MicroRNAs and epigenetics in chemical carcinogenesis : an integrative toxicogenomics-based approach

Valorization

Relevance

The overall aim of this thesis was to investigate what regulatory roles microRNAs and epigenetic changes (i.e. DNA methylation) have in transcriptomic responses induced by genotoxic and non-genotoxic carcinogens in cell models relevant for liver carcinogenicity. Furthermore, the application of the expression patterns of these regulatory molecules (i.e. microRNAs and DNA methylation changes), for the purpose of discriminating between different classes of chemical compounds (i.e. genotoxic carcinogens (GTXC), non-genotoxic carcinogens (NGTXC) and non-carcinogens (NC)), was evaluated in the context of genotoxicity and carcinogenicity testing. Cancer is the number one cause of death worldwide[1]. According to the World Health Organization, liver cancer, specifically hepatocellular carcinoma (HCC) is the sixth most prevalent and the second most lethal type of cancer worldwide[2]. Exposure to hazardous agents (e.g. chemicals, food-additives or pharmaceuticals) appears to be of major influence in the induction of human cancers. For this reason, it is extremely important that the carcinogenic potential of new agents is accurately predicted. Traditionally, carcinogens are divided based on their mode of action (MOA) into DNA-reactive genotoxic (GTX) carcinogens and non DNA-reactive or non-genotoxic (NGTX) carcinogens[3]. Avoidance of exposure to such carcinogens may prevent 30% of all HCC cases[4]. The safe use of these agents and accurate testing of their toxic effects is of economical as well as societal concern.

Valorization of scientific knowledge is defined as the process of value-creation out of knowledge, by making this knowledge suitable and available for economic or societal utilization and to translate this into high-potential products, services, processes and industrial activity.

Economic relevance

Economic utilization of the current obtained scientific knowledge should be focused on developing alternative, improved and less expensive *in vitro* tests which are able to better predict the *in vivo* genotoxic and carcinogenic potential of novel hazardous agents (e.g. chemicals, food-additives or pharmaceuticals). Development, application and commercialization of improved test methods in human risk assessment by industry leads to products which are safer leading to a higher competitive value and are thus more profitable.

Societal relevance

Societal utilization of the scientific knowledge originating from this current thesis should be dedicated to follow-up research focused on the development of so called adverse outcome pathways (AOP) by the global organization, Organization for Economic Co-operation and Development (OECD)[5]. The OECD actually launched a program in 2012 on the development of AOPs that should contribute to an improved risk assessment of human carcinogens. The definition of an AOP, as meant by the OECD, is an analytical concept that describes a linear sequence of causally related key events (KE) within the different molecular layers of the biological organization; starting with a so called molecular initiation event (MIE) that ultimately lead to an adverse outcome (AO), represented by a health or toxicological effect such as the development of HCC. The linkage between the events is described by key event relationships (KER). Ideally these AOPs, and information on these related events, should all be collected

within an open-source web-based interface, namely the AOP Knowledge Base (AOP KB) (https://aopkb.org/). The project focused on collecting this type of information is led by the OECD, the U.S. Environmental Protection Agency (EPA) and the European Commission's Joint Research Center (JRC). AOPs are the central element of a toxicological knowledge framework being built to support chemical risk assessment based on mechanistic reasoning. Such AOPs should also be included in the guidance documents developed by an initiative of the European Union through the Registration, Evaluation, Authorization and restrictions of Chemicals (REACH)[6].

In addition the generation of improved novel alternative *in vitro* test methods could on the long run, replace the use of animals for genotoxicity and carcinogenicity testing, within human risk assessment, thereby complying with the societal demand for reducing animal-based testing.

Target groups

Industry

The mechanistic knowledge obtained in this current thesis might be used within future translational research aimed at developing *in vitro* applications that accurately predict the carcinogenic potential of a novel agent developed by chemical, food or pharmaceutical industry. Especially specific response profiles of genes or other biomarkers may be utilized for developing such alternative test systems. This may fulfill the economic demand of creating products which are safer to use thereby generating more competitive products.

Regulatory organizations

In Europe, specialized organizations such as the European Chemical Association (ECHA)[6], European Food Safety Authority (EFSA)[7] and the European Medicines Agency/U.S. Food and Drug Administration (EMA/FDA)[8,9] are responsible for the safe use of chemicals, food-additives and pharmaceuticals, respectively. Regulations and recommendations are provided by global initiatives such as the guidelines developed by the OECD[5] or by the European Union through REACH[6].

