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Forum

FORUM

A Collaborative Initiative to Establish Genomic Biomarkers for Assessing Tumorigenic Potential to Reduce Reliance on Conventional Rodent Carcinogenicity Studies

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ABSTRACT

There is growing recognition across broad sectors of the scientific community that use of genomic biomarkers has the potential to reduce the need for conventional rodent carcinogenicity studies of industrial chemicals, agrochemicals, and pharmaceuticals through a weight-of-evidence approach. These biomarkers fall into 2 major categories: (1) sets of gene transcripts that can identify distinct tumorigenic mechanisms of action; and (2) cancer driver gene mutations indicative of rapidly expanding growth-advantaged clonal cell populations. This call-to-action article describes a collaborative approach launched to develop and qualify biomarker gene expression panels that measure widely accepted molecular pathways linked to tumorigenesis and their activation levels to predict tumorigenic doses of chemicals from short-term exposures. Growing evidence suggests that application of such biomarker panels in short-term exposure rodent studies can identify both tumorigenic hazard and tumorigenic activation levels for chemical-induced carcinogenicity. In the future, this approach will be expanded to include methodologies examining mutations in key cancer driver gene mutation hotspots as biomarkers of both genotoxic and nongenotoxic chemical tumor risk. Analytical, technical, and biological validation studies of these complementary genomic tools are being undertaken by multisector and multidisciplinary collaborative teams within the Health and Environmental Sciences Institute. Success from these efforts will facilitate the transition from current heavy reliance on conventional 2-year rodent carcinogenicity studies to more rapid animal- and resource-sparing approaches for mechanism-based carcinogenicity evaluation supporting internal and regulatory decision-making.

Key words: cancer; risk assessment; toxicogenomics; biomarkers; adverse outcome pathways; error-corrected sequencing.

Most global regulatory safety assessment standards for agrochemicals and pharmaceuticals require the rodent 2-year bioassay for evaluating the carcinogenic potential of a new chemical entity. However, assessing the carcinogenic potential of a single chemical requires considerable resources: the low-end estimate is 3 years and 600 animals per species at a cost of approximately $2–4M USD. Two-species carcinogenicity testing is routinely conducted for small molecule pharmaceuticals as defined under International Council on Harmonization (ICH) S1 Guidelines (European Medicines Agency, 1996; ICH, 1995). Similarly, global regulatory requirements dictate carcinogenicity testing in 2 rodent species for all agrochemical crop protection substances. Although the 2-year rodent cancer bioassay has successfully identified both human carcinogens and those that are rodent-specific when considering mechanism, exposure, and metabolism (Tomatis *et al.*, 1989; Wilbourn *et al.*, 1986), compelling drivers to move away from automatic application are growing (Goodman, 2018). For example, among human pharmaceuticals associated with development of tumors in 2-year rodent studies, several receive labels indicating that the rat tumors occur through mechanisms that are of questionable human relevance or may likely be considered human irrelevant (Alden *et al.*, 2011; Friedrich and Olejniczak, 2011). There is increasing interest across broad sectors of industry and government to reduce reliance on these studies (National Research Council, 2007). The EPA has stated that all animal toxicity tests will eventually be phased out (Wheeler, 2019), and the European Medicines Agency (EMA) has been requiring those seeking marketing authorization to integrate the 3Rs (replacement, reduction, and refinement) in all aspects of the development of medicines (European Medicines Agency, 2021). Experience over the past 50 years from testing of pharmaceuticals, industrial chemicals, and agrochemicals in short-term tests evaluating endpoints relevant to tumor prediction has fostered growing acceptance of the fundamental concept that a 2-year rat study is not always needed to assess human-relevant carcinogenic potential.

Supported by the above studies and drivers, cross-sector scientific collaborations have formed to support evidence-based modifications of regional and/or global guidelines for carcinogenicity assessment. Formal discussions on the carcinogenicity testing scheme for pharmaceuticals were initiated through the ICH process. A weight-of-evidence (WoE)-based approach was proposed for identifying those drugs for which a 2-year study in rats would or would not add value to the assessment of human carcinogenic risk in S1B(R1) (ICH, 2021). A reporting framework to support a WoE-based assessment for agrochemicals is also being developed in a collaboration between the PETA Science Consortium International (PSCI), the agrochemical industry and governmental agencies. The framework provides support for regulatory review of a WoE assessment by structuring information for consistent data presentation to objectively assess when a waiver of rat and/or mouse carcinogenicity studies is warranted (Hilton *et al.*, 2022).

To help define the strength of the information necessary to predict the outcome of the 2-year bioassay, an ICH Expert Working Group coordinated a prospective evaluation of approximately 50 pharmaceuticals by sponsors and 5 drug regulatory agencies from around the world (ICH, 2016). In the course of this evaluation, as 2-year rat studies were completed, the results were examined against WoE assessment-based predictions submitted within 14 months after the start of the bioassay. Although the WoE assessments from Sponsors predicted study outcomes well, drug regulatory agencies did not concur with certain of the Sponsor's assessments that a 2-year rat study is not warranted to assess human carcinogenic hazard. Importantly, there was agreement by Sponsors and drug regulatory agencies that a significant number of compounds would benefit from a 2-year rodent study,

underscoring that such studies will continue to contribute significant value to human carcinogenicity assessment (ICH, 2016). The outcome of the prospective evaluation indicated that development and validation of additional tools may provide information that will facilitate assessment of human carcinogenic risk of pharmaceuticals in lieu of conducting a 2-year rat study by: (1) providing greater mechanistic understanding and insight into human relevance underlying frequently observed conventional toxicology study endpoints of concern (eg, tumors or preneoplastic events); and (2) increasing confidence in negative 6-month rat study findings with a higher evidentiary data standard that more rigorously informs absence of target related carcinogenic risk.

