Biomedical Research Meets Toxicology: How *In Vitro* Chromosome Instability Methods Can Contribute to Carcinogenicity Prediction

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ABSTRACT

Cancer is a major health concern and a leading cause of mortality. The reliable identification of carcinogens and understanding of carcinogenicity has become a main focus of biomedical research and regulatory toxicology. While biomedical research applies cellular *in vitro* methods to uncover the underlying mechanisms causing cancer, regulatory toxicology relies on animal testing to predict carcinogenicity of chemicals, often with limited human relevance. Exemplified by chromosome instability–mediated carcinogenicity, we discuss the need to combine the strengths of both fields to develop highly predictive and mechanism-derived *in vitro* methods that facilitate risk assessment in respect to relevant human diseases.

Introduction

As cancer is the second leading cause of death worldwide, the identification and classification of carcinogens is of key importance. Although interspecies conflicts and differences in the dose-response make transferability to humans difficult, the evaluation of the carcinogenic potential still highly depends on animal testing. Regulatory toxicology tries to break out of this dilemma by developing an integrated approach, which links exposure to the pathologic effect through a linear chain of molecular and cellular key events. This concept of adverse outcome pathway (AOP; ref. 1) would provide mechanistic insights on carcinogenicity while simultaneously ensuring derivation of predictive *in vitro* methods. Notably, pharmaceutical research successfully applies such mechanistic approaches to develop suitable cancer therapeutics. As biomedical research fills the mechanistic gaps in our understanding of carcinogenicity, it will assist in the development of cancer AOPs and of mechanism-derived *in vitro* methods with a high-predictive value. For instance, oncological researchers revealed ongoing whole-chromosome segregation errors during mitosis, termed whole-chromosome instability (w-CIN; ref. 2), as a key property of human tumors with great promise for cancer prediction. However, biomedical *in vitro* assays measuring w-CIN are not yet available for regulatory use because they are neither tested for predictability of carcinogenicity, nor validated for toxicologic application.

Below we discuss, using the example of w-CIN, how biomedical research can make a significant contribution to the prediction of carcinogenicity, thereby boosting the development of *in vitro* assays for regulatory purposes.

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Carcinogenicity from an Oncological and Toxicologic Point of View

Toxicology and biomedical research handle carcinogenicity with different priorities. While regulatory toxicologists focus on whether a substance harbors a cancer-inducing potential in the context of hazard and risk assessment, biomedical researchers are primarily interested in the mechanisms leading to cancer development to develop promising diagnostic and therapeutic strategies. Despite their different approaches to carcinogenicity, both perspectives have important overlapping strengths. A reliable identification and risk assessment of potential carcinogens undoubtedly requires a basic understanding of the etiology of carcinogenesis. Therefore, in recent years, toxicologists have placed greater emphasis on the mode of action of carcinogens. To develop novel and highly predictive *in vitro* test systems for carcinogenicity, an integrated approach is most appropriate for both, scientists and assay developers. Here, the concept of AOPs promises great potential, because it links the molecular impact of a biomolecule (molecular-initiating event) with several mechanistic key events and describes the key event relationship, resulting in the final pathologic effect, the adverse outcome. On the level of the Organization for Economic Cooperation and Development (OECD), first attempts to develop AOPs for cancer are ongoing, for example, for breast cancer or hepatocellular carcinoma (AOP 200 and 46 https://aopwiki.org/). As carcinogenicity involves multiple complementary steps, regulatory toxicology will benefit from biomedical research, which unveils key pathways implicated in the development of cancer. Indeed, Hanahan and Weinberg defined eight hallmarks of cancer: sustained proliferative signaling, evading growth suppressors, resistance to cell death, replicative immortality, induced angiogenesis, activated invasion and metastasis, reprogrammed cell metabolism, and avoidance of immune destruction (3). All of these unique properties provide mechanistic insight that may inform existing or novel cancer AOPs. Mechanistic understanding of cancer etiology is especially promising in that it enables toxicologists to develop highly predictive quantitative *in vitro* assays for human carcinogenicity, which will be of particular interest for chemical risk assessment. For instance, sustained proliferation of cancer cells is strongly associated with ongoing chromosome missegregation (2). This process of w-CIN causes highly diverse aneuploid karyotypes, which, in turn, drive tumorigenesis and tumor progression as well as therapeutic resistance. It is therefore reasonable to define w-CIN as an important predictive

marker for carcinogenicity and to develop *in vitro* methods, which allow measurement of direct chromosome missegregation.

