The Key Characteristics of Carcinogens

CEBP FOCUS

The Key Characteristics of Carcinogens: Relationship to the Hallmarks of Cancer, Relevant Biomarkers, and Assays to Measure Them

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ABSTRACT

The key characteristics (KC) of human carcinogens provide a uniform approach to evaluating mechanistic evidence in cancer hazard identification. Refinements to the approach were requested by organizations and individuals applying the KCs. We assembled an expert committee with knowledge of carcinogenesis and experience in applying the KCs in cancer hazard identification. We leveraged this expertise and examined the literature to more clearly describe each KC, identify current and emerging assays and *in vivo* biomarkers that can be used to measure them, and make recommendations for future assay development. We found that the KCs are clearly distinct from the Hallmarks of Cancer, that interrelationships among the KCs can be leveraged to strengthen the KC approach (and an understanding of environmental carcinogenesis), and that the KC approach is applicable to the systematic evaluation of a broad range of potential cancer hazards *in vivo* and *in vitro*. We identified gaps in coverage of the KCs by current assays. Future efforts should expand the breadth, specificity, and sensitivity of validated assays and biomarkers that can measure the 10 KCs. Refinement of the KC approach will enhance and accelerate carcinogen identification, a first step in cancer prevention.

See all articles in this *CEBP Focus* section, "Environmental Carcinogenesis: Pathways to Prevention."

Introduction

Carcinogenesis is a multistep process in which normal cells are transformed into cancer cells by acquiring various properties that allow them to form tumors. These acquired properties of cancer cells that distinguish them from normal cells have been classified as a series of Hallmarks by Hanahan and Weinberg (**Fig. 1A**; ref. 1). Originally, six Hallmarks were described in 2000 (1, 2), and two enabling characteristics (genome instability and inflammation) and two emerging Hallmarks (deregulated metabolism and immune system evasion) were added in 2011 (2). By considering cancer as an accumulation of multiple Hallmarks, research has been guided to understand the origins of cancer, to identify targets for prevention and to design strategies to reverse various Hallmarks, individually as well as collectively, to treat cancer. Carcinogens (i.e., agents that induce cancer) are thought to act by inducing multiple Hallmarks in normal cells, thereby transforming them into cancer cells through a variety of mechanisms.

In 2009, Guyton and colleagues (3) described how carcinogenic chemicals act through multiple pathways, mechanisms, and/or modes-of-action to induce cancer. They identified 15 types of "key events" associated with carcinogenesis and documented how known human carcinogens, such as benzene and arsenic, can cause many of these key events (e.g., genotoxicity, immunosuppression). Similarly, Kleinstreuer and colleagues (4) described how chemicals that target multiple Hallmark processes *in vitro* are more likely to be rodent carcinogens *in vivo*. In 2012, participants at two workshops organized by the International Agency for Research on Cancer (IARC) noted that human carcinogens, while operating individually through distinct mechanisms, often share one or more characteristics related to the multiple mechanisms by which agents cause cancer.

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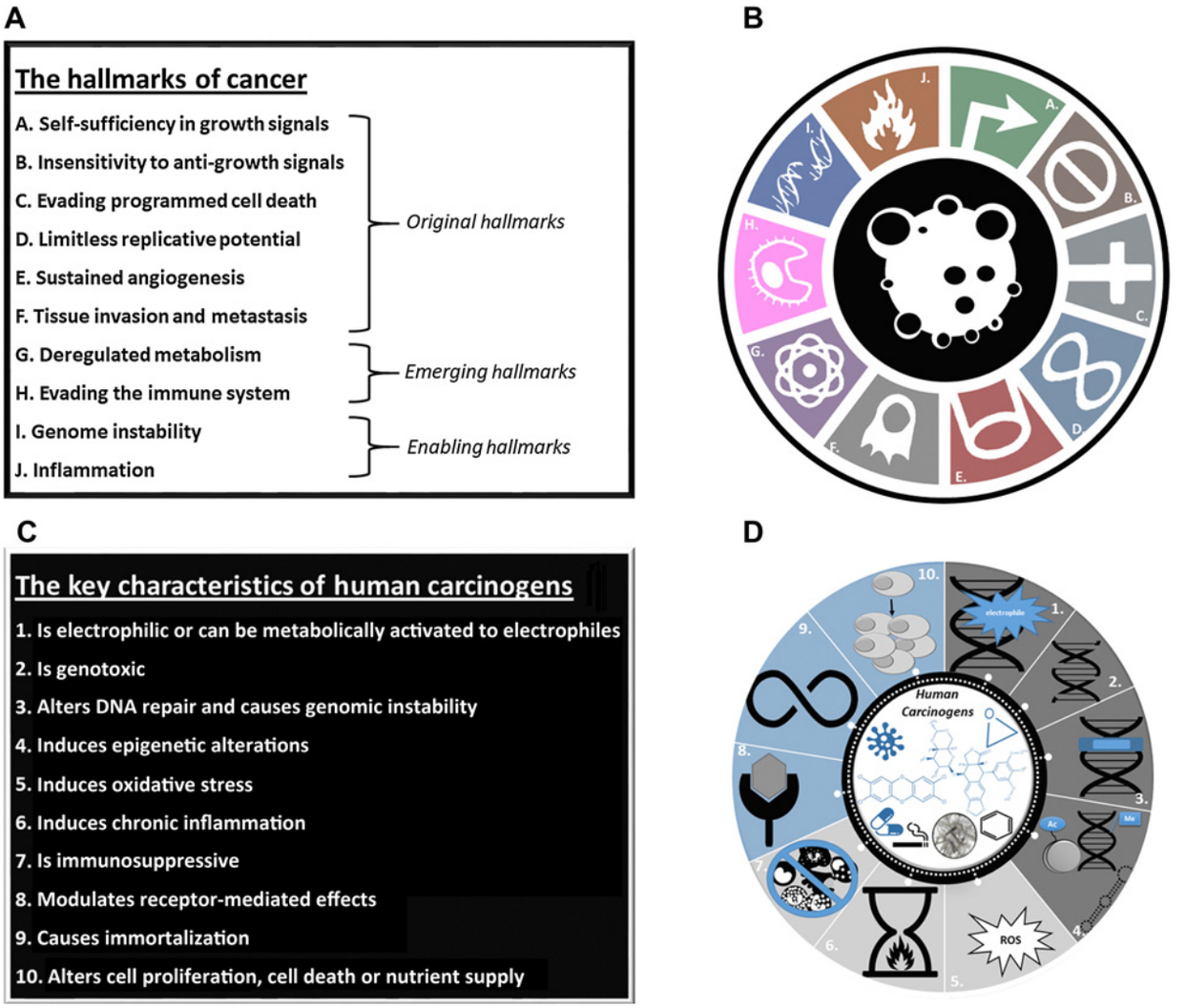

Figure 1.