The Health and Environmental Sciences Institute (HESI)'s Emerging System Toxicology for the Assessment of Risk (eSTAR) Carcinogenomics Workgroup is a multistakeholder scientific collaboration. This group is exploring the hypothesis that for a large proportion of chemicals, measurements of novel genomic biomarkers in subchronic studies can contribute meaningfully to the WoE assessment supporting reductions in the need for 2-year bioassays. These biomarkers can query established mechanisms of early carcinogenic processes, can be applied to samples already collected from required shorter duration studies, and will provide greater insights to relevancy for predicting human cancer risk.

The Carcinogenomics Workgroup envisions that characterized genomic biomarkers can be applied to explain conventional study histopathologic alterations of potential carcinogenic concern including hyperplasia, hypertrophy, foci of cellular alteration, and preneoplastic lesions (Sistare *et al.*, 2011) observed in acute, subchronic, and chronic studies by demonstrating activation of molecular-initiating events (MIE) of well-accepted and commonly encountered tumorigenic modes of action. These modes of action will be considered either human relevant or rodent specific and thus human irrelevant. These biomarkers can also be applied to address hypothetical carcinogenic risk of novel pharmacological agents by querying target tissues for the early emergence of growth-advantaged clonal cell populations with mutations in known cancer driver genes. This multisector collaboration seeks to accelerate the evaluation of such tools to first strengthen internal industrial decision-making across sectors and, subsequently, establish utility in formal sponsor WoE evaluations. The expected result is development and validation of sufficiently robust information to support greater alignment for conducting fewer 2-year mouse and/or rat carcinogenicity studies while maintaining or enhancing human health protection.

In this call-to-action article, we focus initially on the utility of gene expression biomarkers as emerging tools to determine the carcinogenic potential of chemicals and include examples of their use to identify mechanism of action and tumorigenic dose levels. We also discuss the application of error-corrected sequencing to identify early clonal expansion of cells with cancer driver gene mutations. As part of the call-to-action, we encourage interested stakeholder groups to contribute data and computational methods to expedite the translation of these genomic biomarkers into regulatory testing paradigms and improve the process by which chemicals are evaluated for carcinogenic potential.

TRANSCRIPTOMIC BIOMARKERS PREDICTIVE OF TUMORIGENIC MECHANISMS OF ACTION

From Transcriptomic Profiling to Biomarkers

A variety of robust "-omics" approaches, including genomics, epigenomics, transcriptomics, proteomics, and metabolomics are available to query mechanisms underlying carcinogenic responses. Of these approaches, transcriptomics (profiling of global RNA levels) is arguably the most mature and widely used and has provided a robust body of evidence for chemical and genetic factors responsible for perturbing molecular pathways leading to tumors (Hanahan and Weinberg, 2011). Public repositories of transcriptomic data continue to grow in scale and number, providing opportunities to build, test, and incorporate transcriptomics-based molecular tools into cancer assessment strategies (Yauk *et al.*, 2019). A wide range of computational methods are available to identify differentially expressed genes that can be overlaid onto molecular networks of disease or biological pathways at the systems biology level (Rager and Fry, 2013) and used to formulate hypotheses linking exposure to pathology (Waters *et al.*, 2010). For example, a network model of gene coexpression in the rat liver built from gene expression profiling data linked to chemical-dependent and independent pathologies in rats has been used to make predictions of adverse effects upon exposure to new chemicals (Sutherland *et al.*, 2018).

The goal of the HESI eSTAR Carcinogenomics Workgroup is to extend these analyses to develop focused sets of gene expression-based biomarkers that identify specific mechanisms of action and the level of expression change in these biomarkers associated with tumorigenic outcomes. These quantitative mechanistic transcriptomic *biomarker panels* lend themselves to methodical analytical validation, performance evaluation, and a context-of-use-based qualification strategy. The biomarker panels can deliver a well-defined set of pragmatic tools to facilitate assessment of tumorigenic potential, and to assist agrochemical and pharmaceutical discovery, development, and regulation. Here, we define a transcriptomic carcinogenicity biomarker as a set of RNAs providing an accurate and quantitative measure of molecular change associated with specific transcription factors, pathways, or critical genes linked to a carcinogenic mechanism. These mechanism-focused biomarkers will be in contrast to sets of genes identified to broadly differentiate tumorigens from nontumorigens agnostic to carcinogenicity mechanism, except in some cases broadly separating genotoxic from nongenotoxic actions (Auerbach *et al.*, 2010; Ellinger-Ziegelbauer *et al.*, 2009; Fielden *et al.*, 2007, 2008; Nie *et al.*, 2006; Uehara *et al.*, 2008, 2011; Yamada *et al.*, 2013).

Leveraging the Adverse Outcome Pathway Framework to Build Comprehensive Sets of Transcriptomic Biomarkers

Organizing evidence-linking chemical exposure to tumor outcomes has been facilitated by a number of mechanistic pathway frameworks. Compiling pathway information organized by mode of action (MOA)/adverse outcome pathways (AOPs) (Ankley *et al.*, 2010; Boobis *et al.*, 2006; Edwards *et al.*, 2016; Meek *et al.*, 2014; Vinken, 2019) plays a central role in assessing the evidence for effects in animals and people. The chemical-agnostic AOP starts with the sentinel MIE followed by a series of key events (KEs) that proceed through increasing levels of biological complexity (molecular pathway, cell, organ, and individual) to an adverse outcome (AO), in this case a tumor response, to describe how toxicants exert their adverse effects (OECD, 2017). Key event relationships (KERs) describe the causal relationships between KEs qualitatively and quantitatively. The weight of evidence for each KER and the overall AOP is assessed using a set of modified Bradford Hill criteria and a quantitative understanding of the AOP is summarized (Bradford Hill, 1965; Meek *et al.*, 2014).