Methods for Carcinogenicity Testing from a Toxicologic Perspective

Regulatory risk assessment aims to identify carcinogenic substances to avoid exposure to and to ensure public health. Put simply, carcinogens may be grouped as being genotoxic (inducing direct DNA damage) and nongenotoxic (e.g., by affecting gene expression, signal transduction, and/or cell proliferation). The latter has been proposed to act via a number of complex mechanisms including decreased apoptosis, oxidative stress, immune suppression, and peroxisome proliferation, among others. As part of the safety evaluation of chemicals, carcinogenicity studies are based *inter alia* on internationally harmonized test guidelines (TG) provided by the OECD TG program. Because of the inherent biological complexity of cancer development, the *in vivo* rodent bioassay (OECD TG 451) has been the gold standard for predicting human carcinogenicity over the past decades. In detail, 50 or more rodents per sex were exposed to test substances by various administration routes daily. Exposure starts at age 5–6 weeks and lasts up to 2 years, when a complete histopathologic examination is performed. To reduce animal use, two endpoints, carcinogenicity and chronic toxicity, are combined (OECD TG 453). However, species and dose extrapolations might be inappropriate because specific modes of actions might vary between rodents and humans (4). Cancer development in rodents might occur at doses that are irrelevant for human exposure scenarios, or chemicals might exert dose-limiting toxicity before leading to tumors, leaving their carcinogenic potential undetected (5). For these reasons, cell culture–based *in vitro* methods and computational modeling have become increasingly important for the development of regulatory guidelines. Examples of routinely used *in vitro* genotoxicity tests include the *in vitro* mammalian chromosomal aberration test (OECD TG 473) and the *in vitro* mammalian cell micronucleus test (MNT, OECD TG 487), which measure structural chromosomal aberrations or breaks and chromosome missegregation, respectively. In addition, mutagenicity is detected by the bacterial reverse mutation assay (OECD TG 471) and the *in vitro* mammalian cell gene mutation tests (OECD TGs 476 and 490). Compared with animal testing, *in vitro* genotoxicity/mutagenicity assays are rapid and simple, allowing high numbers of replicates and automation. Although they are highly sensitive, their low specificity results in high rates of false positive hits, provoking confirmatory follow-up *in vivo* testing. Thus, highly predictive *in vitro* methods for carcinogenicity that combine a high degree of sensitivity with greatest specificity are urgently required.

Methods for Carcinogenicity Testing from a Biomedical Perspective

Biomedical scientists aim to disclose differences between normal and tumor cells to find novel and promising therapeutic approaches targeting cancer. For this purpose, key features unique to cancer cells must be identified. w-CIN is a well-accepted and important hallmark of human cancers, and thus, *in vitro* methods measuring chromosome instability (or derived features), are particularly promising for risk assessment of substances associated with chromosomally unstable human cancers (e.g., breast, prostate, or colorectal cancer). In biomedical research, various *in vitro* methods are used to detect w-CIN. Among them are karyotype analyses using chromosome counting from metaphase spreads, flow cytometry–based measurements of loss rates of a nonessential human artificial chromosome carrying a constitutively expressed *eGFP* transgene, and interphase FISH using fluorescent centromeric probes bound to the chromosomes (6, 7). Aneugenic substances routinely used to induce w-CIN are also reference chemicals of the OECD testing guidelines for carcinogenicity (e.g., OECD TGs 474 and 487), and include taxol, *vinca* alkaloids, and colchicine (6). One of the great advantages of all these assays is their ability to detect chromosome missegregation on a single-cell level. This reflects the concept of loss of genetic integrity as the first step in human tumorigenesis. While metaphase spreads commonly allow the detection of missegregated chromosomes *per se*, FISH is highly specific for the selected probes and allows the unambiguous detection of chromosomes known to be frequently lost or gained in a specific cancer type. Chromosome flow cytometry–based assays enable the generation of a suitable artificial cell system, facilitating the estimation of the CIN-inducing potency of a certain chemical. These w-CIN–based approaches can specifically detect aneugens, making them highly promising as complementary assays to the routinely applied *in vitro* genotoxicity/mutagenicity tests of regulatory toxicology, which mainly detect mutagens and clastogens. Furthermore, the w-CIN methodology might increase the resolving power of the MNT, which covers both damaged chromosome fragments and whole chromosomes that have become separated from the nucleus proper. Micronuclei might reassociate with the major chromosome population and thus, do not necessarily lead to numerical karyotypic changes. Hence, the MNT uncovers clastogens more reliably than aneugens. Nevertheless, methods detecting w-CIN directly are time consuming and complex because they need to run over several days, making it a challenge to adapt these assays to high content and high throughput (HC/HT) screening.

In recent years, several underlying mechanisms of chromosome instability have been identified, which could facilitate the development of regulatory *in vitro* methods derived from w-CIN. In this context, measuring the rate of lagging chromosomes during anaphase of mitosis might be a promising approach, as such laggards represent an important and well-accepted w-CIN precursor. Lagging chromosomes are randomly segregated to the two daughter cells and represent a common key event in cancer (7). Importantly, lagging chromosomes mechanistically result from transient spindle geometry and orientation defects that facilitate the generation of hyperstable kinetochore-microtubule (KT-MT) attachments. Therefore, mechanisms promoting defects in spindle geometry and KT-MT attachments are also suited for deriving further *in vitro* methods. Here, the persistence of supernumerary centrosomes is a prime example, as multipolar mitotic spindles are associated with faulty KT-MT attachments. Consequently, it represents a well-accepted precursor of w-CIN and a hallmark of human cancer. A further underlying mechanism of lagging chromosomes is increased microtubule (MT) plus end growth rates in living cells, resulting in mitotic spindle disruption (6). This live cell–based approach requires a rather slow and complex analysis method, but a promising, simpler method might be the phenotypic screening assay. This is based on the analysis of monopolar mitotic spindle structures and measures the increased MT polymerization rates and chromosome missegregation (8). Importantly, all of these *in vitro* methods can be adapted to a variety of mammalian cells with different tissue origin and are potentially adaptable to primary tissue samples, thereby improving the transferability to the *in vivo* situation with high validity. Integration into HC/HT systems enables a variety of chemicals and conditions to be tested simultaneously, allowing data to be acquired within a few days. In addition, w-CIN assays are of high biological

