicity, a common animal model of chemical hepatocarcinogenesis was used. N-Nitrosomorpholine (NNM) was administered to adult male Wistar rats for 7 weeks to induce hepatocarcinogenesis. Specimens of each treatment group (vehicle-control + NNM low and high dose) were killed at different time points between 1 day and 50 weeks after the start of the study and left liver lobes from five animals were sampled for simultaneous biochemical and histopathological processing. Gene expression was analysed using the Affymetrix rat genome chip U34A. Proteomic analysis was based on 2D-electrophore-

sis and mass spectrometry. Results demonstrated characteristic deregulations of genes and proteins by complementary use of transcriptomics and proteomics, which can be related to mechanisms of carcinogenicity. It was shown that comparably few genes were deregulated at both levels of expression and each approach contributes different parts of information, which can be used to analyse mechanistic toxicity of genotoxic carcinogens and to identify early biomarkers of carcinogenesis. By this approach, adverse long-term effects could be detected in short-term bioassays and, in consequence, the number of long-term animal studies can be reduced. New approaches focus on the identification of predictive molecular profiles for non-genotoxic carcinogens.

**Poster**

Can the number of animal experiments be reduced by the application of transgenic models? A case study for testing estrogenic effects using the ChgH-GFP medaka strain

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Reporter gene constructs of GFP (Green Fluorescent Protein) are ideal tools to visualise the gene expression in living animals. They are in particular useful for transparent, early life stages of fish and can be applied to analyse the individual response of transcript abundance to a certain stimulus, such as the exposure to toxic chemicals or hormones. In contrast to other techniques, that require a certain amount of RNA, the fluorescence of the non-toxic GFP can be analysed conveniently in early life stages of a single organism. In the present study we have developed a technique to quantify the GFP levels in living ChgH-GFP transgenic medaka by image analysis. The ChgH-GFP strain was developed by Kurauchi et al. (2005, EST, in press) and harbours a regulatory region of the estrogen-responsive choriogenin H

gene fused to the GFP gene. A series of calibration measurements was performed to develop a technique that allows to calculate GFP fluorescence intensity independent of the photo exposure time. ChgH-GFP showed a strong induction in 14 day-old fish at exposure to ≥ 183.5 pM (50 ng/L) 17-beta-estradiol. Time course and recovery experiments indicated an accumulation of GFP in the liver. The ChgH-GFP medaka is a very useful tool to analyse water contamination and to identify environmental chemicals with estrogenic activity. However, the quantification of GFP could be easily adopted for any other GFP-reporter strain. If applied for the purpose of gene regulation studies in early life stages, it will contribute to the refinement and reduction of animal experiments.

Poster

A two-dimensional gel database of rat liver proteins useful for detection of toxic and carcinogenic effects of chemicals

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Although promising approaches during the past decade, the availability of publicly accessible databases of rat liver proteins became limited. Since emerging proteomic technologies still depend on data generated by two-dimensional electrophoresis (2-DE), we decided to establish a new 2-DE map of male Wistar rat liver proteins in the context of a molecular toxicological joint research project. This information is useful to quickly retrieve protein information from 2-DE experiments.

2-DE separation was performed using the common IPG technique. Spots were excised from 2-D gels using a spot picker (ProPic, Genomic Solutions) and the proteins were identified after in-gel digestion with trypsin by using peptide mass fingerprint (MALDI-TOF-MS) and Tandem MS (LC-ESI-Quad-TOF).

At present, the map contains about 621 proteins and could be the basis for a publicly accessible HTML-database, providing

information to identify protein expression patterns of toxicological relevance.

Protein spots are linked with detailed information, i.e. protein identity, EC-number, function, localisations and molecular weight. Among the annotated protein spots we found several of proteins with relevance to mechanistic toxicological endpoints and some of them are well known as toxicological marker proteins. Furthermore we could assign many of them to toxicological categories like DNA damage response, detoxification, stress response or apoptosis. Expression patterns of marker proteins could contribute mechanistic data to assess toxic and carcinogenic effects of chemicals in short-term rodent bioassays.

In perspective, our data should be integrated in comprehensive, publicly available toxicogenomic databases.



Lecture

Extrapolating the toxicogenomics data derived from rat to human – the roles of bioinformatics in liver cancer analysis using cross species mapping

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Liver cancer is a public health concern in many parts of the world. We used albumin-SV40 transgenic rats, which spontaneously develop hepatic neoplasms within 6-9 months, as a model for the liver cancer study. Data from microarray analysis of liver tumours from these animals demonstrated an altered gene expression. A wide range of bioinformatics methods was used to determine the relevance of the findings in rat to human with respect to chromosomal aberration, pathways and functions (Gene Ontology). Using these bioinformatics tools, particularly ArrayTrack that is developed in house and available to the public, we found that genes related to cell cycle control, cell prolifer-

ation, apoptosis, transcriptional regulation, and protein metabolism were altered. We also closely examined gene expression in regions of previously identified chromosomal aberrations associated with early hepatic neoplasms in this transgenic rat model using a novel visualisation tool. The utility of ArrayTrack was demonstrated in this project for analysis of gene expression data derived from microarray experiment. Analysis indicates that the altered gene expression associated with rat liver tumour development may be useful in the analysis of human liver cancer.