We postulate that an AOP-guided approach to build sets of biomarkers that query MIEs in AOPs that are known to lead to

tumors in specific tissues will be more informative for predicting cancer than seeking to identify a single convergent downstream gene set expected to be predictive of the many diverse mechanisms of tumor induction. Biomarkers accounting for diverse upstream MIEs would capture changes at early timepoints across the target cell population enhancing the predictive power of short-term studies (terminated well before tumors are observed) and would also provide mechanistic understanding for derisking and assessment of human relevance. For example, constitutive androstane receptor (CAR) altered specific gene expression in hepatocytes leads to increased cell mitogenic proliferation, then increased preneoplastic foci, leading to increased hepatocellular adenomas (AOP-Wiki, 2016a). Similar KEs leading to liver cancer occur for chemicals activating peroxisome proliferator-activated receptor α (PPARα) (AOP-Wiki, 2016b), and the aryl hydrocarbon receptor (AhR) (AOP-Wiki, 2016c) and inducing cytotoxicity (AOP-Wiki, 2017) pathways under tumorigenic exposure conditions. Further, identification of genes involved in MIEs and KEs *in vivo* could support more biologically relevant *in vitro* screening paradigms for predicting AOPs (Ring *et al.*, 2021). It should be noted that there are parallel efforts to use 10 key characteristics of carcinogens (KCC) to identify potential carcinogens in *in vitro* screening or short-term animal studies (Guyton *et al.*, 2018). This approach differs from an AOP-based approach in that KCC are not necessarily MIEs or KEs in well-defined AOPs and the KCCs can be used in a weight of evidence approach but have not demonstrated accurate prediction (Becker *et al.*, 2017). Our efforts seek to readily identify mechanisms and tumorigenic dose thresholds as well. The KCC weight of evidence-based approach could be tested for the chemicals that are identified as liver tumorigens using the biomarkers.

A central premise of the AOP concept is that while MIEs/KEs are required at a qualitative level, an MIE and downstream KEs must be activated to a sufficient level and duration to cause an AO. Progression through an AOP depends upon a level of disruption that goes beyond that causing normal adaptation in order to initiate the downstream KEs (Conolly *et al.*, 2017). This premise has generated interest in the computational derivation of quantitative effect levels, or "molecular tipping points," that can be used as tools for adversity determinations using shorter-term data (Hill *et al.*, 2017; Julien *et al.*, 2009; Knudsen *et al.*, 2015). The challenge is not only how to identify those levels, taking into consideration both dose and duration, but also to understand how best to apply the tumorigenic threshold levels for cancer prediction, especially considering that global gene expression changes are measured through the use of different platforms and different durations of exposure.

Rat Liver Transcriptomic Biomarkers Identify the AOP Through Which a Chemical Causes Tumors

Below we introduce and summarize some of the scientific background supporting the proposed strategy for selection of MIE-based *in vivo* transcriptomic biomarkers being advanced by the Carcinogenomics Workgroup (Figure 1). The HESI collaborative strategy will: (1) centralize, quality control, and consistently process existing publicly available transcriptomic data derived from several global profiling platforms (microarrays, RNA-Seq, targeted RNA-Seq); (2) adjudicate the carefully selected prototype chemicals activating each MIE; (3) identify training and test data sets; (4) align on transcriptomic biomarker optimization approaches for each MIE; (5) identify the consensus panel of RNAs for each biomarker; (6) identify activation levels that determine MIE activation; (7) identify activation levels associated with tumorigenicity for each biomarker; (8) define each transcriptomic biomarker's strengths and limitations; and (9) rigorously test and publicly disseminate performance metrics justifying applications in specific contexts of use and consensus interpretations of data from *in vivo* rat studies.

Our initial focus will be on identifying biomarkers of rat liver tumors for a number of reasons. In conventional pharmaceutical chronic rat toxicology studies, the liver is the most frequent target for neoplasia (Sistare *et al.*, 2011; Van Oosterhout *et al.*, 1997). The liver, along with being a first-pass organ for the oral route of exposure, is also one of the most common tumor sites in bioassays of industrial chemicals and agrochemicals (Heusinkveld *et al.*, 2020; Hill *et al.*, 2017). The extensive set of xenobiotic receptors in liver can serve as transcriptional sensors and sentinels of activity of a chemical that may or may not be tumorigenic in rat liver but would be predicted to be tumorigenic in distant tissues, such as the thyroid or gonads (Foster *et al.*, 2021; Klaunig *et al.*, 2003; Murphy and Korach, 2006; Ohara *et al.*, 2018; Sistare *et al.*, 2011; Vansell and Klaassen, 2002). Liver transcriptomics can inform if a compound with direct or indirect (anti)estrogenic (Singhal *et al.*, 2009; Ståhlberg *et al.*, 2005) or (anti)androgenic activity (Goetz and Dix, 2009; Klaunig *et al.*, 2003) may cause tumors in sex organs (Coulson *et al.*, 2003). The public availability of an extensive amount of microarray and RNA-Seq data generated from the livers of chemically treated rats support the rat liver as the priority tissue for building transcriptomic biomarkers of established MIEs and KEs associated with carcinogenicity (Bushel *et al.*, 2018; Sequencing Quality Control Consortium, 2014; Wang *et al.*, 2014; Yeakley *et al.*, 2017). The MicroArray/Sequencing Quality Control (MAQC/SEQC) project led by the U.S. Food and Drug Administration (FDA) and others have demonstrated that the overall agreement of transcriptomic signatures is highly reproducible across platforms for rat liver transcriptomics from toxicological studies (Wang *et al.*, 2014; Xu *et al.*, 2016). It is anticipated that our initial approach will serve as a template for future identification and validation of transcriptomic biomarkers that could inform carcinogenic potential in additional, nonliver organs. Future efforts could be expanded beyond the liver to include, for example, urinary bladder and thyroid. Although these tissues are not in the top 3 most frequent human sites in either sex, they are frequent sites in rat 2-year bioassays.

