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Review

Improving prediction of chemical carcinogenicity by considering multiple mechanisms and applying toxicogenomic approaches☆

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ABSTRACT

While scientific knowledge of the potential health significance of chemical exposures has grown, experimental methods for predicting the carcinogenicity of environmental agents have not been substantially updated in the last two decades. Current methodologies focus first on identifying genotoxicants under the premise that agents capable of directly damaging DNA are most likely to be carcinogenic to humans. Emphasis on the distinction between genotoxic and non-genotoxic carcinogens is also motivated by assumed implications for the dose–response curve; it is purported that genotoxicants would lack a threshold in the low dose region, in contrast to non-genotoxic agents. However, for the vast majority of carcinogens, little if any empirical data exist to clarify the nature of the cancer dose–response relationship at low doses in the exposed human population. Recent advances in scientific understanding of cancer biology—and increased appreciation of the multiple impacts of carcinogens on this disease process—support the view that environmental chemicals can act through multiple toxicity pathways, modes and/or mechanisms of action to induce cancer and other adverse health outcomes. Moreover, the relationship between dose and a particular outcome in an individual could take multiple forms depending on genetic background, target tissue, internal dose and other factors besides mechanisms or modes of action; inter-individual variability and susceptibility in response are, in turn, key determinants of the population dose–response curve. New bioanalytical approaches (*e.g.*, transcriptomics, proteomics, and metabolomics) applied in human, animal and *in vitro* studies could better characterize a wider array of hazard traits and improve the ability to predict the potential carcinogenicity of chemicals.

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1. Introduction

The short-term tests currently used to predict a chemical's ability to induce cancer were implemented based on scientific evidence that emerged in the 1970's linking DNA damage and mutation with cancer. Accordingly, these screening methodologies aim to identify genotoxic agents (*e.g.*, as described in Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use guidance [1,2]) under the premise that such agents would most likely pose cancer risk in humans. Typically, the tests include a battery of three assays: (a) a test for gene mutation in bacteria, (b) an *in vitro* test for mutation and/or chromosomal damage in mammalian cells and (c) an *in vivo* test for chromosomal damage using rodent hematopoietic cells. In addition to their use as a screening tool and surrogate for carcinogenicity data in the development of drugs and other chemicals, genotoxicity data constitute part of the weight of evidence evaluation in regulating environmental chemicals [3]. In practice, environmental contaminants have not been regulated as carcinogens on the basis of positive genotoxicity results alone. Nonetheless, chemicals testing positive in standard genotoxicity assays are generally assumed to contribute to cancer induction *via* a genotoxic or mutagenic mode of action that is indicative of human risk [3,4].

An accompanying assumption is that the dose–response curve for such genotoxicants lacks a threshold in the low dose region. Moreover, in recent guidance, US Environmental Protection Agency (US EPA) has ascribed increased early-life susceptibility to compounds with a mutagenic mode of action and, in the absence of chemical specific data, adjusts cancer risk estimates for such environmental contaminants [5]. Except in the very few cases where specific data are available that the chemical poses an increased risk due to early life exposures, no adjustment is made by US EPA to account for childhood susceptibility to environmental exposures from compounds that do not have a mutagenic mode of action.

The standard test battery is highly sensitive for detecting genotoxicity, and only a few *in vivo* genotoxicants are not detected *in vitro*. However, the ability of these genotoxicity tests to predict the outcome of an animal carcinogenicity bioassay is imprecise [6–8]. For example, an analysis of a selected group of marketed pharmaceuticals found that 20% of the 124 non-carcinogens were positive in at least one genotoxicity assay while two-thirds of the 77 rodent carcinogens lacked activity in the genotoxicity tests employed [8]. The discrepancy between genotoxicity and rodent carcinogenicity findings arises from non-genotoxic mechanisms of carcinogenicity and limitations of genotoxicity endpoints and assays. Indeed, there are no routine screening tests for mechanisms other than genotoxicity, including the epigenetic effects that can also play a critical role in induction and progression of human cancer [9]. Moreover, the tests do not readily accommodate the concept that a single chemical may have multiple impacts on the carcinogenic process.

Development of additional short-term assays and better methods to detect effects associated with a range of toxicity pathways, mechanisms and modes of action would be of considerable interest [10,11]. More than 100,000 chemicals are used in commerce in the US and Europe. Data about chemicals in commerce is extremely limited [12–14]. In 1988 US EPA reported that almost 2000 chemicals were used in quantities exceeding 1 million pounds per year, of which 43% had no testing data available and only 7% had a relatively complete set of data [12]. Fewer than 1,000 compounds have been systematically evaluated for carcinogenicity by the National Toxicology Program, the International Agency for Research on Cancer (IARC) or the US EPA [15–17] (Table 1).