Poster

Review tool for pharmacogenomics data submission: ArrayTrack

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DNA microarray is a key technology in pharmaco- and toxicogenomics, a field that has been identified in the U.S. FDA Critical Path document as a major opportunity for advancing medical product development and personalised medicine. It is expected that the regulation of microarray-based medical diagnostics and the review of microarray data submitted as part of an IND or NDA will become an essential regulatory responsibility for the FDA. A single microarray experiment generates a large volume of data. The management, analysis and interpretation of this vast amount of data are the most critical components in realising the value of the technology. Many solutions are available, but, unfortunately, most of them, if not all, deal with these com-

ponents separately, posing a great challenge for reviewers to efficiently handle microarray data. ArrayTrack, developed at NCTR/FDA, integrates these three essential components into a single application with a user-friendly and intuitive interface, and thus provides a single solution for reviewers to analyse microarray data, interpret the information and verify the results submitted by sponsors. In addition, ArrayTrack provides the capability to develop an aggregate genomics knowledge base to support scientifically sound future regulatory policies. Currently, ArrayTrack is being integrated and further refined at the U.S. FDA as a review tool for pharmaco- and toxicogenomic data submission.



Lecture

The H295R adrenocortical cell line as alternative *in vitro* system for screening of endocrine disruptors: A microarray based approach

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Developing screening methods is an important effort to bypass initial needs to identify individual endocrine disruptors (EDs). However, screening methods for EDs will have to accommodate a wider variety of diverse chemicals than ever been subjected to screening methods before. The urge for more comprehensive *in vitro* systems that make multiple endpoint detection possible, is however in contrast with the few hormonal tissues currently analysed. So far, testing strategies have omitted the adrenal gland and therefore do not adequately cover the process of steroidogenesis, critical in adrenocortical, testicular and ovarian function. The present study combines the advantage of a pluripotent adrenocortical cell line with the capacity of the microarray technique to analyse thousands of genes at ones. The H295R cell line covers the entire biochemical pathway respon-

sible for steroidogenesis and therefore presents multiple molecular targets for toxicity, ranging from general effects on all steroidogenic tissues (e.g. aromatase) through to specific targets affecting only adrenocortical function. The idea of the project is to develop a cell line specific microarray, which allows classification of EDs according to their mode of action and is an important step in selecting potential biomarkers. In preparation of this custom array, gene expression profiles of H295R cells exposed to model chemicals will be analysed using an extensive human array, covering 21,000 genes. The group of differentially expressed genes form the basis of the custom array. Here, first results of gene expression profiles in preparation of the custom array are presented and discussed.

Poster

Differential gene expression patterns in the zebrafish (*Danio rerio*) embryo – development of a test system for the prediction of chronic toxicity

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Acute, prolonged and chronic fish tests are used for ecotoxicity assessment in the process of the registration of chemicals, biocides, pesticides and veterinary pharmaceuticals. As alternative for the acute fish test, the zebrafish embryo test (DarT – *Danio rerio* Toxicity test) is available. Until now, no alternative assay methods have been developed for prolonged and chronic fish tests. The objective of the research project Gen-DarT (gene expression *Danio rerio* Toxicity test) is to develop a gene expression-based test system for zebrafish embryos to predict toxic effects in chronic and prolonged fish tests. Sensitive marker genes were identified by analysis of selected, potential candidate genes by RT-PCR and by using a 14k-oligonucleotide-microarray with cDNA of embryos exposed for 48 h to the

model substances cadmium chloride and 3,4-dichloroaniline. We identified 8 marker genes (*cyp1a1*, *ahr2*, *hsp70*, *fzr1*, *nrf2*, *maft*, *hmox1*, *mt2*), of which the altered expression could be confirmed by quantitative RT-PCR in independent embryo tests. The lowest observed effect concentrations for differential gene expression and toxicity were compared in embryos (48 h) as well as 5 and 30 day-old fish from an early life stage test (ELST). Toxicity in the ELST was the most sensitive endpoint, followed by gene expression in 5 day-old (post fertilisation) fish larvae. Our studies are continued by the analysis of additional chemicals, the identification of further marker genes with higher sensitivity and by comparison of gene expression patterns in embryos and fish larvae.



Lecture

Application of toxicogenomics towards drug safety evaluation

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Introduction: Toxicity remains a major hurdle for the successful discovery and development of new pharmaceuticals. Drug safety studies are generally not conducted until the later stages of the drug discovery process. Ideally, the evaluation of the toxic properties of a compound should be conducted early before a significant amount of time or resources has been spent. However, the traditional *in vivo* toxicologic approach is not suited for an early evaluation, due to compound requirement. At Abbott, we are using gene expression signatures generated using isolated rat hepatocytes to evaluate compounds *in vitro* for their toxic properties.

Materials and methods: Isolated rat hepatocytes were treated with over 100 reference compounds at a TC20 concentration for 24 hours. RNA was harvested, amplified and analysed using Affymetrix microarray chips.

Results: Expression signatures were developed by profiling over 100 reference compounds in isolated rat hepatocytes. The signatures are being used to identify compounds with the potential to induce a variety of toxic changes, including mitochondrial damage, phospholipidosis, microvesicular steatosis and peroxisome proliferation with a high degree of sensitivity and specificity. These signatures were originally developed using microarray technology, but have since been transferred to a gene expression platform with higher throughput, lower cost, and amenable to customisation.

Summary: Identifying and characterising compounds with the potential to cause toxicity early in the drug discovery process leads to a more rationale selection of compounds in discovery, improved productivity of the research and development process, and ultimately should result in drugs with a better safety profile.