The Hallmarks of cancer and the key characteristics of carcinogens. **A,** List of the Hallmarks of cancer. The Hallmarks of cancer, acquired properties of cancer cells that distinguish them from normal cells, have been described by Hanahan and Weinberg (1, 2). Six Hallmarks were described in 2000 (1), and two emerging hallmarks (deregulated metabolism and immune system evasion) and two enabling characteristics (genome instability and inflammation) were added in 2011 (2). **B,** Symbolic illustration of the Hallmarks of cancer. **C,** List of the key characteristics of human carcinogens. The 10 key characteristics of human carcinogens describe the properties of human carcinogens that induce cancer (5). Expert participants at two IARC-led workshops initiated the development of the key characteristics based on empirical observations of the chemical and biological properties associated with the human carcinogens identified by the IARC Monographs program up to and including Volume 100. **D,** Symbolic illustration of the key characteristics of human carcinogens. Reprinted with permission from (203) Guyton KZ, Rieswijk L, Wang A, Chiu W, Smith MT (2018). Key characteristics approach to carcinogenic hazard identification. Chemical Research in Toxicology. 31(12):1290–1292. Copyright (2018) American Chemical Society.

This led to the identification of 10 key characteristics (KCs) of human carcinogens (**Fig. 1C**; ref. 5). These KCs, such as "is genotoxic," "is immunosuppressive," or "modulates receptor-mediated effects," are based on empirical observations of the chemical and biological properties associated with the human carcinogens identified by the *IARC Monographs* program up to and including Volume 100. Hence, whereas the Hallmarks are the properties of cancer cells, the KCs are the properties of human carcinogens that induce cancer. Thus, while the Hallmarks describe what biology exists in a cancer, the KCs describe the actions of carcinogens that can cause those Hallmarks to become acquired.

Relationship of the key characteristics to the hallmarks of cancer

Some of the KCs produce effects analogous to the Hallmarks, for example, carcinogens that induce genome instability can produce this Hallmark in both normal and cancer cells, and those that induce immunosuppression produce an effect analogous to cancer cells evading immune destruction by suppressing immune surveillance mechanisms. However, as Stewart recently noted (6), there often is not a one-to-one relationship. Indeed, several of the KCs can produce genetic and epigenetic alterations that could cause almost all of the Hallmarks, whereas other Hallmarks, such as "reprogramming energy

metabolism," which occur in many cancer cells, have no real equivalent in the KCs. It is therefore generally not possible to identify individual relationships between specific KCs of carcinogens and a single Hallmark of cancer.

Use of the key characteristics in cancer hazard identification

Mechanistic evidence is an important contributor to hazard identification, the first step in human health risk assessment. For example, it can add biological plausibility to epidemiologic findings, thereby strengthening causal inference, and contribute to understanding the human relevance of findings in experimental animal studies. Prior to the introduction of the key characteristics approach, however, there was no widely accepted method to systematically search for and organize relevant mechanistic evidence. The KCs now provide a common basis for assembling and evaluating mechanistic evidence to support cancer hazard identification and are increasingly being used by multiple authoritative bodies including the California EPA (CalEPA) OEHHA, who sponsored this project. One important advantage of using the KCs to assemble data relevant to carcinogenic mechanisms is that an *a priori* hypothesis about a specific mechanism of action is not required. Instead, as noted by a recent National Academies report, the KCs are based on the empirically observed, common properties of human carcinogens, thus avoiding "a narrow focus on specific pathways and hypotheses" and instead "providing for a broad, holistic consideration of the mechanistic evidence" (7). The key characteristics approach therefore presents a comprehensive and inclusive approach to using mechanistic data in cancer hazard identification, in contrast to more narrow, reductionist approaches such as adverse outcome pathway and mode-of-action frameworks that focus on singular events.

IARC has applied the KCs in mechanistic data evaluations for more than 50 diverse chemicals and complex exposures since 2015 (8), and have now formally incorporated them into the January 2019 Preamble of the IARC Monographs (9). Other authoritative bodies are increasingly using the KCs, such as the National Toxicology Program's Report on Carcinogens (NTP, RoC), who used them in recent evaluations of antimony trioxide and haloacetic acids (10, 11). As the KCs of carcinogens have been applied by IARC, CalEPA OEHHA, NTP, and the U.S. Environmental Protection Agency (EPA) in their hazard identification and evidence integration efforts, several opportunities for refinement have been identified (8). For instance, some members of various IARC Monograph Working Groups and the Carcinogen Identification Committee of the State of California and several participants at the AACR conference, Environmental Carcinogenesis: Potential Pathway to Cancer Prevention, requested that they be more clearly defined and asked how they relate to the Hallmarks of Cancer. In addition, specific questions have arisen about which assays and biomarkers measure each of the KCs, for example, whether certain measures of hematotoxicity belong under KC7 (immunosuppression) or KC10 (altered cell proliferation).

Materials and Methods

With funds provided by the CalEPA OEHHA, we assembled an expert working committee consisting of academics, regulators, and scientists in the government and pharmaceutical industry, from the United States, Canada, France, Netherlands, and Japan. IARC, NTP, U.S. EPA, and CalEPA were all represented. The committee was charged with: (i) more clearly describing each KC based on their knowledge and experience in applying them in cancer hazard identification; (ii) describing the endpoints that best define each KC and the current and emerging assays and *in vivo* biomarkers that can be used to measure these endpoints; and, (iii) making recommendations for future assay development to improve how agents (e.g., chemicals, therapeutics) can be systematically evaluated for cancer hazard *in vitro* and *in vivo*. A comprehensive list of all assays that measure each of the KCs and Hallmarks to differing degrees is being compiled in a separate project, and we note that Menyhart and colleagues (12) recently cataloged the functional assays which measure most of the Hallmarks of Cancer. Here, we describe each of the KCs and document representative assays that can be used to measure them *in vitro, in vivo* in experimental animals, and as biomarkers in humans. The committee selected assays and biomarkers that are well-validated or widely used and identified emerging assays and biomarkers that could be used now or in the future (**Tables 1** and **2**).

Results

Descriptions and assays to measure the key characteristics of carcinogens

KC1: Is electrophilic or can be metabolically activated to an electrophile

Electrophiles are reactive, electron-seeking molecules capable of binding to electron-rich cellular macromolecules including DNA, RNA, lipids, and proteins, forming covalent adducts. The measurement of covalent adducts on DNA and proteins is the most common method of assessing electrophilic activity both *in vitro* (**Table 1**) and *in vivo* (**Table 2**). Electrophiles and their nucleophilic targets can be described by their strength, which can help predict their reactivity. "Hard" electrophiles, so called due to the relatively greater polarizability of their electrophilic center, are of high concern, having electron-withdrawing groups capable of binding nucleophilic N and O sites in DNA (e.g., epoxides; ref. 13). "Soft" electrophiles primarily target nucleophilic sites in proteins, such as thiol groups, rather than DNA. This can cause glutathione depletion and functional inhibition of critical proteins, such as tubulin (14).