The paucity of experimental animal or human data on most chemicals, some of which constitute significant environmental contaminants and have human exposures, provides motivation for improved screening and predictive methods that reflect current knowledge and are less time consuming and costly. The development of experimental approaches and predictive tools enabling the use of mechanistic information in risk assessment is increasingly being identified as a priority. Two recent National Academy of Sciences (NAS) panels noted key areas such as identifying toxicity pathways, exploring simultaneous alteration of multiple pathways, and developing a plan for validation of new approaches and assays as important for future toxicity testing strategies [10,11]. The need for development of mechanism-based assays is also fully recognized by the European Union's Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) initiative [18]. The US (Interagency Coordinating Committee), European and Japanese Centers on the Validation of Alternative Methods (ICCVAM, ECVAM, JaCVAM) are also involved in coordinating the development and evaluation of new alternative testing methods. Such methods are intended to address the data needs arising from the general decline in the number of conventional animal bioassays being conducted and increased emphasis on non-animal tests; for example, EU's cosmetic directive aims, among other objectives, to end animal testing of cosmetic products and ingredients by 2009 irrespective of the availability of non-animal tests.

As an initial step to evaluating current methods and exploring future approaches, we undertook a broad review of recent

Table 1
Chemicals in commerce and their evaluation.

Chemicals in US commerce	
On Toxic Substances Control Act Chemical Substance Inventory	~75,000
Chemicals in US commerce between 25,000 and 1,000,000 lbs/year	6500–7000
Chemicals in US commerce >1,000,000 lbs/year	>2200
Notable publicly available reviews	
Number of chemicals evaluated by IARC	>900
Number of chemicals listed on IRIS	545
Number of chemicals reviewed by Center for the Evaluation of Risks to Human Reproduction (CERHR)	23
Proposition 65 list of carcinogens and reproductive toxicants	~775

advances in cancer biology focusing on scientific evidence of health significance supporting regulatory decisions. Current approaches to using mechanistic information were reviewed, including those of IARC and US EPA. To provide insight into the mechanisms especially relevant for chemical carcinogenesis in humans, data for eight known human carcinogens (a subset of the 105 agents currently classified by IARC in Group 1: carcinogenic to humans) were examined to assess the potential contribution of a range of possible mechanisms to carcinogenicity. The analysis considered whether chemical carcinogens may act through multiple mechanisms and found that simple dichotomous characterization regarding whether a chemical is "genotoxic" or "non-genotoxic," though often part of risk assessment approaches, is not particularly informative. We also examined the extent to which *in vitro*, *in vivo* and human biomarker studies have the potential to provide mechanistic insights relevant to chemical carcinogenesis. Finally, future implications for research and risk assessment were addressed. These topics and their associated implications are discussed in detail in the sections below.

2. Current uses of mechanistic data

Mechanistic data have several applications in health risk assessments. The key issues that mechanistic data can inform in hazard identification include the relative sensitivity of humans to effects observed in animals, and human inter-individual variability and susceptibility to effects (including the identification of sensitive subpopulations). Mechanistic data are also used to inform the dose–response relationship, especially to support arguments regarding the expected existence or absence of thresholds at low doses. US EPA's *Guidelines for Carcinogen Risk Assessment* [5] particularly emphasize the use of mechanistic data to inform the mode of action of a chemical. The mode of action is used in the hazard characterization and dose–response components of a risk assessment, as well as in the identification of susceptible populations and life stages. These guidelines define mode of action as "a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes and resulting in cancer formation". Mode of action is distinguished from a "mechanism", with the latter implying the full series of events that lead from exposure to disease. The guidelines acknowledge that the "possible involvement of more than one mode of action at the tumor site should be considered", but suggest that one mode of action may predominate depending on tumor site, conditions of exposure, dose, *etc.* Rather than an integrated analysis of all mechanistic data, the guidelines specify a separate, weight of evidence-based evaluation of each hypothesized mode of action. The general framework for analyzing mechanistic data and hypotheses is similar to those published by the International Life Sciences Institute [19,20] and World Health Organization International Programme on Chemical Safety [21,22]. However, an examination is warranted of the extent to which these evolving approaches have utility in informing either the cancer hazard identification or the shape of the population dose–response curve at low doses [23].

The IARC takes a different approach of using mechanistic data as one component of an integrated evaluation. In assessing carcinogenicity, IARC relies on epidemiologic studies, cancer bioassays and other relevant data including mechanistic information. While epidemiologic studies offer clear relevance, causality inferences rely on ruling out chance, bias and confounding with reasonable confidence. Limitations include difficulties in estimating exposure, attributing cause to a single factor, and ruling out small risks that may not be detectable due to small sample sizes. In addition, human studies must necessarily accommodate the long latency of the cancer disease process. Animal cancer bioassays complement these limitations with clearly defined exposure, reduced bias and confounding, and a more rapid availability of results. However, the correspondence between animals and humans is not always clear, leading to the use of mechanistic data to make judgments about the human relevance of animal results. Thus, the relevance of animal studies is often challenged, with a trend towards identifying mechanisms of carcinogenesis in experimental animals and using this to evaluate relevance to humans. For these reasons, the role of mechanistic studies generally has been limited to supporting or discounting the relevance of bioassay results.