New chemicals, food-additives or drugs first need to be extensively tested for their potential hazards and risks to human health and the environment, before they enter the public market. Human risk assessment is based on toxicological tests performed with short-term *in vitro* assays and (sub-) chronic animal experiments. The animal experiments, mainly using mice and rats, are used to estimate the human cancer risks, but are very expensive and time consuming as they require many animals and a large amount of test compound during a period of usually 2 years. The effectiveness, reliability and relevance of these *in vivo* animal experiments are questioned, especially with regards to the extrapolation of data from animal to humans and from short-term experiments in animals to long-term real-life exposure in man[10]. Due to inconsistencies within the translation of testing results from rodents to humans, certain undetected carcinogenic compounds may enter the market while other compounds are incorrectly withdrawn from the market due to their exclusive carcinogenic potential in rodents[11].

Withdrawal of a novel agent from the public market may have tremendous financial consequences for industry. Therefore, the demand for alternative testing methods, for better predicting human cancer risks, is increasing and new high throughput *in vitro* methods need to

be developed. Numerous relatively cheap *in vitro* tests are available (e.g. bacterial Ames test, mouse lymphoma test, micronucleus test and the chromosomal aberration test) to determine if a compound or drug has carcinogenic potential, however these are characterized by a high false positive rate (falsely predicted to be carcinogenic when compared with *in vivo* data)[12,13]. Many compounds that are tested positively *in vitro* therefore need additional animal tests, which thus may be unnecessary. Further development of reliable standardized *in vitro* tests is therefore requested by the OECD and REACH as well as industry which correctly predict human cancer risks[6]. The focus of the development of these alternative models may very well be on liver-based systems. The liver is responsible for the processing of chemical compounds to potential carcinogens and is also a major target organ. In the last decade the development of new alternative test systems has focused on the application of novel technologies, thereby enabling complete monitoring of the effects of carcinogens in a cell which allows us to better understand why these compounds cause cancer in the long run. The field of toxicogenomics, in which this so called systems biology approach may be applied, can be extremely helpful in improving the translation of the *in vitro* setting to the *in vivo* situation[14-16].

Furthermore, toxicogenomics-based alternative *in vitro* assays may help in a better understanding of the regulatory mechanisms (e.g. epigenetic events such as DNA methylation or via non-coding microRNAs) underlying the carcinogenic potential of certain types of agents. The findings from Chapter 3 might be useful in developing a cross-omics based AOP for GTXC versus NGTXC, in which the roles of microRNAs in chemically-induced carcinogenesis leading towards HCC may provide an added value. Especially the findings, presented in Chapter 5, 6 and 7, regarding the persistent effects on the microRNA and gene expression and DNA methylation level after, in this case, termination of AFB1 treatment in primary human hepatocytes (PHH) may add a new perspective on AOPs for carcinogenicity in particular and toxicity in general. An increased understanding of chemically-induced carcinogenesis and especially the persistency of the induced effects due to carcinogen treatment, may lead towards an improved risk assessment of chemical carcinogens within humans by contributing to a controlled regulatory environment in which the safe use of products and processes is guaranteed. This may ultimately lead to an improvement of health of patients and consumers.

The development of AOPs may therefore facilitate in a better prediction of for example the onset of HCC [17]. In the AOP Knowledge Base wiki (https://aopkb.org/aopwiki/index.php/Main_Page) there is currently an AOP under review named "AFB1: Mutagenic Mode-of-Action leading to Hepatocellular Carcinoma (HCC)" developed by the working group of OECD Project 2.8 focused on developing AOPs for Mutagenic Modes of Action for Cancer. Our obtained regulatory networks could provide added value to this AOP and may therefore be uploaded to the AOP Knowledge Base wiki and reviewed by the OECD. The epigenetic- and microRNA-directed regulatory networks obtained within this current study might therefore be used for further development of AOPs characteristic for a particular type of exposure but could also be useful in generating AOPs representative for more general responses (GTX versus NGTX carcinogen exposure)[18].

Activities or products

Commercialized in vitro test system

mRNA expression-based *in vivo* and *in vitro* tests have already proven to be successful in separating, specifically, human cells or rats treated with GTX from NGTX carcinogens [19-21].