Computational chemistry tools can be used to calculate characteristics of chemical structures that can aid in identifying hard electrophiles (15). Structural alerts for reactive organic functional groups requiring metabolic activation have also been developed (16). While *in silico* calculations can identify potential DNA-reactive electrophiles, *in vitro* approaches can provide confirmation as well as information on chemical potency and/or reactivity. The direct measurement of electrophilicity involves approaches that determine a rate constant (k) as a measure of reactivity. This can be carried out through adduct measurement of deoxyribonucleosides, although this requires a high-performance liquid chromatography method that limits throughput (17). High-throughput, *in chemico* assays are available, but these target primarily soft electrophiles (18) and there is a need for equivalent, high-throughput formats for hard electrophiles.

Biomarkers of reactive, electrophilic chemicals in humans have consisted primarily of measuring protein adducts reflecting the parent chemical structure through sampling of the readily accessible blood proteins hemoglobin and albumin (19). Various analytic techniques have been employed to measure adducts at known, susceptible amino acid residues in these proteins. Measurement of adducts on DNA is now less frequent, but new approaches can

Table 1. Representative *in silico* and *in vitro* assays to measure the key characteristics of carcinogens.

Endpoint	*In silico* or nonhuman *in vitro* assay	Human *in vitro* assay
KC1: Is electrophilic		
Electrophilic reactivity	*In silico* prediction (111)	—
Protein adducts	Fluorescence-based (MSTI) assay (18)	Fluorescence-based (MSTI) assay (18)
	Glutathione depletion assay (112)	Glutathione depletion assay (112)
	[a]Chemoproteomics (113)	[a]Chemoproteomics (113)
DNA adducts	DNA adduct measurement by HPLC (114)	DNA adduct measurement by HPLC (114)
KC2: Is genotoxic		
Mutation/single nucleotide variants	*In silico* prediction (115)	—
	Bacterial reverse mutation (Ames; OECD 471)	
	Mouse lymphoma assay (OECD 476)	Human HPRT mutation assay
	[a]Error-corrected next-generation sequencing (116)	[a]Error-corrected next-generation sequencing (116)
Structural chromosome alterations/ DNA strand breaks (clastogenicity); aneugenicity	Chromosome aberration assay (OECD 473/475)	Chromosome aberration assay (OECD 473/475)
	Micronucleus assay (OECD 487)	Micronucleus assay (OECD 487)
	Comet assay (OECD 489)	Comet assay (OECD 489)
	—	[a]TGX-DDI biomarker in human TK6 cells (117)
KC3: Alters DNA repair or causes genomic instability		
Copy number variations (duplications, deletions, amplifications, insertions)	Comparative genome hybridization (CGH, array based; ref. 118)	Comparative genome hybridization (CGH, array based; ref. 118)
	Next-generation high-throughput sequencing for somatic mutation detection (43)	Next-generation high-throughput sequencing for somatic mutation detection (43)
Inter-/intrachromosomal translocations	Spectral karyotyping (SKY), karyotyping (50)	Spectral karyotyping (SKY), karyotyping (50)
	Next-generation high-throughput sequencing for somatic mutation detection (43)	Next-generation high-throughput sequencing for somatic mutation detection (43)
Microsatellite instability	Fluorescent multiplex PCR-based method using DNA (119)	Fluorescent multiplex PCR-based method using DNA (119)
DNA repair capacity	Unscheduled DNA synthesis (120)	Unscheduled DNA synthesis (120)
	Host cell reactivation for evaluation of nucleotide excision repair, mismatch repair, base excision repair, nonhomologous end joining, homologous recombination, and methylguanine methyltransferase (121, 122)	Host cell reactivation for evaluation of nucleotide excision repair, mismatch repair, base excision repair, nonhomologous end joining, homologous recombination, and methylguanine methyltransferase (121, 122)
	Topoisomerase I and II enzymatic activity analysis using gel electrophoresis (123)	Topoisomerase I and II enzymatic activity analysis using gel electrophoresis (123)
KC4: Induces epigenetic alterations		
Global and locus-specific DNA methylation	High-performance liquid chromatography and ELISA-based methods (124)	High-performance liquid chromatography and ELISA-based methods (124)
	Enzyme activity assays for "writers, erasers, editors and readers" (125)	Enzyme activity assays for "writers, erasers, editors and readers" (125)
	Bisulfite sequencing (BS-seq; ref. 126)	Bisulfite sequencing (BS-seq; ref. 126)
Histone modifications	ChIP-Seq (61)	ChIP-Seq (61)
	Stable isotope labeling by amino acid in culture (SILAC; ref. 127)	Stable isotope labeling by amino acid in culture (SILAC; ref. 127)
	High-throughput histone mapping (HiHiMap; ref. 128)	High-throughput histone mapping (HiHiMap; ref. 128)
Chromatin remodeling	Transposase-accessible chromatin using sequencing (ATAC-seq; ref. 129)	Transposase-accessible chromatin using sequencing (ATAC-seq; ref. 129)
Changes in noncoding RNAs	RNA-Seq (130)	RNA-Seq (130)
	In situ/FISH detection of small RNAs (131) and long non-coding RNAs (132, 133)	*In situ*/FISH detection of small RNAs (131) and long non-coding RNAs (132, 133)
KC5: Induces oxidative stress		
Oxidative damage to DNA	8-OHdG adducts via HPLC-electrochemical detection (134, 135)	8-OHdG adducts via HPLC-electrochemical detection (134, 135)
	Comet assay modified with lesion-specific repair endonucleases [e.g., 8-oxoguanine DNA glycosylase (OGG1), formamidopyrimidine (fapy)-DNA glycosylase (FPG), Endonuclease III (Nth; ref. 136)]	Comet assay modified with lesion-specific repair endonucleases [e.g., 8-oxoguanine DNA glycosylase (OGG1), formamidopyrimidine (fapy)-DNA glycosylase (FPG), Endonuclease III (Nth; ref. 136)]
Reactive oxygen species (ROS) formation	Electron paramagnetic resonance (137)	Electron paramagnetic resonance (137)
Glutathione oxidation	Measurement of GSH/GSSG ratio (138)	Measurement of GSH/GSSG ratio (138)
NFE2L2/ARE-dependent gene expression response	Antioxidant enzyme activity (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase; ref. 139)	Antioxidant enzyme activity (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase; ref. 139)
	NFE2L2/ARE-dependent gene expression (140)	NFE2L2/ARE-dependent gene expression (140)

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Table 1. Representative *in silico* and *in vitro* assays to measure the key characteristics of carcinogens. (Cont'd)