Scientifically, it may be more appropriate to identify key mechanisms involved in human carcinogenesis as a means to identify the agents that can play a role in advancing these mechanisms—rather than starting with an animal tumor and evaluating whether each event is similar or different in humans. Indeed, IARC plans to review all human carcinogens identified to date in order to support development of the necessary information base to identify these key mechanisms involved in human carcinogenesis. These mechanisms are expected to be described at the organ, cellular and/or molecular level. IARC also recently modified its guidelines to allow an agent to be classified as possibly carcinogenic to humans (Group 2B) "solely on the basis of strong evidence from mechanistic and other relevant data" [16]. Before this change, a classification in Group 2B required at least limited evidence of carcinogenicity in experimental animals; chemicals that had not been tested in an animal bioassay and found to induce tumors were not considered for classification as possibly carcinogenic. Among the several prominent challenges that remain, new mechanistically based predictive approaches will require development and validation, and must gain acceptance for use to support regulatory action. The question also remains whether novel approaches should attempt to predict animal bioassay results or to predict human carcinogenicity directly. A related issue is whether, in order to be considered a known human carcinogen, an agent must be observed to cause tumors in animals or increase the incidence of human cancer.

3. Identifying mechanisms or modes of action that can contribute to cancer

The number of mechanisms by which chemicals are known to contribute to carcinogenesis is extensive, but most can be grouped into a limited number of categories (*e.g.*, mutation, immunosuppression, *etc.*). When this is done, it is important to keep in mind that the full series of events that lead from exposure to cancer is unknown for essentially all chemicals, even for extensively studied chemicals such as benzene, aflatoxin B_1 and arsenic. The US EPA has attempted to overcome this lack of knowledge by categorizing chemicals according to their "mode of action", the "key events" associated with a toxicological outcome [5]. Table 2 lists 15 types of "key events" associated with carcinogenesis that collectively represent the majority of known carcinogenic modes of action.

In constructing a set of identifiable modes of action for carcinogenesis, several challenges were identified. Of note, the key events identified comprise multiple levels of biological organization ranging from molecule (*e.g.*, DNA mutation) to pathway (*e.g.*, cell signaling alteration) to cell (*e.g.*, mitogenicity) to organ (*e.g.*, immune response modulation) to organism (*e.g.*, nutrient deficiency). Indeed, the current approaches for identifying and studying modes of action involve multiple components. Moreover, data requirements for characterizing cancer risk to humans are rarely specified in regulatory guidance, nor is the integration or synthesis of data across levels of biological

Table 2
Fifteen example key events representing diverse carcinogenic modes of action.

Key events associated with carcinogenesis
DNA reactivity (covalent binding)
Gene mutation
Chromosomal breakage
Aneuploidy
Enzyme-mediated effects on DNA damage or repair
Epigenetic effects
Cell signaling: nuclear receptor-mediated
Cell signaling: other than nuclear receptor-mediated
Immune response modulation
Inflammation
Cytotoxicity and compensatory cell proliferation
Mitogenicity
Chronic metabolic or physiologic overload
Nutrient deficiency related
Interference with intercellular communication (*e.g.*, gap junctions)

organization required. To date, little effort has been devoted to clarifying these issues.

4. Interpreting information relevant to modes or mechanisms of action

One of the biggest challenges in utilizing mode of action information in chemical health risk assessments is the fact that it is difficult to establish a unique mode of action for any chemical. Chemical-specific mechanistic data are often absent or limited, and mode of action data are completely lacking for the vast majority of chemicals in commerce. Available mechanistic data typically come from high dose experiments that have rarely been conducted in the context of or under conditions that are comparable to animal bioassays or epidemiologic studies in which increased cancer risk was observed. Thus, reaching conclusions about the mode of action of a chemical at low doses is almost always difficult and controversial.

Another challenge for establishing the mode of action is the difficulty discriminating causative from associated key events in the cancer process. An example of a study providing data that differentiates association from causation is the recent report that di(2-ethylhexyl)phthalate (DEHP) induces liver tumors in peroxisome proliferator activator receptor-α (PPARα)-null mice, with significantly increased adenomas in knockout compared with wild-type mice [24]. In addition, a significant dose-dependent trend in adenomas and carcinomas combined was observed in the PPARα-null mice. Other data have associated PPARα agonism with DEHP hepatocarcinogenesis, and some have concluded that this represented the sole mode of action for DEHP and other chemicals [25]. However, the study by Ito et al. [24] disproves the hypothesis that PPARα agonism is the sole causative factor for DEHP carcinogenesis. As discussed in further detail later, chemical carcinogens can affect biology in many ways to contribute to cancer. Thus, the approach of centering the decision-making on a single mode of action may lack scientific justification and may limit interpretation of systems-level information covering multiple mechanistic pathways. Indeed, the hypothesis that carcinogens can and usually do have multiple modes of action may be the more appropriate starting supposition.

5. Do carcinogens typically have multiple modes of action?

To examine the hypothesis that carcinogens typically have multiple modes of action, we classified eight IARC Group 1 carcinogens, two IARC Group 2A carcinogens, the model carcinogen diethylnitrosourea, and two non-carcinogens according to the key events listed in Table 2. The results are shown in Table 3. The table shows that key events representing multiple modes of action are operative for each of the chemical carcinogens examined. For example, benzene and arsenic have been shown to cause almost every effect listed. This underscores the need to consider interactions among a carcinogen's multiple modes of action, which may in turn be highly informative of the complex interactions among different carcinogens. In addition, the relative importance of a given mode of action may vary with life stage, genetic background, and dose.

This exercise identified several key challenges. First, using even this abbreviated list of key events, we noted that the mechanistic information about these well-studied compounds is incomplete. Despite the large number of publications, covering decades of research, on the IARC Group 1 compounds (*e.g.*, >4000 publications on aflatoxin B_1 with >200 specifically focusing on mechanisms), it is evident that information gaps still exist regarding their effects on some of the postulated key events in carcinogenesis. For other carcinogens, the information gaps are more pronounced; moreover, basic information is completely lacking for tens of thousands of chemicals.