relevance and therefore promising for cancer prediction, although their sensitivity and specificity for carcinogenicity for regulatory purposes remains to be demonstrated. While none of these assays can serve as a standalone method for predicting carcinogenicity, they are a previously unappreciated but powerful tool for regulatory toxicology in combination with additional assays within a testing strategy.

Implementation of w-CIN–Derived *In Vitro* Assays in Regulatory Toxicology

The plethora of assays detecting w-CIN or its precursors clearly illustrates the variety of biomedical *in vitro* methods available with which to characterize key human cancer attributes. Even if w-CIN is considered to be predictive for carcinogenesis, its role as a bystander of tumor progression is currently under discussion. Nonetheless, chromosome instability represents a hallmark of human cancers, with roughly 90% of solid tumors and over 50% of hematopoietic cancers exhibiting chromosomal abnormalities and aneuploidy (9). Thus, unveiling underlying mechanisms of w-CIN could provide vital information on the general etiology of carcinogenicity. Of note, the majority of existing assays detecting w-CIN are only incipiently developed towards carcinogenicity prediction. To assess whether they can actually predict carcinogenicity, they must be validated in a toxicologic framework by using reference chemicals, which are defined by regulatory authorities such as EURL ECVAM, the OECD or US EPA, EFSA, and ECHA. Because most of the reference chemicals have been identified in animal test systems, it will be of great interest to see whether their carcinogenic potential can be confirmed in the human cell system as well. At the end of this process, it will become clear whether the existing methods can reliably predict carcinogenicity. Only validated and predictive methods will gain final acceptance in the field of regulatory toxicology and prospectively replace or at least complement, the standard *in vivo* tests for carcinogenicity.

As carcinogenicity is a complex process, it may be rewarding to combine several *in vitro* assays in a test strategy addressing different key events or pathways. Such a test strategy is likely to provide a more reliable prediction of carcinogenicity in comparison with a single test method. To this end, it is essential to understand the processes leading to cancer development and to define underlying key events promoting tumorigenesis. As w-CIN represents an important, but not the only property of human cancer, regulatory toxicology agencies should propagate the integrated AOP concept. The overriding challenge is whether it will be feasible to develop a general AOP network covering carcinogenicity as a whole. This strategy is currently being pursued by using the 2-year rodent bioassays and chronic toxicity studies, aiming to target all kinds of emerging tumors instead of specific ones. However, in the last years, we have learned from clinicians and biomedical researchers that the molecular properties of different cancer subtypes are more distinct than previously assumed. Consequently, a paradigm shift is required in the discussion of carcinogenicity assessment. A recently initiated debate proposed a more focused approach on the most prevalent cancer types such as breast, lung, or colorectal cancer (10). This strategy requires a fruitful interaction of biomedical researchers, toxicologists, clinicians, and regulators that could grow on the level of an AOP framework to implement specific *in vitro* assays, which are highly predictive for carcinogenicity assessment in a regulatory environment. In combination with further biomarkers or key events, various AOPs for specific cancer types could be generated. Finally, based on these AOPs, the development of an *in vitro* test strategy could be evolved to assess the risk potential of a chemical carcinogen.

Conclusions

Both, biomedical researchers and toxicologists aim to understand the molecular mechanisms of human carcinogenicity. The former focuses on the improvement of cancer diagnosis and therapy, while the latter aim to identify substances with carcinogenic activity and avoid their exposure to humans. Most importantly, both recognize the urgency to push predictive *in vitro* methods forward. In comparison with existing *in vivo* methods, cell culture–derived *in vitro* methods may allow HC/HT screening of putative carcinogens or novel therapeutics. This would allow entire chemical libraries to be screened for toxicologic purposes within a short span of time, including a high number of different conditions and chemical combinations. *In vitro* methods are adaptable to complex models such as human organoids and primary tissue samples, moving closer to the human situation and substantially reducing interspecies inconsistencies. Prospectively, the available validated *in vitro* methods must be combined in a valuable test strategy for the prediction of carcinogenicity in humans. From this point of view, a close collaboration between biomedical scientists, toxicologists, clinicians, and regulatory authorities is required to paint a complete picture of cancer diseases and to protect human health. Consequently, AOP development expert groups should involve more clinicians and biomedical scientists in the development of risk assessment tools and guidelines. Vice versa, biomedical researchers should contribute their scientific knowledge to the open source AOP development framework. Ultimately, the constructive collaboration of both fields has the potential to bring about a significant improvement in preserving human health.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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