To construct and characterize such predictive mechanistic biomarkers, the Carcinogenomics Workgroup will be guided by published studies describing a growing number of gene expression biomarkers shown to be useful in a variety of chemical evaluation contexts. Mouse liver biomarkers were previously built to predict some of the same MIEs outlined in Figure 2. These efforts capitalized on available microarray data from chemically treated wild-type and transcription factor-null mice allowing for the identification of well-defined mechanistic gene sets (Corton, 2019; Oshida *et al.*, 2015a–c, 2016a,b; Rooney *et al.*, 2018b, 2019). These biomarkers have been applied to sets of chemicals to identify the most likely AOP responsible for rodent liver tumors (Peffer *et al.*, 2018; Rooney *et al.*, 2017) or to understand the relationships between exposure and hazard (Rosen *et al.*, 2017). In response to the growing emphasis on tiered screening of chemicals using high-throughput transcriptomics in human cell lines (Harrill *et al.*, 2021; Thomas *et al.*, 2019), a number of groups have constructed biomarkers that identify important molecular targets underpinning *in vivo* toxicity including estrogen receptor (ER) activation (Ryan *et al.*, 2016), androgen receptor activation (Rooney *et al.*, 2018c), histone deacetylase inhibition (Cho *et al.*, 2021), stress factor induction (Cervantes and Corton, 2021; Jackson *et al.*, 2020; Rooney *et al.*,

Figure 1. The structure of the HESI eSTAR Carcinogenomics Project. The Carcinogenomics Workgroup is made up of separate teams including the Mechanisms of Carcinogenicity and Compound Classification Team, the Data Compilation Team, and the Gene Signature Development Team. The major activities of each team are listed. The project is coordinated by the Strategy and Outreach Leadership Team. This HESI collaboration strategy: (A) centralize, quality-control, and consistently process existing publicly available transcriptomic data derived from several global profiling platforms (microarrays, RNA-Seq, targeted RNA-Seq); (B) adjudicate the carefully selected prototype chemicals activating each MIE; (C) identify training and test data sets; (D) align on transcriptomic biomarker optimization approaches for each MIE; (E) identify the consensus panel of RNAs for each biomarker; (F) identify activation levels that determine MIE activation; (G) identify activation levels associated with tumorigenicity for each biomarker; (H) define each transcriptomic biomarker's strengths and limitations; and (I) rigorously test and publicly disseminate performance metrics justifying applications in specific contexts of use and consensus interpretations of data from *in vivo* rat studies.

2020) and DNA damage (TgX-DDI biomarker) (Buick *et al.*, 2020; Corton *et al.*, 2019; Li *et al.*, 2017, 2019).

Resultant signatures in many cases have been further refined to genes directly downstream of KEs of interest, adding crucial evidentiary data supporting the genes selected to comprise the biomarkers. To this end, many of the biomarker genes selected have been confirmed to be physically associated with the expected transcription factor using chromatin immunoprecipitation-Seq experiments (Podtelezhnikov *et al.*, 2020; Rooney et al., 2018, 2019; Ryan *et al.*, 2016) or have the expected activity changes after the factor is knocked down *in vitro* by factor-specific siRNAs (Corton *et al.*, 2019; Rooney et al., 2018c; Ryan *et al.*, 2016). For example, the p53 dependence of genes in the TGx-DDI biomarker (Corton *et al.*, 2019; Li *et al.*, 2019), and the ER dependence of genes in the ER biomarker were confirmed (Rooney *et al.*, 2021) by comparing transcriptional responses in wild-type cells versus knock-out/knock-down cells. The Carcinogenomics Workgroup will leverage similar sets of available evidence to further validate the mechanistic basis for regulation of the identified genes, and also include wild-type versus factor-null rodents where dependence of the carcinogenic phenotype on ligand-activated transcription factors has been confirmed, complemented with a large body of published *in vitro* trans-activation, electromobility shift, and direct binding data to further support specific compound MIEs. Data from such additional experimental strategies enhance rigor and increase confidence that relevant gene sets comprising a MIE transcriptomic biomarker have been identified, and that tumorigenic compound linkages to MIEs are strong and plausible. For some transcriptomic MIE biomarkers, there is more alignment on specific RNAs than on others. Although such published demonstrations from individual laboratories exist, scientific consensus is needed on the specific RNAs to optimize both sensitivity and specificity across the full target set of MIEs, alignment is needed on setting tumorigenic activation levels for each transcriptomic MIE biomarker, and guidance is needed to establish and communicate strengths and limitations before more general and widespread use for internal decision making as well as for regulatory applications.