6. Incorporating knowledge about the carcinogenic process in humans in consideration of modes of action

The focus in current risk assessment methods is on the animal mode of action and its relevance. This can inhibit fuller consideration of the chemical's potential impact on the human disease process. This can falsely dichotomize complex decisions about whether—and how—to use animal mode of action data to inform human risk. The potential for interaction among a chemical's multiple mechanistic effects, and the likelihood that some of the causative factors may already exist in susceptible populations, may constitute the more important determinants of the dose–response relationship at the population level. Centering the decision process on human disease mechanisms can take advantage of increased understanding of individual and population heterogeneity of disease mechanisms. Moreover, the approach would be more amenable to incorporating systems biology-level tools and data. Although the current approach explicitly encourages the use of mechanistic information, it largely reflects the knowledge and thinking from the early 1990s. This underscores the need to update these approaches to reflect current knowledge.

Cancer involves the accumulation of multiple genetic mutations over time (average 11 for solid tumors) and epigenetic alterations [26,27]. Examination of a large number of genes in a panel of breast and colorectal cancers identified somatic mutations in 1149 genes [28]. This raises the question of whether or not these are all key events. This seems unlikely, and actually demonstrates the different point that there are multiple genetic pathways to the development of a specific type of cancer. For example, an average of 63 genetic changes were found in a recent study of 24 pancreatic cancers; these comprised 12 signaling pathways and processes that were altered in 67–100% of the tumors studied [27]. Pedersen-Bjergaard et al. recently characterized 8 different genetic pathways for the development of acute myeloid leukemia [29]. In two of these pathways, mutations affecting the *P53* gene play key roles, but in the other 6 they do not. Thus, a reported key event, *i.e.* mutations affecting *P53*, can be bypassed by other genetic and/or epigenetic changes such as the loss of chromosome 7 or the hypermethylation of the promoter of *P15*. The current US EPA guidelines acknowledge that multiple events may be involved in cancer induction. However, the guidelines recommend an independent evaluation of each "alternative" mode of action and

Table 3
Carcinogens operate via multiple key events representing diverse modes of action for carcinogenesis.[a].

	Key events															
	DNA reactivity/ covalent binding	Gene mutations	Chromosomal breakage	Aneuploidy	Enzyme-mediated DNA damage/ repair	Epigenetic	Cell Signaling: Nuclear receptor-mediated	Cell Signaling: other	Immune response modulation	Inflammation	Cytotoxicity/ compensa -tory cell proliferation	Mitogenicity	Chronic metabolic or physiologic overload	Nutrient deficiency related	Interference with GJIC	Other
IARC Group 1																
Aflatoxin B1	+	+	+	–	+	+	–	–	+	+	+	–	+	–	+	
Arsenic +3	–		+	+	+	+	+	+	+	+	+	+	–	+	+	
Asbestos	+	+	+	–		+		+	+	+						
Benzene	+	+	+	+	+	+		+	+	+	+	–	–	–	+	
DES	+	–	+	+	–	+	+	+	+		–	+	+		+	
Formaldehyde	+	+	+	+	+			+	+	+	+					
TCDD, 2,3,7,8-	–	–	–	–	–	–	+	–	+	–	+	–	–	–	+	+
Vinyl chloride	+	+	+	–	+	–	–	–	–	+		–		+	+	
IARC Group 2A																
Acrylamide	+	+	+	+							–	–	–	–		+
PCBs	+	–	+	–	+		+	+	+	+		–	+		+	
Model Carcinogen																
Diethylnitrosourea	+	+	–	–	–		–	–			–	–	–	–		
Non-Carcinogen																
Toluene			+												+	
Acetaminophen	–	–	+	+	+			+		+	+		+		–	

[a] Blank entry indicates no information, equivocal or conflicting information; +, evidence for; –, evidence against. Classification requires scientific judgment; differences of scientific opinion about individual entries were noted in the literature.

provide no guidance for integrating these separate assessments. This approach fails to recognize that recent research shows that for most human cancers there is not one key or unique event.

Recent research has also highlighted the importance of epigenetic changes in cancer. These epigenetic alterations encompass a variety of effects such as altered CpG island methylation, direct and indirect modification of histones and alterations in non-coding RNA expression. It is increasingly recognized that altered methylation in key regulatory genes can be an early and prominent event in human carcinogenesis (see [30,31]). Alterations in non-coding genes can also contribute to cancer pathogenesis. Non-coding microRNAs can silence cognate target genes and have been implicated in the regulation of a variety of cellular processes, including apoptosis and hematopoietic differentiation. Some microRNAs can function either as oncogenes or tumor suppressors, controlling functions such as tumor invasion and metastasis [32]. Expression profiling analyses have revealed characteristic microRNA signatures in certain human cancers [33]. Environmental contaminants can affect multiple types of epigenetic changes (for reviews see [34,35]). In addition to the more immediate deleterious effects, epigenetic effects of environmental exposures (especially during critical developmental windows) are important determinants of differential susceptibility to disease pathologies. For example, covalent histone modifications are a key determinant of responses to DNA damage such as double-stranded breaks [36]. Epigenetic changes, including the examples described above, therefore cannot be ignored in any current understanding of how chemicals cause cancer in humans.