Endpoint	*In silico* or nonhuman *in vitro* assay	Human *in vitro* assay
Lipid peroxidation	Thiobarbituric acid reactive substances assay for detection of malondialdehyde, 4-Hydroxynonenal hydroperoxides, and isoprostanes (141)	Thiobarbituric acid reactive substances assay for detection of malondialdehyde, 4-Hydroxynonenal hydroperoxides, and isoprostanes (141)
KC6: Induces chronic inflammation		
Inflammatory signaling	[a]Colonic organoid-based cell transformation assay (142)	—
KC7: Is immunosuppressive		
T-cell activation and proliferation	Mitogen and/or antibody-mediated proliferation (143)	Mitogen and/or antibody-mediated proliferation (143)
Cytotoxic T-lymphocyte (CTL) activity	[a]BiTE-mediated CTL assay (144)	—
Natural killer cell activity	Missing-self cytotoxicity assay (145)	Missing-self cytotoxicity assay (145)
KC8: Modulates receptor-mediated effects		
Interacts with receptors	—	Androgen and estrogen receptor binding assay (ref. 146; OECD TG 493)
Receptor activation	Estrogen receptor transactivation (147) Androgen receptor transactivation (148) Aryl hydrocarbon receptor transactivation (149)	Estrogen receptor transactivation (147) Androgen receptor transactivation (148) Aryl hydrocarbon receptor transactivation (149)
Alters ligand synthesis	—	Aromatase enzyme activity (US EPA 890.1200) H295R steroidogenesis assay (OECD TG 456)
KC9: Causes immortalization		
Alters *in vitro* transformation activity	Cell transformation assays (150)	—
Alters cellular senescence markers	Changes in B-galactosidase, CDKN2A, CDKN1A, and TP53 protein levels (151)	Changes in B-galactosidase, CDKN2A, CDKN1A, and TP53 protein levels (151)
Telomere length and telomerase activity	Telomerase activity assay (152)	Telomerase activity assay (152)
Alterations in stem cell genes	Expression of MYC, POU5F1, Klf4, Sox2 (153, 154)	Expression of MYC, POU5F1, Klf4, Sox2 (153, 154)
KC10: Alters cell proliferation, cell death, or nutrient supply		
Cell proliferation	DNA labeling (e.g., EdU, 3H-thymidine; ref. 155) Cell-cycle markers (e.g., Ki-67, propidium iodide; ref. 155) Metabolic activity (e.g., MTT; ref. 155) Cell number/microscopy (e.g., Hemocytometer; ref. 155) Colony formation (156)	DNA labeling (e.g., EdU, 3H-thymidine; ref. 155) Cell-cycle markers (e.g., Ki-67, propidium iodide; ref. 155) Metabolic activity (e.g., MTT; ref. 155) Cell number/microscopy (e.g., Hemocytometer; ref. 155) Colony formation (156)
Evasion or reduction of apoptosis	Evasion of apoptosis (157) by TUNEL, Annexin-V, PARP1 cleavage, or others [a]Changes in expression of pro- and antiapoptotic factors (158)	Evasion of apoptosis (157) by TUNEL, Annexin-V, PARP1 cleavage, or others [a]Changes in expression of pro- and antiapoptotic factors (158)
Angiogenesis	Endothelial cell proliferation, migration, and differentiation (159) Transwell cell invasion (Boyden) assay (160) Aortic ring assay (161)	Endothelial cell proliferation, migration, and differentiation (159) Transwell cell invasion (Boyden) assay (160) Aortic ring assay (161)
Glycolytic (Warburg) shift	Cellular respiration and acidification (Seahorse) assay (162)	Cellular respiration and acidification (Seahorse) assay (162)

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; BiTE, bispecific T-cell engager; ChIP-Seq, chromatin immunoprecipitation-sequencing; ELISA, enzyme-linked immunosorbent assay; GSH, glutathione; GSSG, glutathione-S-S-glutathione; HPLC, high-performance liquid chromatography; Klf4, Krüppel-like factor 4; LC, liquid chromatography; MSTI, (E)-2-(4-mercaptostyryl)-1,3,3-trimethyl-3H-indol1-ium; NEF2L2/ARE, nuclear factor, erythroid 2 like 2/antioxidant response element; PARP1, poly (ADP-ribose) polymerase 1; POU5F1, POU class 5 homeobox 1; Sox2, SRY-box transcription factor 2; TGx-DDI, toxicogenomics-DNA damage-inducing; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
[a]Emerging assay or biomarker.

determine DNA adducts in human biopsy samples by liquid chromatography–mass spectrometry, requiring only several milligrams of tissue (20, 21). Methods for protein and DNA adductomics, which identify multiple adducts, are emerging (22, 23), as is chemoproteomics, which assesses the sites on proteins adducted by electrophilic small molecules (24).

KC2: Is genotoxic

Genotoxicity is generally defined as the capability of an external agent to cause DNA damage, alteration to the genome (mutation), or both. The link between genotoxicity and chemical carcinogenesis is well established and has shaped standardized testing batteries of carcinogens for decades (25). Genotoxicity can arise from, for example, DNA strand breaks, DNA adducts, DNA–DNA cross-links, and DNA–protein cross-links, as well as from oxidative damage to DNA (see also KC5, below); all of these types of damage may give rise to permanent changes in the nucleotide sequence (mutation) as the cell attempts to repair the damage. Genotoxicity can give rise to single-nucleotide variants (point mutations), or it can manifest over a larger span of the genome at the chromosome level, for example, structural (clastogenicity) or numerical chromosomal aberrations (aneuploidy). For practical

Table 2. Representative endpoints/biomarkers and corresponding examples of *in vivo* assays and biomarkers that can be used to measure the key characteristics of carcinogens.