The approach being taken by the Carcinogenomics Workgroup is to build transcriptomic MIE biomarkers that will require gene expression profiles of reference chemicals with well-defined molecular targets adjudicated to be linked to tumor outcomes. These reference chemicals will be used to train and test predictive models, where several types of machine learning algorithms will be leveraged to evaluate the predictive power of these gene signatures and identify which models to carry forward based on overall model performance metrics. Recent studies have described building biomarkers that predict effects in the rat liver entirely from reference chemical profiles. For example, in 2 studies, a set of 6 transcriptomic biomarkers were characterized that predict the major MIEs in AOPs by which chemicals cause liver tumors in rats (Corton *et al.*, 2020a; Rooney *et al.*, 2018a). These biomarkers predict genotoxicity, cytotoxicity, and activation of AhR, CAR, ER, and PPARα. Each of the biomarkers was built using a set of gene expression profiles from the livers of rats treated with known inducers of the respective receptor, DNA damage, or cytotoxicity, and could predict the MIE of a test set of chemicals with excellent predictive accuracies (91%–97%) across a wide range of doses and times of exposure. This study thus serves as an example of an approach that can be taken to identify highly predictive gene sets.

Recent studies have used an alternative approach to identify and optimize sets of biomarker genes. Using a multivariate regression approach, genes were identified with overlapping transcriptional responses associated with the different MIEs, as well as the polytropic effects of most drugs on gene expression networks to optimize biomarker specificity (Podtelezhnikov *et al.*,

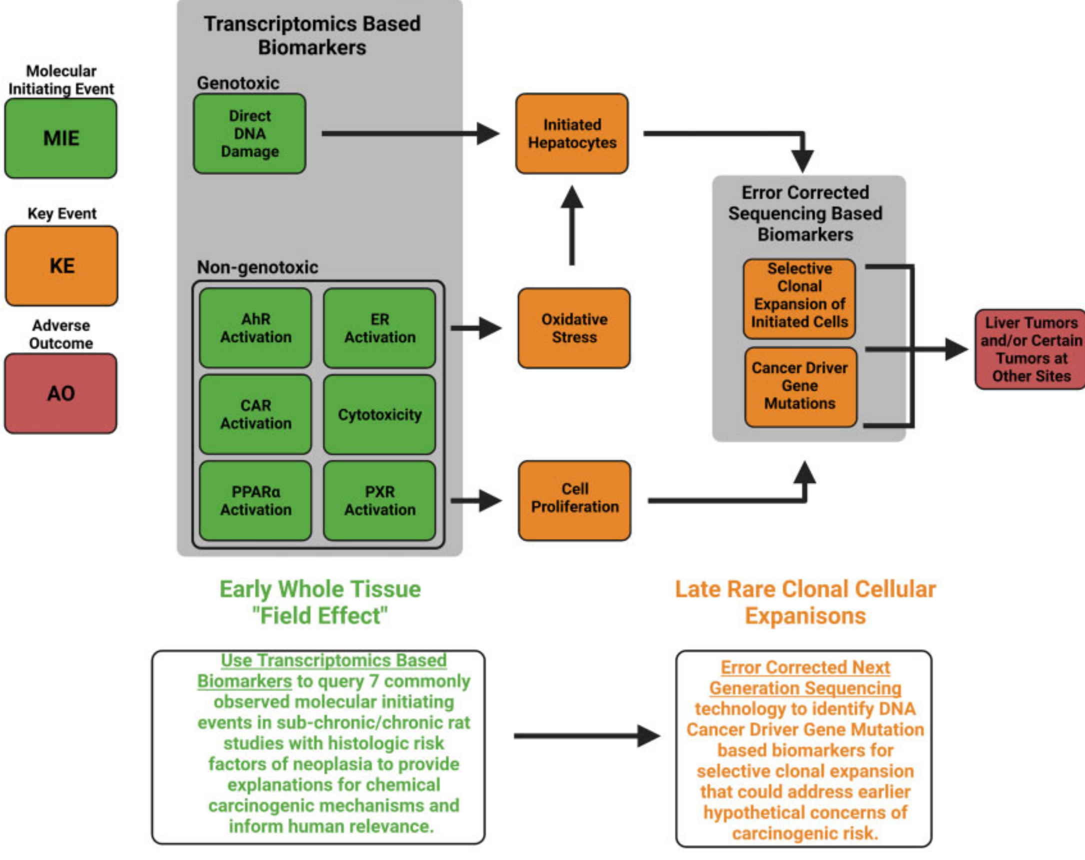


Figure 2. Strategy to reduce the reliance on the 2-year rodent bioassay to identify carcinogens. The strategy is put into the context of the molecular initiating events (MIEs) and key events (KEs) critical for induction of liver tumors in rats as an example. The overall strategy could be applied to other tissues with sufficient information about AOPs important in tumor induction. The figure outlines the MIEs (left side) and certain KEs (middle) that could be measured using genomic interrogation techniques including gene expression biomarkers and error-corrected sequencing.