In summary, cancer in humans is far too complex a long-term process to conceptualize in terms of one simple mode of action and arises from multiple genetic and epigenetic changes, many of which are difficult to measure *in vivo*.

7. Impact of recent knowledge on dose–response assessment

Significant effort has been devoted to trying to distinguish between modes of action for cancer denoted as "genotoxic" and those denoted as "non-genotoxic." The distinction has received considerable attention because the point of view has been advanced that "genotoxic" or "mutagenic" agents would have a dose–response curve that is linear in the low dose region and without a threshold, while "non-genotoxic" agents would have a dose response curve that has a threshold. Few data from large, diverse human populations exposed to carcinogens are available to discern the general nature of the dose–response relationship for these two classes of compounds. Indeed these assumptions have not been scientifically established, and both theoretical considerations and experimental data refute them.

For example, the concept that mechanisms inform the shape of the dose–response curve at low doses has been challenged by several experimental studies including the recent demonstrations that the dose–response curves for the *HPRT* assay for gene mutations shows a non-linear or apparent threshold response for some (MMS and EMS) but not all (MNU or ENU) mutagens [37,38]. On the other hand, no threshold has been demonstrated for diverse effects including non-genotoxic effects, such as receptor binding [39] and clastogenesis (chromosome breakage leading to micronucleus formation) from mitomycin C and diepoxybutane [40]. DNA adduct formation following exposure to benzene and other chemicals in rodents also lacks an apparent threshold [41–44].

Theoretical considerations have been put forward to suggest that genotoxic or mutagenic carcinogens could have a threshold at the single cell level. DNA is one of only a few biomolecules known to be repaired, rather than replaced, when damaged. All forms of DNA damage from mismatched base-pairs to double strand breaks can be repaired. In fact, DNA repair involves hundreds of proteins, with approximately 220 of 4279 *E. coli* genes (>5%) being involved in DNA replication and repair. Thus, it has been theorized that a level of exposure could exist below which DNA repair efficiently removes all deleterious DNA damage; however, the existence of a such a threshold is challenging if not impossible to demonstrate experimentally because it could occur below the limits of detection for DNA damage assays. On the other hand, the proposal that non-genotoxic chemicals such as the dioxins that act through receptors would always exhibit threshold behavior has also been challenged on theoretical grounds. Fifteen years ago Lucier et al. stated that the dose–response curve would in fact be linear in the low-dose region because the "occupancy of one receptor would produce a response, although it is unlikely that this response could be detected" experimentally [39].

Several other investigators have also pointed out that human variability and background exposure tend to broaden and increase the linearity of the dose–response curve [45–47]. As Lutz stated in 1999, "The human population is very heterogeneous with respect to both genetic and life-style factors that modulate the process of tumor formation. Therefore, individuals are expected to show widely variable susceptibility to carcinogenic factors, and the dose–response curve is in fact a reflection of the tolerance distribution. Each modulating factor divides the population up into subpopulations of different susceptibility so that nonlinearities that could be present in a homogeneous population are flattened out. A linear extrapolation of a human cancer risk to low dose might therefore be appropriate under certain conditions even if the dose–response curve in animals has a strongly sigmoidal (non-linear) shape." [48].

Hattis [49] was one of the first to suggest that, "Better definition of the sources and magnitude of variability in susceptibility in the human population is a central issue for making more quantitative estimates of both cancer and non-cancer risks from occupational and environmental exposures". Recently, Lutz and Gaylor proposed that because of advances in molecular epidemiology individual risk factors can be assessed today [50]. Indeed, many epidemiological studies have examined susceptibility to chemical exposures using the candidate gene approach (*e.g.*, [51,52]). However, with the advent of whole genome scans, molecular epidemiology studies could identify all the major genetic factors contributing to toxicological risk from exposure to a single chemical or to chemical mixtures [53,54]. The extent of susceptibility contributed by genetic variability can also be informed by other novel approaches, such as genetic analysis of resistant and sensitive isogenic mouse strains, parallel genetic analysis of yeast and human deletion mutants [55,56] and generation of small hairpin RNA libraries covering the mouse and human genome [57,58].

In summary, several concepts are pertinent to the consideration of the shape of the dose–response curve at low doses in the human population. It may not be possible to conclusively discern the presence or absence of thresholds for *in vitro* or *in vivo* effects in the low dose region, at which the magnitude of effect may be at or near the limit of detection or measurability. In some cases, the data supporting apparent thresholds do not statistically preclude the hypothesis of low-dose linearity. This was recently demonstrated using Bayesian statistical analysis of the dose–response data for induction of foci of hepatocytes expressing glutathione-S-transferase placental form by the carcinogen 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline [59]. Lutz et al. [45] noted several reasons for which apparently thresholded non-linear curves can result spuriously, including random sampling variation or small differences in individual susceptibility. Also, a linear dose–response curve that is evident on a linear scale is well known to exhibit an apparent threshold when the dose (*x*-axis) is