Endpoint	*In vivo* assay in experimental animals	*In vivo* biomarker in humans
KC1: Is electrophilic		
Protein adducts	Protein adduct measurement by LC/mass spectrometry (163) [a]Chemoproteomics (113)	Protein adduct measurement by LC/mass spectrometry (164) Hemoglobin or albumin adducts in blood (164) [a]Protein adductomics (165)
DNA adducts	DNA adductomics (166, 167) Nuclease P1-enhanced (32)P-postlabeling method (168) Mass spectrometry (21)	DNA adductomics (166, 167) Nuclease P1-enhanced (32)P-postlabeling method (168) Mass spectrometry (21)
KC2: Is genotoxic		
Mutation/single nucleotide variants	Transgenic rodent assay (e.g., Big Blue; OECD 488) Pig-a assay (169) Next-generation high-throughput sequencing for somatic mutation detection (43)	Hypoxanthine-guanine phosphoribosyltransferase (HPRT) mutation assay (170) Glycophorin A (GPA) assay (171) Next-generation high-throughput sequencing for somatic mutation detection (43)
Structural chromosome alterations/ DNA strand breaks (clastogenicity); aneugenicity	Micronucleus assay (OECD 474) Chromosomal aberration test (OECD 475) Alkaline comet assay (OECD 489; ref. 175) Chromosomal aberration (44) Interphase and metaphase FISH (176)	Micronucleus assay (172, 173) OctoChrome FISH (174) Alkaline comet assay (OECD 489; ref. 175) Chromosomal aberration (44) Interphase and metaphase FISH (176)
KC3: Alters DNA repair or causes genomic instability		
Copy number variations (duplications, deletions, amplifications, insertions)	Comparative genome hybridization (CGH, array based; ref. 118) Next-generation high-throughput sequencing for somatic mutation detection (43)	Comparative genome hybridization (CGH, array based; ref. 118) Next-generation high-throughput sequencing for somatic mutation detection (43)
Inter-/intrachromosomal translocations	Spectral karyotyping (SKY), karyotyping (50) Next-generation high-throughput sequencing for somatic mutation detection (43)	Spectral karyotyping (SKY), karyotyping (50) Next-generation high-throughput sequencing for somatic mutation detection (43)
Microsatellite instability	Fluorescent multiplex PCR-based method using DNA (119)	Fluorescent multiplex PCR-based method using DNA (119)
DNA repair capacity	Unscheduled DNA synthesis (120) Host cell reactivation for evaluation of DNA repair (121, 122) Topoisomerase I and II enzymatic activity analysis using gel electrophoresis (123)	Unscheduled DNA synthesis (120) Host cell reactivation for evaluation of DNA repair (121, 122) Topoisomerase I and II enzymatic activity analysis using gel electrophoresis (123)
Increased expression of activation-induced cytidine deaminase (AICD)	Western blotting using antibodies (177)	—
KC4: Induces epigenetic alterations		
Global and locus-specific DNA methylation	High-performance liquid chromatography and ELISA-based methods (124) Enzyme activity assays for "writers, erasers, editors and readers" (125) Bisulfite sequencing (BS-seq; ref. 126)	Illumina Methylation EPIC 850k Beadchip (178) High-performance liquid chromatography and ELISA-based methods (124) Enzyme activity assays for "writers, erasers, editors and readers" (125) Bisulfite sequencing (BS-seq; ref. 126)
Histone modifications	ChIP-Seq (61) Stable isotope labeling by amino acid in culture (SILAC; ref. 127) High-throughput histone mapping (HiHiMap; ref. 128)	ChIP-Seq (61) Stable isotope labeling by amino acid in culture (SILAC; ref. 127) High-throughput histone mapping (HiHiMap; ref. 128)
Chromatin remodeling	Transposase-accessible chromatin using sequencing (ATAC-seq; ref. 129)	Transposase-accessible chromatin using sequencing (ATAC-seq; ref. 129)
Changes in noncoding RNAs	RNA-Seq (130) *In situ*/FISH detection of small RNAs (131) and long non-coding RNAs (132, 133)	RNA-Seq (130) *In situ*/FISH detection of small RNAs (131) and long non-coding RNAs (132, 133)
KC5: Induces oxidative stress		
Oxidative damage to DNA	8-OHdG adducts via HPLC-electrochemical detection (134) Comet assay modified with lesion-specific repair endonucleases [e.g., 8-oxoguanine DNA glycosylase (OGG1), formamidopyrimidine (fapy)-DNA glycosylase (FPG), Endonuclease III (Nth; ref. 136)]	8-OHdG adducts via HPLC-electrochemical detection (134) Comet assay modified with lesion-specific repair endonucleases [e.g., 8-oxoguanine DNA glycosylase (OGG1), formamidopyrimidine (fapy)-DNA glycosylase (FPG), Endonuclease III (Nth; ref. 136)]
Reactive oxygen species (ROS) formation	Electron spin resonance imaging (179)	—

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Table 2. Representative endpoints/biomarkers and corresponding examples of *in vivo* assays and biomarkers that can be used to measure the key characteristics of carcinogens. (Cont'd)

Endpoint	*In vivo* assay in experimental animals	*In vivo* biomarker in humans
Glutathione depletion	NMR-based non-targeted global metabolomics (180)	Magnetic resonance spectroscopy (181)
NFE2L2/ARE-dependent gene expression response	Antioxidant enzyme activity (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase; ref. 139)	Antioxidant enzyme activity (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase; ref. 139)
	NFE2L2/ARE-dependent gene expression (140)	NFE2L2/ARE-dependent gene expression (140)
Lipid peroxidation	Thiobarbituric acid reactive substances assay for detection of malondialdehyde, 4-Hydroxynonenal hydroperoxides, and isoprostanes (141)	Thiobarbituric acid reactive substances assay for detection of malondialdehyde, 4-Hydroxynonenal hydroperoxides, and isoprostanes (141)
KC6: Induces chronic inflammation		
Tissue inflammation	Histologic examination	Histologic examination (limited in human)
KC7: Is immunosuppressive		
Hematology	White blood cell counts and examination of lymphoid tissues (182)	White blood cell counts and examination of lymphoid tissues (182)
Immunophenotyping of T cells and NK cells	Enumeration of NK cells, and $CD4^+$ and $CD8^+$ T cells (183)	Enumeration of NK cells, and $CD4^+$ and $CD8^+$ T cells (183)
T-cell activation and proliferation	Mitogen and/or antibody-mediated proliferation (143)	Mitogen and/or antibody-mediated proliferation (143)
Cytotoxic T-lymphocyte (CTL) activity	BiTE-mediated CTL assay (144)	—
	Virus-specific CTL function in mouse (184)	—
Generation of antigen-specific $CD8^+$ T cells	[a]Measurement of endogenous or vaccination-induced antiviral immunity (185)	[a]Measurement of endogenous or vaccination-induced antiviral immunity (185)
NK cell activity	Missing-self cytotoxicity assay (145)	Missing-self cytotoxicity assay (145)
Systems immunology	Mass cytometry (186)	Mass cytometry (186)
KC8: Modulates receptor-mediated effects		
Activates or antagonizes receptors	Posttranslational and/or transcriptional changes associated with the estrogen, androgen, and aryl hydrocarbon receptor activity (187)	Posttranslational and/or transcriptional changes associated with the estrogen, androgen, and aryl hydrocarbon receptor activity (187)
Alters receptor expression	Immunohistochemistry or Western blotting in animal or human tissue (187)	Immunohistochemistry or Western blotting in animal or human tissue (187)
Alters ligand synthesis, clearance, distribution, or levels	Circulating steroid hormone levels (188–190)	Circulating steroid hormone levels (188–190)
	Alteration in sex hormone–binding globulins (188, 191, 192)	Alteration in sex hormone–binding globulins (188, 191, 192)
KC9: Causes immortalization		
Alters cellular senescence markers	Changes in B-galactosidase, CDKN2A, CDKN1A, and TP53 levels (151)	Changes in B-galactosidase, CDKN2A, CDKN1A, and TP53 levels (151)
Telomere length and telomerase activity	Telomere length by real-time PCR	Telomere length by real-time PCR (193, 194)
	Telomerase activity assay (152)	Telomerase activity assay (152)
Alterations in stem cell genes	Expression of MYC, POU5F1, Klf4, Sox2 (153, 195)	Expression of MYC, POU5F1, Klf4, Sox2 (153, 195)
KC10: Alters cell proliferation, cell death, or nutrient supply		
Proliferation/hyperplasia	Histology/microscopy	Cell cycle/cell number markers (e.g., Ki-67, propidium iodide; ref. 197)
	DNA labeling (e.g., EdU, 3H-thymidine; ref. 196)	Cell number/microscopy (155)
	Cell cycle/cell number markers (e.g., Ki-67, propidium iodide; ref. 197)	
	Cell number/microscopy (155)	
Evasion or reduction of apoptosis	Histology/microscopy	Histology/microscopy
	[a]Changes in expression of pro- and antiapoptotic factors (198)	[a]Changes in expression of pro- and antiapoptotic factors (198)
Angiogenesis	Factor VIII stains for capillary basement membrane (199)	Tissue vascular permeability by magnetic resonance imaging (MRI; ref. 200)
Glycolytic (Warburg) shift	F-18-fluorodeoxyglucose (FDG) by computed tomography (CT) and positron emission tomography (PET; ref. 201)	F-18-fluorodeoxyglucose (FDG) by computed tomography (CT) and positron emission tomography (PET; ref. 201)
	MRI and spectroscopy (MRS; ref. 202)	MRI and spectroscopy (MRS; ref. 202)