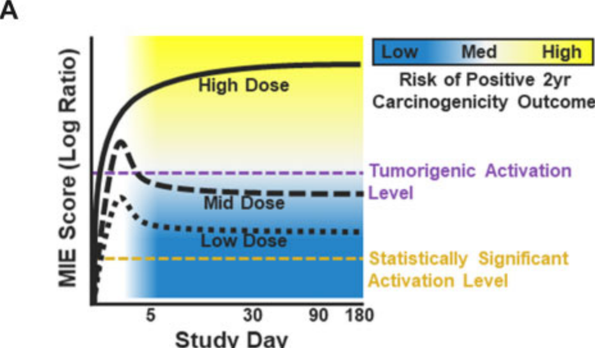
2020). Multivariate modeling using liver RNA-Seq data derived from rats exposed to a diverse reference chemical set enabled the identification and refinement of genes specifically regulated by each MIE individually. The resulting biomarkers are predictive of agonists for 5 different canonical xenobiotic receptors (AhR, CAR, Pregnane X Receptor [PXR], PPARα, ER), 3 mediators of reactive metabolite-mediated stress responses (NRF2, NRF1, p53), and activation of the innate immune response. A composite transcriptional biomarker of tissue injury and regenerative repair response was discovered by the same group to be conserved across 8 different tissues (Glaab *et al.*, 2021). These 10 biomarker sets have been deployed for routine monitoring in initial rat tolerability studies just prior to entering drug development in an integrated manner to identify drug candidate potential for activating these MIEs to trigger certain liver and other organ toxicities with strong (>90%) sensitivity and/or specificity (Glaab *et al.*, 2021; Monroe *et al.*, 2020). Application of these 10 biomarkers by the same group to 2-year rat carcinogenicity study outcome prediction has been explored with preliminary sensitivity exceeding 70% at >95% specificity among a set of approximately 60 rat liver and non-liver carcinogens and 40 non-carcinogens (Tanis *et al.*,, in preparation). The gene sets identified as biomarkers in the 2 independent studies by Corton *et al.* (2020a) and Podtelezhnikov *et al.* (2020) demonstrate a high degree of overlap. This supports the concept that multiple computational methods can converge on optimized mechanistic gene sets and provide increased confidence that the HESI workgroup will be able to identify and align on a comprehensive set of transcriptional biomarkers with high predictive accuracy for induction of MIEs associated with carcinogenicity.

Transcriptomic Biomarkers Have Identifiable Activation Levels Associated With Tumor Induction

One goal of the HESI eSTAR Carcinogenomics project is to identify activation levels of the MIEs that can distinguish between adverse and non-AOs. It will be important to identify not only statistically significant altered activity but also sufficient MIE perturbation that can propagate growth signals leading to tumors (Figure 3). Indeed, there is support in the literature that relative activation levels of the MIEs can be measured and used to predict tumorigenesis in rat livers even after short-term exposures (Hill *et al.*, 2020; Lewis *et al.*, 2020; Qin *et al.*, 2019). These studies required careful annotation of the known liver tumorigenic outcomes of each chemical-dose combination derived from 2-year bioassay data archived in a number of databases such as the Lhasa database (https://carcdb.lhasalimited.org/, last accessed April 22, 2022; based on the Carcinogenicity Potency Database). One challenge that the Carcinogenomics Workgroup faces is how to best move from tumor incidence data across chemical doses that do/do not induce tumors to establishing a convincing connection to levels of MIE activation predictive of carcinogenic outcome.

Initial studies integrating measurement of 5 gene expression biomarkers with nongenomic endpoints such as liver to body

A



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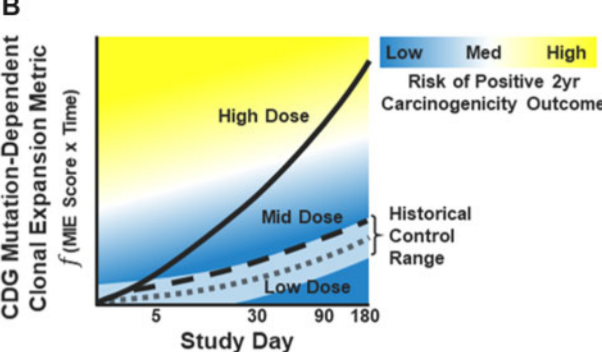

Figure 3. Relationships between chemical exposure and levels of activation of MIEs in AOPs and clonal expansion of Cancer Driver Gene mutations. A, The relationship of chemical dose to MIE signature response within the liver across time reaches a steady state with continued daily dosing. The sustained steady-state activation of a certain MIE may exceed a statistically significant change at low doses but never achieve sufficient activation to result in tumorigenesis via this MIE, resulting in no tumor risk via this MIE, and little evident MIE-associated biological effects. At the mid dose activation of this MIE may only transiently achieve sufficient activation to result in tumorigenesis and drop to levels of sustained activation at steady state resulting in no tumor outcome but significant biological effects (eg, enzyme induction, histopathology, organ weight gain). High doses result in excessive levels of sustained MIE activation exceeding the tumorigenic activation level. B, Critical Cancer Driver Gene (CDG) mutations supporting growth advantaged clonal expansion increases with time and can differentiate when MIE activation is being sustained at tumorigenic levels. Low doses of the rat liver tumorigen result in levels of clonal expansion with CDG mutations that do not exceed the upper range of variability seen among historical control values (HCV). Mid doses may exceed the upper range of HCV but not reach levels associated with drugs causing liver tumorigenesis via this MIE in two-year (2yr) rat studies. The high dose results in levels of targeted CDG mutated clonal expansion known to be caused by this MIE that clearly exceed the tumorigenic activation level.

weights and clinical chemistry associated with MIEs in liver tumor AOPs, found that a WoE approach could perform reasonably well (85%–89% predictive accuracy) to identify chemical-dose combinations that lead to liver tumors (Rooney *et al.*, 2018a). Using a more precise approach, activation levels of 6 MIEs associated with liver tumor induction had a predictive accuracy of 91%–97% depending on the activation levels used (Hill *et al.*, 2020), with low false negative rates—a highly desirable characteristic for regulatory determination of hazard. In follow-up studies from the same lab (Lewis *et al.*, 2020), an analysis of approximately 50 chemicals profiled in rat liver from an independent dataset yielded approximately 90% predictive accuracy.