represented on a logarithmic scale. Moreover, empirical data are unavailable to inform or update statistical approaches for extrapolating to the diverse human population from true threshold or non-threshold responses *in vitro* or in rodent bioassays, even if discernable with statistical confidence. The human population cancer dose–response curve reflects multiple mechanistic effects and outcomes, including cancers arising from different scenarios of exposure (*e.g.*, in adulthood from lifetime exposures as well as in childhood or in later life following single or more limited exposures during vulnerable windows of susceptibility). Importantly, both mutagenic as well as non-mutagenic compounds are known to exhibit lifestage susceptibility [60]. Given the fact that these and other aspects of human variation flatten the dose–response curve in human populations, and the lack of scientific evidence for making clear distinctions between extrapolation methods for genotoxic and non-genotoxic carcinogens, one wonders why so much effort has been focused on separating these types of carcinogens. It is especially puzzling when one considers that carcinogens that are thought to act through receptors such as estrogens and dioxins, while commonly assumed to be non-genotoxic, enhance cell division, disrupt the mitotic spindle, or inhibit apoptosis, thereby increasing the possibility of mutations. Thus, separating carcinogens into mutagenic (genotoxic) and non-mutagenic modes of action can be an artificial dichotomy that may have little or no impact on dose–response assessment.

8. Need for new methods of chemical characterization

National and international governments and organizations recognize the need for improved data to characterize the hazards of chemicals. As previously noted, the EU has adopted a new regulatory paradigm that requires the assessment of chemicals in commerce and any proposed for use [61]. Testing will be staged by volume of production. The Canadian government is engaged in an effort to categorize substances in use and to better manage those of highest risk [62]. While the US federal government has not undertaken new regulatory approaches in this area, it has recognized the importance of acquiring data for assessments and established a voluntary program to obtain data for high production volume chemicals. The Government Accountability Office concluded that amendments are needed to the 1976 Toxic Substances Control Act (TSCA) to achieve a scientifically credible program for testing and characterization of chemicals [63]. It was noted that the US EPA has used its TSCA authorities to require testing of "fewer than 200 of the 62,000 chemicals in commerce", and had issued regulations to ban or limit production of only five existing chemicals or groups of chemicals [63]. A recent review of 128 high production volume chemicals showed that 60% were rodent carcinogens [64].

In addition to testing and risk characterization of many more chemicals, there is also a need to characterize a wider array of hazard traits. To achieve this would likely require development of new methods that go beyond the current testing methods developed decades ago. Faster methods providing qualitative and quantitative approaches for better characterization of hazard and risk are needed. Such tests and approaches should aim to provide comprehensive information for multiple audiences, including the regulatory bodies and regulated communities as well as the public at large. In addition, the study of molecular endpoints in humans exposed at low levels may be able to provide empirical data necessary to clarify the shape of the population dose–response relationship. Below we address the predictive potential of animal and *in vitro* toxicogenomic and molecular epidemiology studies.

9. Using toxicogenomics and molecular epidemiology to predict chemical carcinogenicity

9.1. Toxicogenomics studies to predict carcinogenicity

Toxicogenomics and statistical classification methods have progressed such that it may be possible to use them in acute or subchronic studies to predict carcinogenicity. Several research groups have recently identified cancer-relevant gene sets that appear to discriminate carcinogens vs. non-carcinogenic compounds. In general, the strategy involves selecting a training set of known carcinogenic and non-carcinogenic compounds to which to expose rodents *in vivo* or cultured cells *in vitro* from 1 to 90 days. Toxicogenomic studies are performed in target organs or cells, and statistical classification approaches are used to identify genes that discriminate possible outcome categories. The training set genes are then used in classifying untested chemicals.

Four recent *in vitro* and *in vivo* studies using a variety of microarray platforms illustrate the utility of the approach. In the first study, microarray analysis was performed following 3-day exposure of MH1C1 rat hepatoma cells to non-toxic concentrations of 17 known hepatocarcinogens, 8 carcinogens at other sites, or 14 non-carcinogens [65]. Sets of genes were identified that could differentiate hepatocarcinogens from non-carcinogens, and all carcinogens from non-carcinogens. The best differentiation was achieved using 207 genes, but a smaller set of 26 genes afforded a concordance of 84.6% and correct prediction of carcinogenicity of 88.9%. In the second study, hepatic gene expression profiling following 28-day repeat gavage exposure of groups of 4 male F-344 rats was used to distinguish carcinogenic compounds from their non-carcinogenic isomers (*e.g.*, 2-acetylaminofluorene from 4-acetylaminofluorene) [66]. The study applied hierarchical clustering analysis using sets of 54 or 28 genes, and was able to discriminate between three of the four carcinogen/non-carcinogen isomer pairs examined. In the third study, male Sprague–Dawley rats were exposed for 1 day to a high dose of 24 non-genotoxic carcinogens or 28 non-carcinogens [67]. Microarray analysis revealed a 6-gene signature of non-genotoxic carcinogens that was 88.5% accurate in classifying the non-genotoxic carcinogens. The genes identified (nuclear transport factor 2, progesterone receptor membrane component 1, liver uridine diphosphate glucuronyltransferase (phenobarbital-inducible form), metallothionein 1A, suppressor of lin-12 homolog, Sel1h, and methionine adenosyltransferase 1, alpha) are not coordinately regulated. A fourth study exposed female B6C3F1 mice to 7 lung carcinogens and 6 non-carcinogens for 13 weeks via gavage or feed [68]. The lung carcinogens were diverse in terms of chemical structure, genotoxicity, and potential modes of action. Microarray analysis of right lung lobes identified six genes whose expression identified the lung carcinogens with 93.9% accuracy, 95.2% sensitivity and 91.8% specificity. The six genes identified comprised primarily endogenous and xenobiotic metabolism enzymes as well as a gene encoding a growth factor receptor involved in lung development.