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; BiTE, bispecific T-cell engager; ChIP-Seq, chromatin immunoprecipitation-sequencing; EdU, 5-ethynyl-2′-deoxyuridine; Klf4, Krüppel-like factor 4; LC, liquid chromatography; NEF2L2/ARE, nuclear factor, erythroid 2 like 2/antioxidant response element; NK, natural killer; NMR, nuclear magnetic resonance; PARP1, poly (ADP-ribose) polymerase 1; POU5F1, POU class 5 homeobox 1; Sox2, SRY-box transcription factor 2.
[a]Emerging assay or biomarker.

purposes, we define KC2 as encompassing these forms of genotoxicity, which are commonly evaluated using classical *in vitro* assays referenced in some regulatory testing guidelines (**Table 1**) and can be measured preclinically using biomarkers in experimental animals and in humans (**Table 2**). Although measurements of endpoints such as DNA cross-links can provide valuable mechanistic information, more apical endpoints such as mutation or chromosome loss, which reflect the consequence of damage to

daughter cells, are considered more relevant and useful for hazard identification. Indeed, chromosome aberrations and micronuclei have been shown to be associated with future cancer risk (26, 27). A large proportion of established human Group 1 carcinogens are genotoxic (IARC Monographs Volume 100 A–F).

There is clear overlap between the genotoxic effects described here in KC2 and other KCs that can lead to genotoxicity. For example "KC1 is electrophilic," for agents that can covalently bind DNA and form adducts; "KC3 alters DNA repair or causes genomic instability" for agents that inhibit DNA repair or induce error-prone repair pathways; and "KC5 induces oxidative stress" which can cause oxidative damage to DNA. The intent of considering multiple distinct KCs with seemingly similar effects on genome integrity is to functionally distinguish the mechanisms by which the agent can cause genotoxicity, which in turn may facilitate a more biologically accurate understanding of how the agent causes cancer. For example, benzene, an established human leukemogen, has all four of these characteristics [see also (28): its reactive metabolites are electrophilic (23); it is genotoxic, causing both structural and numerical chromosomal changes in humans (29); it inhibits topoisomerase II (30) causing genomic instability (31); and its metabolites induce oxidative damage to DNA (8-hydroxydeoxyguanosine, 8-OHdG) and micronuclei from oxidative stress (32)].

With the rapid development of next-generation sequencing (NGS) technologies, it is becoming possible to directly evaluate the result of genotoxicity at the nucleotide level with high resolution. However, further work is needed to validate these systems for detecting rare somatic mutations that approach the background level of mutations in humans (33). DNA sequencing–based measurements are broadly applicable to *in vitro* and *in vivo* model systems, and human-based investigations, providing an adequate amount of target tissue DNA, or a valid surrogate, can be obtained.

KC3: Alters DNA repair or causes genomic instability

Genomic instability is a Hallmark of Cancer and is an enabling characteristic of tumorigenesis (2). DNA damage response (DDR) pathways maintain genomic stability by ensuring the fidelity of DNA replication and by activating cell-cycle checkpoints and/or DNA repair pathways in response to DNA damage caused by endogenous processes and exogenous agents (34). Disruptions in DDR pathways and DNA repair can lead to elevations in mutagenesis and genomic instability, defined by an increasing rate of the accumulation and clonal expansion of genomic alterations, and characterized by gene mutation, microsatellite instability (MSI), and genomic/chromosomal instability (CIN; ref. 35).

Genomic instability has been hypothesized to result from the expression of a mutator phenotype driven by mutations in DNA repair genes (36). Altered DNA repair and/or induction of genomic instability can result from agents that are genotoxic (KC2), induce oxidative stress (KC5), or induce epigenetic alterations (KC4) and thereby disrupt the expression of genes involved in DNA repair. Several chemical and physical agents have been shown to impede or inhibit high-fidelity DNA repair and/or activate error-prone DNA repair pathways leading to genomic instability and cancer, including metals (37), aldehydes (38), and ionizing radiation (39).

DNA repair activity is a useful human biomarker for mutagenic agents. Repair activity can be detected by measuring the expression, location, or recruitment of DNA repair proteins, but these measurements may not reflect the end outcome or consequence of a change in mutagenic repair capacity. The comet assay (i.e., single-cell gel electrophoresis) can be used to measure the rate or capacity of DNA repair, including high-throughput applications (40). Other high-throughput approaches include molecular beacon assays that can detect multiple DNA repair enzyme activities (41), and fluorescence-based, multiplex flow-cytometric host cell reactivation assays that have been used to measure interindividual differences in DNA repair capacity in humans (42). Newer higher-resolution NGS-based methodologies can measure the consequences of activated mutagenic DNA damage repair (e.g., large deletions/amplifications and inter-/intrachromosomal translocations), which provides more direct evidence of the repair outcome itself (43, 44).