The optimal number of genes constituting a specific MIE biomarker is expected to vary and requires careful deliberation and empirical support. Just 2 genes (*Cyp1a1*, *Cyp1a2*) were selected for a transcriptomic biomarker of AhR activation in rat liver (Qin *et al.*, 2019; Taylor *et al.*, 2015). The same 2-gene AhR biomarker was systematically derived by Podtelezhnikov *et al.* (2020) and used to differentiate between tumorigenic and nontumorigenic dose levels of AhR-activating chemicals. Tumorigenic activation levels of AhR biomarker activation in rat liver (Qin *et al.*, 2019) were derived that could distinguish nontumorigenic dose levels of chemicals that exhibit high-level sustained induction (eg, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]) from AhR-activating but nontumorigenic pharmaceuticals (Hu *et al.*, 2007; Jin *et al.*, 2012) that exhibit more modest and transient inductions (eg, omeprazole) at the dose levels used in published rat carcinogenicity studies. The AhR biomarker and tumorigenic activation levels were established for internal use in a WoE approach to determine whether a novel drug candidate with AhR activity would likely cause sustained high-level tumorigenic level induction of AhR when administered at dose levels destined for use in future 2-year rodent carcinogenicity registration studies (Qin *et al.*, 2019; Taylor *et al.*, 2015). In another study examining 12 genes (2 from each of the 6 biomarkers described above) (Hill *et al.*, 2020), all genes exhibited activation levels associated with liver tumorigenicity for individual genes that were similar across training and test sets of chemicals in the TG-GATES dataset. The 12 individual activation levels when used collectively resulted in high predictive accuracies for 77 chemicals (TG-GATES) or 86 chemicals (DrugMatrix) analyzed (up to 94%) (Hill *et al.*, 2020). In addition to transcriptional biomarkers, the concept of tumorigenic activation levels can be applied to liver: body weight and clinical chemistry markers that have been used to accurately identify chemical-dose pairs that would lead to a tumor outcome (Corton *et al.*, 2020b). Thus, these studies provide a high degree of confidence of the feasibility of using gene expression biomarkers to not only identify the AOP responsible for liver tumor induction but also the dose levels that would or would not lead to liver tumor induction.

Gaining Regulatory Acceptance of the Biomarkers

The long-term goal of the HESI eSTAR Carcinogenomics Workgroup is to gain regulatory acceptance of the rat liver biomarkers. The Workgroup will capitalize on experience the group has with the TGx-DDI biomarker, a transcriptomic biomarker currently under regulatory review by the FDA through the Center for Drug Evaluation and Research Biomarker Qualification Program (FDA, 2021). This 64-gene biomarker was developed to enable differentiating true positive DNA damage-inducing (DDI) agents from non-DDI irrelevant positive agents using human TK6 cells (Li *et al.*, 2015, 2017) and human liver HepaRG cells (Buick *et al.*, 2020, 2021; Corton *et al.*, 2018, 2019) and is composed mostly of genes that are under control of p53 (Corton *et al.*, 2019). Experience with the TGx-DDI biomarker by the HESI collaborative group has been invaluable in understanding the process required to build toward acceptance of any new biomarker for regulatory drug development applications, which first requires precisely defining and acquiring consensus on the need statement and regulatory context of use, followed by assessment of the benefits and risks of biomarker use (Leptak *et al.*, 2017). The level of evidence required for qualification is then determined and is based on both biological (empirical testing that the biomarker predicts the endpoint of concern, appropriate study designs, etc.) and technical studies (technical standards, independent cross-laboratory validation, etc.). These learnings will be valuable for internal business decision-making

and may be appropriate for case-by-case regulatory application, well before broad regulatory acceptance is attained.

COUPLING ERROR-CORRECTED SEQUENCING WITH TRANSCRIPTOMIC BIOMARKER ANALYSES TO ESTABLISH EARLY DNA BIOMARKERS OF NONGENOTOXIC CARCINOGENESIS

Although DNA sequencing has been used for decades to characterize the mutational landscape of tumors, its application for identifying rare *de novo* mutational events and clonal expansion of cancer driver genes has been hampered by the inherent error rates of conventional next-generation sequencing technologies. Specifically, current sequencing error rates per nucleotide are on the order of 10^{-2}–10^{-3}, whereas somatic mutation frequency is on the order of 10^{-7} (Salk *et al.*, 2018). Emerging error-corrected sequencing technologies that individually barcode double-stranded DNA molecules to dramatically reduce the technical error rates now enable the detection of extremely rare mutations with exceptional accuracy (Salk and Kennedy, 2020). One of the most promising technologies available commercially today is Duplex Sequencing, which can detect spontaneous and induced *de novo* mutations following exposure to mutagenic agents (Salk and Kennedy, 2020). Duplex Sequencing has achieved unprecedented accuracy and sensitivity, on the order of less than 1 error per 10^8 nucleotides sequenced. It offers the opportunity to accurately detect the early clonal expansion of mutations in genes that provide growth and survival advantages within rodent tissues after relatively short-term exposures prior to the formation of preneoplastic foci (Martínez-Jiménez *et al.*, 2020). These cancer driver gene mutation biomarkers could provide further early molecular evidence of a chemical tumorigenic risk when found to occur in cells of a tissue from treated animals at frequencies indicative of a growth advantaged clonal selection process, when observed in multiple animals within a dose group that exceeds background control levels, or when frequencies occur in a dose-dependent manner. Most importantly, the absence of mutations in cancer driver genes may provide assurances that hypothetical scenarios of tumor development in tissues known to express the chemical target are not in fact reasonable.