Furthermore, distinct cellular pathways were affected in rat livers treated with genotoxic and non-genotoxic carcinogens [69]. In this study, DNA damage response and the activation of proliferative and survival signaling was characteristic to genotoxic carcinogens, whereas non-genotoxic carcinogens featured mainly oxidative stress and effects on cell cycle including signs of regenerative response. The multifactorial nature of observed changes provide further evidence that a combination of pathway-associated gene expression profiles that provide insights into underlying mechanisms might be advantageous for prediction of chemical carcinogenesis. Together, these studies demonstrate that toxicogenomics is becoming robust and able, using statistical

classification methods and pathway analysis tools, to discriminate gene expression profiles of carcinogens acting by diverse modes of action from those of non-carcinogens.

9.2. In vitro testing for carcinogenicity—moving beyond batteries

Responses to a single or multiple chemicals are multidimensional, with deleterious effects evident at genetic (*e.g.*, DNA damage, genome instability), epigenetic (*e.g.*, DNA methylation alterations) and signaling (*e.g.*, changes in DNA damage response, apoptosis, proliferation, pathways) levels. The genetic toxicology battery identifies changes at one of these levels, focusing on endpoints such as mutations, chromosome damage, DNA damage (*e.g.*, Comet assay, DNA adducts), cell transformation, and genome instability. Although the battery has been widely implemented, it does not detect carcinogens that act primarily by epigenetic or signaling perturbations. Additional methods should be explored to address these deficiencies. One possibility that affords high genomic sequence homology to humans as well as high throughput potential is a DNA deletion recombination assay in yeast. This test was recently shown to identify a variety of carcinogens, including those testing both negative and positive in the standard *Salmonella* mutation assay (reviewed in [70]). A second avenue to pursue is toxicogenomics/systems approaches that detect pathway-level changes, providing opportunities for evaluating the relevance of genotoxicity findings to humans, thereby informing risk assessment [71]. In human TK6 cells, gene expression signatures have been shown to discriminate between four broad general mechanisms of stress agents, including non-DNA-damaging stresses (heat shock, osmotic shock, and 12-O-tetradecanoylphorbol 13-acetate), oxidative stress (arsenite and hydrogen peroxide), ionizing radiation, and variety of DNA-damaging agents (ultraviolet radiation, methyl methanesulfonate, adriamycin, camptothecin, and cis-Platinum(II)diammine dichloride) [72]. Toxicogenomic analysis of the genotoxic stress response provides one approach that could serve to characterize stress agents including non-covalent DNA interacting agents [73]. A recent toxicogenomic analysis identified a gene expression signature in yeast that could differentiate genotoxic from cytotoxic stress [74]. Due to the common network of genes for DNA-reactive agents, this pathway data should be able to be translated from cell to organ to animal to human. Moreover, gene networks characterizing the cellular responses to exposure can address the multidimensional mechanisms of action. Thus, these mechanistic assays can help to address several challenges as a bridge between screening tests and carcinogenesis bioassays by providing insights into mechanisms most relevant to evaluating human risk. The approach reflects cancer biology rather than phenomena such as broken chromosomes. In addition to providing context for the current genotoxicity paradigm, they can also be used as the basis for new assays and testing paradigms to explore relevance and risk. Finally, knowledge of pathways can also inform the selection of appropriate molecules as biomarkers that can be used in clinical trials or studies of human environmental exposures.

9.3. Using biomarkers of cancer risk in molecular epidemiology

Human biomarker studies could potentially be used to identify possible carcinogens as well as susceptibility factors. A flowchart in Fig. 1 illustrates how cytogenetic changes (type and level), and gene expression and epigenetic profiles could be used to determine the carcinogenic status of a chemical by comparing these endpoints to those of known carcinogens. Perhaps the most useful biomarker in such a classification scheme is chromosome aberrations (CA), the only truly validated biomarker of future cancer risk in humans. Increased CA levels are biomarkers of early biological effect in high-risk populations exposed to carcinogenic chemicals and multiple studies show they are predictive of increased future cancer risk in humans [75–79]. Recently, micronuclei (MN) have also been shown to be predictive of overall future cancer risk in humans [76] but further studies are needed to confirm this. According to our schematic, agents that elevate CA in the peripheral blood cells of humans could be considered possible carcinogens (equivalent to Group 2B under IARC classification) (Fig. 1). This cytogenetic approach covers at least two of the multiple key events listed in Table 3. For other biological events, toxicogenomic or epigenetic approaches should be alternatively considered.