Other potential contributors to genomic instability have been identified. Oncogene-induced DNA replication stress, generated by sustained cell proliferation, may confer a selective advantage that drives tumorigenesis (45). In addition, genomic instability can be induced by chronic inflammation (KC6), independently of DNA damage, following exposure to pathogens, chemicals, direct/indirect DNA mutagens, radiation, hypoxia, or starvation (39, 46–48). Several inflammatory mediators involved in proinflammatory signaling and activation of NFκB have been implicated, including reactive oxygen species (ROS), cytokines, TNF, and aberrant expression of activation-induced cytidine deaminase (AICD), a DNA mutator enzyme (39, 49).

Spectral karyotyping (SKY) is a useful technique for identifying and characterizing genomic instability exhibited by CIN (50). Higher resolution NGS technologies have been established that can measure genomic instability at the DNA level (**Tables 1** and **2**), although they have been largely restricted to studying tumor heterogeneity (51). Further characterization with expanded numbers of drugs/xenobiotics is needed to validate these genomic endpoints for the purposes of nonclinical and clinical safety studies.

KC4: Induces epigenetic alterations

Epigenetic modifications occur without changing DNA sequence and lead to stable and mitotically heritable changes in gene expression. Epigenetic modifications include alterations to DNA (e.g., DNA methylation), noncoding RNAs (e.g., altered expression of miRNA), chromatin (e.g., histone modifications), and 3D structures (e.g., nucleosome positioning; ref. 52). These alterations are surprisingly common (53), and some cancer types show widespread losses in DNA methylation with small gains in specific genomic areas and genes (54). The downstream effects depend on the type of epigenetic modification and the location in the genome where they impact (e.g., promoter/exon/intron/inter-/intra-genic region of oncogenes or tumor suppressor genes). Epigenetic marks are hypothesized to serve as mediators of cancer etiology and progression, in many cases preceding cancer (55).

A variety of methods exist to measure DNA methylation status at the global and locus-specific level. In human samples and cell cultures, the most commonly used method is to measure locus-specific methylation using Illumina 450K and 850K bead chip arrays. A more comprehensive but costly method is bisulfite sequencing, usually in the form of reduced representation bisulfite sequencing (RRBS), which can generate genome-wide methylation profiles at a single nucleotide level. Total genomic 5methylcytosine can be quantified by using a variety of antibody kits with ELISA, high-performance liquid chromatography, or liquid chromatography/mass spectrometry. Because more than one third of DNA methylation occurs in repetitive elements, analyzing the methylation

of repetitive elements can also serve as a surrogate marker for global genomic DNA methylation. Many carcinogens alter DNA methylation status. For example, arsenic exposure is associated with global DNA hypomethylation and increased DNA methylation of proto-oncogenes such as *RAS* (56), *TP53*, and *CDKN2A* (57).

Mass spectrometry is the gold-standard method for analyzing histone modifications, as it enables the quantification of specific modifications with high resolution (58). However, it is quite difficult to apply in practice and many researchers have used antibody-based methods. A variety of carcinogenic metals, including arsenic, chromium, and nickel have been shown to cause significant modifications of histone proteins (59). The Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq), as the name suggests, uses a transposase to insert sequencing adapters into accessible regions of chromatin, followed by sequencing, to assess genome-wide DNA accessibility (60). It is a rapid and sensitive alternative to DNase-Seq and has been used with multiple cell types and species. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is extensively used to map epigenetic proteins such as histone proteins, chromatin regulators, and transcription factors in the genome (61).

Changes in noncoding RNA expression are now usually measured by small RNA sequencing, but a variety of arrays also exist. *In situ* hybridization/FISH are also used to detect both small RNAs and long noncoding RNAs. Chemical carcinogens alter the expression of multiple miRNAs and this has been postulated to play a role in chemical carcinogenesis (62, 63).

The difficulty with interpreting the KC "induces epigenetic modifications" is that the relevance of a particular epigenetic change for carcinogenesis by a specific agent may not be clear and causality is hard to establish. More validation is clearly needed to fully understand which epigenetic endpoints are most indicative of carcinogenic risk.

KC5: Induces oxidative stress

The induction of oxidative stress and subsequent injury is a characteristic of diverse carcinogens, including radiation, asbestos, some chemicals metabolized to quinones, and carcinogenic infectious agents (64). Specifically, these carcinogens are capable of influencing redox balance within target tissues, leading to an imbalance favoring formation of ROS at the expense of their detoxification, and may also be accompanied by the production of reactive nitrogen species. Oxidative stress can lead to oxidative damage to DNA, including base modification, DNA–protein cross-links, and other lesions (65, 66). For instance, 8-OHdG is a DNA adduct that is considered a critical biomarker for carcinogens that cause oxidative stress. Other biomolecules, including proteins and lipids, are also subject to oxidative damage. Thus, oxidative stress is directly related to many other KCs, notably KC2 and KC3 via DNA damage leading to genotoxicity and alteration of DNA repair, as well as others including chronic inflammation (KC6) and altered cell proliferation (KC10). For example, oxidative stress affects cellular proliferation (e.g., EGFR regulation, MAPK3/MAPK1 and MAPK activation), evasion of apoptosis (e.g., Src, NF-κB and PI3KCA/Akt1 activation), tissue invasion and metastasis (e.g., MMP secretion, Met overexpression, and Rho–Akt1 interaction), and angiogenesis (e.g., VEGF release; refs. 67, 68).

A wide variety of assay systems, exemplified in **Tables 1** and **2**, are available to measure an increase in ROS formation, changes in oxidative enzymatic activity, defects in the antioxidant defense system, lipid peroxidation, protein oxidation, and oxidative damage to DNA. These methods and associated biomarkers are widely used across *in vivo* studies in humans and experimental animals (69), small model organisms like *C. elegans* (70), and *in vitro* cell-based assays. This KC is often nonspecific, as noncarcinogens can also induce oxidative stress. Therefore, oxidative stress is most informative in hazard identification when there is concordance among increases in oxidative stress markers and additional KCs. For instance, such concordance has been shown previously when oxidative stress was accompanied by genotoxicity in strains of yeast and bacteria known to be more sensitive to oxidative damage to DNA (8). Furthermore, evidence is strengthened when these effects can be shown to be attenuated with coexposure to antioxidants or in knockout animals (8). Overall, it appears that ROS production alone is not specific to carcinogens in the absence of other KCs. Thus, future efforts should be focused on further identifying ROS endpoints and markers that are specifically associated with induction of other KCs, such as DNA damage and inflammation.