The gene expression found within Chapter 4 could therefore be used for the development of an alternative *in vitro* test system which could be detected by a dedicated PCR- or microarray-based test system within PMH. Such an alternative test system should then be accompanied with a standard operating procedure (SOP) concerning: 1) how to effectively culture PMH and 2) how to design a sophisticated experiment. This concept could then be commercially sold as a quick and standardized ready-to-go test system for the evaluation of carcinogenic properties of novel chemical compounds.

Adverse Outcome Pathways (AOP)

AOPs might be developed representing a set of characteristic responsive molecules and their behavior or expression within a cell, in response to a given exposure with a particular compound. By defining particular classes of toxicity, using prototypical model compounds, chemical AOPs may be established which can be used for a better mechanistic understanding and prediction of toxicity of new compounds, food-additives or pharmaceuticals. Generation of AOPs representing important events in genotoxicity and carcinogenicity should be included in regulatory guidance documents for the testing of novel agents. Therefore, the obtained persistently affected genes (non-methylated/methylated and/or differentially up/down regulated) and microRNAs, which were found in this current thesis after termination of AFB1 treatment in PHH (Chapter 5 – 7), could be form the basis for the development of such an AOP.

Innovation

Dedicated gene expression platforms

The main innovative value of the obtained results is to be found in the applicability of a gene expression pattern, more specifically the differentially expressed genes, within an commercialized test system which may improve the *in vitro*-based prediction of *in vivo* genotoxicity and carcinogenicity. In Chapter 4 we initially aimed at developing a similar test system which was preferably based on microRNA expression profiles; however, we were not successful. Instead, we were able to establish such a predictive signature for genotoxicity and, specifically, non-genotoxic carcinogenicity which was based on gene expression profiles. Such an expression profile based on mRNA data has already been proven successful in predicting *in vivo* genotoxicity with the use of *in vitro* cultured HepG2 cells by Magkoufopoulou and colleagues[19]. This *in vitro*-based expression profile has been patented and licensed out to the spin off company ToxGenSolutions (http://www.toxgensolutions.eu/) to develop a commercialized PCR-based platform. A comparable platform may thus be developed focused on an improved prediction of *in vivo* genotoxicity or non-genotoxic carcinogenicity. Our robust gene expression profile could be applied which is based on *in vitro* transcriptomics data originating from exposed primary mouse hepatocytes. The availability of a more accurate *in vitro* test system may greatly benefit big partners in industry since such a method would lead to the production of safer and more competitive products with a higher market value.

Improvement of mechanistic understanding

Furthermore, within this thesis we were able to establish the basis for the development of AOPs, important within genotoxicity and carcinogenicity related events, using a set of prototypical model compounds. These AOPs could improve the current understanding of

how chemicals provoke toxic events leading to a pathological outcome. Therefore the results of this thesis improve the current knowledge with regards to mechanism of toxicity.

Implementation

The development of an appropriate AOP for chemically-induced carcinogenesis could improve the mechanistic understanding of toxicological endpoints related with certain pathologies. Replacing animal experiments with an improved and more mechanistically relevant alternative test system may generate major societal as well as economic impact. Furthermore, better insights into carcinogenic mechanisms might lead to more efficient and cost-effective future experiments.

Economic implementation

Therefore, in order to further validate the use of potential biomarker genes or microRNAs for the development of an alternative *in vitro* test system, the current results from this thesis need to be further expanded with more test chemicals. Also for the development of potential biomarkers of genotoxicity or carcinogenicity this is relevant in order to further develop the training set of compounds. The training set of compounds is used to establish the chemical specific biomarkers. When the training set is sufficiently large and accurate biomarkers are established, the tests needs to be reviewed and validated by an independent laboratory with a group of test compounds. The data from these extended experiments then need to be used within appropriate prediction software programs that are able to accurately predict the toxic potential of the tested compound. For achieving economic impact, preferably, the biomarkers need to be patented. This may be commercialized by developing a standardized dedicated analytical platform as well as software for the analysis of the data.

Societal implementation

Furthermore, in order to get the new test generally accepted in chemical safety assessment, recognition by regulatory authorities is required. Validation of this new method therefore needs to be reviewed by the European Centre for the Validation of Alternative Methods (ECVAM)[22] in order to evaluate the reproducibility and the robustness of this assay. When the new tests finds acceptance by ECVAM it may be implemented as an EU test method and included in the EU Test Methods Regulation or as an OECD Test Guideline[5]. The OECD Test Guidelines are a collection of internationally agreed test methods used by government, industry and independent laboratories. They are used to determine the safety of chemicals and chemical preparations, including pesticides and industrial chemicals.