In the toxicogenomic approach, gene expression profiles associated with the chemicals under evaluation would be compared to those of known carcinogens (Fig. 1). Microarray studies using peripheral blood (*e.g.*, lymphocytes) or target tissues (*e.g.*, liver) have illustrated that fewer than 1–10% of genes screened are differentially expressed in populations exposed to carcinogens acting via a diversity of mechanisms including benzene [80], dioxin [81], arsenic [82–84], welding fumes [85], or metal fumes [86]. A recent review by Sen et al. [87] supports the utility of microarray studies for identifying mechanisms and biomarkers of exposure to complex mixtures (*e.g.*, cigarette smoke). These data indicate the potential of gene signatures associated with carcinogen exposures to be validated as predictive cancer biomarkers. Also in the toxicogenomic approach, analyses of specific pathways or sets of genes known to be involved in cancer could be conducted (Fig. 1). Potential candidates include the

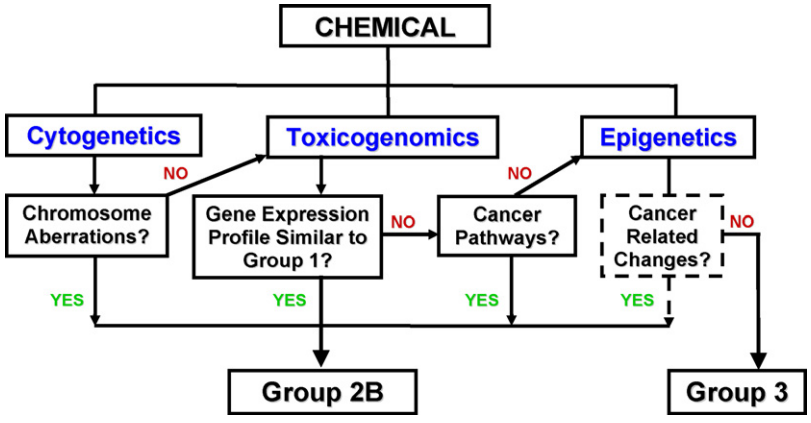

Fig. 1. Chemical evaluation in human molecular epidemiology studies.

set of genes altered in arsenic-induced skin lesions [82], the AhR pathway for dioxins and polycyclic aromatic hydrocarbons, the DNA damage response, and aneuploidy-associated genes [28]. This would allow multiple mechanisms or modes of action to be examined. Lastly, comparable epigenetic approaches, including profiling of DNA methylation, histone modifications and microRNAs [9] also hold promise. However, the genome-wide methodology (*e.g.*, for DNA methylation and histone modifications) has only recently become available and is more technically challenging than toxicogenomics. Consequently, data from exposed populations are lacking.

Our approach proposes that cytogenetic (*e.g.*, CA, MN), toxicogenomic (profile comparisons, cancer pathways) and epigenetic profiles of known carcinogens (*e.g.*, IARC Group 1 compounds) could be used to develop a paradigm to classify compounds as possible human carcinogens (IARC Group 2B). As well as classification of compounds for hazard identification, the relationships between dose and response at low levels of exposure could also be explored by applying these methods. Finally, cataloguing an increased number of chemicals according to these endpoints will further our understanding of the mechanisms underlying carcinogenesis.

10. Conclusions and future directions

Scientific knowledge of the potential health significance of chemical exposures is increasing at a rapid rate, but the approaches used to predict whether exposure to chemicals increases risk of carcinogenicity have not been substantially updated in more than two decades. This highlights the need to generate new ideas and strategies to predict and assess the carcinogenic risks of environmental chemicals. Investment in research and development of methods is also motivated by the paucity of data on most chemicals used in commerce. Improved screening and predictive methods amenable to high throughput application should be developed. In addition, approaches to address the challenges of multiple, simultaneous exposures are required.

It is increasingly evident that multiple biological alterations or sets of different perturbations are necessary to convert a normal cell to a transformed cell and ultimately a tumor [88]. Chemicals appear to impact this complex process in multiple ways. Rather than relying on a simple translation of binary (+/−) response profiles, future approaches should reflect an appreciation of the genomic biology of chemical carcinogenesis. Proof-of-concept studies using advances in toxicogenomic and systems biology approaches have provided an initial demonstration of the utility of these assays as predictive tools [65–69,71,72]. However, further exploratory research as well as validation efforts are needed.

A broad consideration of how chemicals change biology may help to foster novel approaches to identify likely carcinogens. Characterization of the multiple mechanisms of known human carcinogens, an effort to be undertaken by IARC, will help to identify a profile of mechanistic effects predictive of carcinogenic risk. This, in turn, will aid development of novel predictive *in vitro* and *in vivo* assays to evaluate the carcinogenic potential of agents to which humans have not been exposed (*e.g.*, those being developed for pharmaceutical applications). In addition, it will encourage the development of biomarkers that can be directly studied in human populations exposed to carcinogenic compounds. Especially in light of the reduction in number of animal bioassays being performed, approaches for categorizing chemicals as to their human carcinogenic risk based on the results of such assays are needed. This includes *in vitro* and laboratory animal assays to predict human risk, as well biomarker-based approaches to assess risk and exposure in the exposed human population. Moreover, research characterizing the multiple mechanisms of chemical carcinogenesis can help to identify susceptibilities, clarify human dose–response relationships and, accordingly, lead to improved scientifically based public health decisions [89]. Decision-making frameworks must be flexible enough to accommodate advances in the mechanistic understanding of chemical carcinogenesis, as well as the data from novel approaches that aim to address these scientific developments.

Conflicts of interest

The authors declare no conflicts of interest.

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