The potential of this exciting technology has been described for *in vivo* genetic toxicology, for chemical carcinogenesis research, and numerous emerging clinical applications for early cancer diagnoses (Merrick, 2019; Parsons, 2018; Valentine *et al.*, 2020). The approach would be especially impactful if shown to rapidly detect MIE-agnostic nongenotoxic tumorigens as well. One HESI Workgroup within the eSTAR Committee will be evaluating proof-of-principle experiments with a small set of nongenotoxic rodent carcinogens in the tumor accelerated rasH2-Tg mouse model, whereas another HESI Committee—the Genetic Toxicology Technical Committee—leads rigorous analytical validation and systematic exploration of applications of this technology for *in vivo* mutation detection of genotoxic chemicals (eg, Valentine *et al.*, 2020). The mutational spectra of genotoxic versus nongenotoxic carcinogens are expected to be very different (Balmain, 2020) and mutations associated with chemical MOA that do not directly induce mutations may likely more closely resemble the spectra of spontaneous tumors (McKim *et al.*, 2021). Accordingly, a recent study analyzing whole genomes of tumors from mice chronically exposed to various known or suspected human carcinogens including genotoxic and nongenotoxic agents revealed that only a subset of the carcinogens yielded mutation signatures that are distinct from tumors arising spontaneously due to age. Surprisingly, tumors from a majority of the tested carcinogens exhibited mutation signatures that are similar to the tumors arising spontaneously due to various endogenous mutagenic processes (Riva *et al.*, 2020). Interestingly, mutations in various cancer driver genes are present in all the tumors regardless of the type of exposure and were not limited to any tumor etiology. Studies are in progress to determine if the mutation spectra and the mutated cancer driver genes from rat tumors due to various carcinogen exposures also mimic the mouse tumors. Further studies are needed to correlate the genomic mutational alterations with the transcriptomic changes and determine if the mutation spectra and cancer driver genes can be linked to one or more AOPs. It is known that for some nongenotoxic MOAs, such as CAR/PXR, clonal populations harboring mutations in the cancer driver gene *beta-Catenin* are preferentially promoted (Aydinlik *et al.*, 2001; Hoenerhoff *et al.*, 2013). Opportunities would open up to better understand, for human-relevant nongenotoxic MOAs, where clonal expansion of cells in target organs (that are histologically normal) harboring mutations in one or more cancer driver genes may be detected in nontumor tissues from subchronic studies using highly sensitive technologies such as error-corrected duplex sequencing. We expect that insights will be gained about mechanisms underlying the mutational spectra and as the technology evolves and becomes less expensive, routine assessment could be used in dose response modeling to derive a point of departure.

THE HESI ESTAR COMMITTEE WILL ENSURE SCIENTIFIC RIGOR AND WILL ENCOURAGE REGULATORY ACCEPTANCE OF GENOMIC TOOLS FOR EVALUATING THE CONTEXT IN WHICH A CHEMICAL COULD EXHIBIT TUMORIGENIC POTENTIAL

The HESI eSTAR Committee provides the strategic direction, collaborative framework, and resources to facilitate industrial incorporation and regulatory acceptance of genomic tools. The Committee aims to catalyze adoption of new translational and predictive tools that guide decision-making based on mechanistic understanding of toxicological response. The committee is divided into a number of Workgroups that are tasked with various aspects of advancing applications of genomics in risk assessment. To implement the goals of this "call-to-action" article, the eSTAR Committee launched the Carcinogenomics Workgroup (Figure 1) to implement genomic strategies for possible application within the evolving WoE-based ICH and industrial and agrochemical frameworks for cancer risk assessment. Transcriptional biomarkers provide a clear example of viable opportunities for immediate use driven by proposed changes in ICH S1 carcinogenicity testing guidance for pharmaceuticals, and analogous changes evolving for evaluating industrial and agrochemicals. The availability of quantitative, predictive toxicogenomic biomarkers aligned against cancer-outcome AOPs will have many applications in assessment of tumorigenic potential in both the pharmaceutical and chemical sectors. Collaborating across >20 institutions, the Carcinogenomics Workgroup's immediate goal is to develop a set of mechanistic gene expression biomarkers for prediction of carcinogenic effects in the livers of rats to address critical testing and data gaps.

SUMMARY

When early biomarkers of MIEs associated with tumorigenic AOPs are coupled with error-corrected duplex sequencing strategies, the interpretation of the carcinogenic potential of later effects including histopathology changes would be robust and predictive. As scientific evidence expands, there will be increased confidence that lack of effects on cancer driver gene mutations can be concluded to indicate the absence of tumorigenic potential and will contribute to the carcinogenicity WoE assessment. Initially, transcriptomic and DNA mutational biomarkers are expected to inform internal decision-making and assist with explanations of outcomes of already completed rodent carcinogenicity studies. Over time, the integration of such biomarkers with proven sensitivity, specificity, and positive and negative predictivity into short-term rodent studies will reduce the need for rodent cancer bioassays in regulatory decision-making contexts in the assessment of pharmaceuticals, and industrial and agricultural chemicals.

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