Other ways of valorization by then also include instructing and informing personal from governmental institutes, industry and independent laboratories about these new guidelines in order to allow correct application of the new test method.

The results from this thesis may form the preliminary basis for developing such AOP- or biomarker-focused alternative tests systems thereby contributing to a more accurate prediction of carcinogenicity for human risk assessment.

References

1. Stewart, B.W., Wild, C., International Agency for Research on Cancer & World Health Organization. *World cancer report 2014*, xiv, 630 pages (International Agency for Research on Cancer; WHO Press, Lyon, France; Geneva, Switzerland, 2014).
2. Ferlay, J. *et al.* Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136, E359-86 (2015).
3. Ashby, J. Use of short-term tests in determining the genotoxicity or nongenotoxicity of chemicals. *IARC Sci Publ*, 135-64 (1992).
4. World Health Organization. *National cancer control programmes : policies and managerial guidelines*, xxiii, 180 p. (World Health Organization, Geneva, 2002).
5. Organisation for Economic Co-operation and Development (OECD). OECD Guidelines for the Testing of Chemicals http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm, (accessed on July 15, 2015) (2015).
6. European Chemicals Agency (ECHA). Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). http://echa.europa.eu/regulations/reach, (accessed on July 15, 2015) (2015).
7. European Food Safety Authority (EFSA). http://www.efsa.europa.eu/, (accessed on July 15, 2015) (2015).
8. European Medicines Agency (EMA). http://www.ema.europa.eu/ema/, (accessed on July 15, 2015) (2015).
9. U.S. Food and Drug Administration (FDA). http://www.fda.gov/, (accessed on July 15, 2015) (2015).
10. Walmsley, R.M. & Billinton, N. How accurate is in vitro prediction of carcinogenicity? *British Journal of Pharmacology* 162, 1250-1258 (2011).
11. Ennever, F.K. & Lave, L.B. Implications of the lack of accuracy of the lifetime rodent bioassay for predicting human carcinogenicity. *Regul Toxicol Pharmacol* 38, 52-7 (2003).
12. Kirkland, D. & Speit, G. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing in vivo. *Mutat Res* 654, 114-32 (2008).
13. Kirkland, D. *et al.* How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: Report of an ECVAM Workshop. *Mutat Res* 628, 31-55 (2007).
14. National Research Council (U.S.). Committee on Applications of Toxicogenomic Technologies to Predictive Toxicology. *Applications of toxicogenomic technologies to predictive toxicology and risk assessment*, xxii, 275 p. (National Academies Press, Washington, D.C., 2007).
15. Kleinjans, J. *Toxicogenomics-based cellular models : alternatives to animal testing for safety assessment*, xviii, 348 p. (Elsevier/AP, Amsterdam, 2014).
16. Piersma, A.H. *et al.* A critical appraisal of the process of regulatory implementation of novel in vivo and in vitro methods for chemical hazard and risk assessment. *Crit Rev Toxicol* 44, 876-94 (2014).
17. Ankley, G.T. *et al.* Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem* 29, 730-41 (2010).
18. Mirbahai, L. & Chipman, J.K. Epigenetic memory of environmental organisms: a reflection of lifetime stressor exposures. *Mutat Res Genet Toxicol Environ Mutagen* 764-765, 10-7 (2014).
19. Magkoufopoulou, C. *et al.* A transcriptomics-based in vitro assay for predicting chemical genotoxicity in vivo. *Carcinogenesis* (2012).
20. Fielden, M.R. *et al.* Development and evaluation of a genomic signature for the prediction and mechanistic assessment of nongenotoxic hepatocarcinogens in the rat. *Toxicol Sci* 124, 54-74 (2011).
21. Ellinger-Ziegelbauer, H., Stuart, B., Wahle, B., Bomann, W. & Ahr, H.J. Comparison of the expression profiles induced by genotoxic and nongenotoxic carcinogens in rat liver. *Mutat Res* 575, 61-84 (2005).
22. European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM). https://eurl-ecvam.jrc.ec.europa.eu/, (accessed on July 15, 2015) (2015).