KC6: Induces chronic inflammation

Chronic inflammation associated with the development of cancer is a prolonged response to persistent infections or irritants that inflict cell death and tissue injury followed by deregulated compensatory cell proliferation and aberrant repair (71). The type, intensity, and timing of the inflammatory response varies depending on the host immune status, the initiating stimulus, and the target organ. For example, infection of the gastric mucosa with the bacterium *Helicobacter pylori* induces epithelial cell death and acute and chronic inflammation leading to atrophic gastritis. Eventually, an adaptive response characterized by intestinal metaplasia of the gastric epithelium occurs and in approximately 3% of persistently infected patients, intestinal-type gastric adenocarcinoma develops after a long latent period (72). In contrast to this complex inflammatory response, *Schistosoma haematobium* infection leads to chronic irritation and granulomatous inflammation in response to the helminth eggs in the lining of the urinary bladder. Over years or following repeated helminth infections, these eggs induce metaplasia and hyperplasia of the transitional epithelial lining of the bladder, potentially leading to squamous cell carcinoma (73). During these chronic inflammatory reactions, production of reactive oxygen and nitrogen species by infiltrating inflammatory cells contributes to oxidative stress and epigenetic alterations (KC4 and KC5). In addition, repeated episodes of cell necrosis or immune-mediated apoptosis (e.g., persistent hepatitis B infection in the liver) and altered parenchymal cell differentiation and proliferation links chronic inflammation with other KCs (e.g., KC 10). Sustained release of growth factors, cytokines, angiogenic factors, and matrix metalloproteinases perpetuate the chronic inflammatory response and facilitate tumor growth, progression, and invasion (74).

The best measure of chronic inflammation is by histopathology following repeated *in vivo* exposures (**Table 2**). Studies in rodents have provided strong evidence for the involvement of chronic inflammation in cancers induced by welding fumes, malathion, tetrachloroazobenzene, indium tin oxide and melamine in recent IARC evaluations (8). Because of the short-term nature of most *in vitro* assays, it is difficult to assess persistent, chronic inflammatory responses in these systems. Some "long-term" *in vitro* coculture models better mimic chronic inflammation, especially in regard to cytokine/chemokine profiles, but it is difficult to model inflammatory cell recruitment. It is also difficult to establish a causal relationship between inflammatory biomarkers and development of cancer, especially in epidemiologic studies (75, 76). Proinflammatory cytokines such as TNF or IL6, C-reactive protein, fibrinogen,

and high mobility group box 1 (HMGB1) have been investigated as potential circulating biomarkers for chronic inflammation in a variety of diseases, including asbestos-related diseases (77, 78). Unfortunately, these biomarkers are not very specific for diagnosis of chronic inflammation due to poor sensitivity, confounding exposures, and variability in individual patients over time (76, 79). Because of its stability, IL6 is perhaps the best current biomarker available (80).

KC7: Is immunosuppressive

Immunosuppression is a reduction in the capacity of the immune system to respond effectively to foreign antigens, including antigens on tumor cells. Epidemiologic data from patients with congenital immunodeficiencies, virally induced immunodeficiencies (e.g., HIV mediated), and from patients treated with immunosuppressive therapies (e.g., organ transplant rejection prevention therapies) indicate that profound immunosuppression is associated with an increased cancer risk. Immunosuppression-associated cancer types can include hematologic malignancies and solid tumors and these cancers are often but not always associated with an oncogenic virus etiology. Examples include cyclosporine-induced non-Hodgkin lymphoma (81) and lung cancer from welding fumes (82), agents which are both immunosuppressive. The interplay between immunity and tumorigenesis is complex and different components of the immune system are either pro- or antitumorigenic. While profoundly altered immunity, such as that achieved during organ transplant, can promote cancer risk, not all components of the immune system are equally important in defense against or promotion of cancer, and a similar cancer hazard for all immunomodulatory molecules (e.g., for anti-inflammatory agents) should not be assumed (83).

Immunosuppressive agents are molecules that interfere with key steps of the "cancer-immunity cycle" which can be divided into several processes, starting with the release of antigens from the cancer cell, followed by the presentation of cancer antigens to immune effector cells, the priming/activation of these effector cells (e.g., T lymphocytes), infiltration/migration of immune effector cells into tumors, and ending with the killing of cancer cells (84). T-cell function plays a particularly important role in antitumor immunity and some components of innate immunity. Natural killer (NK) cells can also play an important role. Several immunosuppressive agents (such as cyclosporine A or 7,12-dimethylbenz[a]anthracene) directly inhibit T-cell activation leading to decreased immune surveillance of precancerous cells/lesions.

Rodent tumor models or rodent bioassays are generally poor models to assess the cancer hazard associated with immunosuppression (85). Endpoints or assays that can assess the degree of interference with antitumor immunity should ideally interrogate various key steps of the cancer–immunity cycle. Certain phenotyping and functional assays and endpoints listed in **Tables 1** and **2** are representative of assays that can inform that hazard. These include standard hematology and anatomic pathology (e.g., evidence of marked lymphodepletion and hematotoxicity), lymphocyte population enumeration by flow cytometry, and NK-cell activity. There are also some less routine but important endpoints such as Th and cytotoxic T-cell functions (including endpoints relating to factors that govern killing mechanisms such as cytokine production and evidence of degranulation). Other assays or models such as host resistance models may detect immunomodulation (e.g., decreased resistance to bacterial infection or even certain viruses) but these effects may not translate to decreased immune surveillance of tumors. Efforts are ongoing to identify and develop new approaches and methods to evaluate systemic immune status *in vivo*. Systems immunology approaches can leverage methods such as mass cytometry to interrogate multiple cell types and functions concomitantly (**Table 2**).

KC8: Modulates receptor-mediated effects

Receptor-mediated effects can occur at the cell surface (through ligand binding) or intracellularly (via the disruption of signaling cascades or actions on nuclear/cytosolic receptors), all of which can modulate transcriptional changes in the nucleus. Outcomes of transcriptional changes are varied and often regulate critical cellular pathways. Some receptors promote cell proliferation [e.g., hormone nuclear receptors such as estrogen (ER; ref. 86), androgen (AR; ref. 87), and progesterone (PR; ref. 86) receptors and growth factor receptors such as EGFR (88) and ERBB2 (89)]. Activation of the aryl hydrocarbon receptor (AhR; KC8) can lead to immunosuppression (KC7), in addition to effects on cell proliferation and survival (KC10; refs. 90–92). Finally, the activation of certain nuclear receptors, including peroxisome proliferator activated receptor alpha (93) and constitutive androstane receptor (94), is associated with rodent liver carcinogenesis (94, 95).

Nuclear and cytosolic receptors are generally regulated by small molecules and are thus of higher priority for evaluating xenobiotics as carcinogens compared to peptide-regulated growth factor receptors. Binding of drugs/xenobiotics to receptors is readily measurable but does not completely inform how downstream signaling activity is modulated. Hence, the best approach is to evaluate both receptor binding and receptor functional activity. Radioligand binding and luciferase reporter gene assays in human cells are the current respective gold-standard assays. For example, internationally harmonized guideline assays exist for binding and transcriptional activation of both ER and AR, although not all use human cells. Other binding and reporter assays are available for several aforementioned receptors although they generally lack extensive validation. As many of these receptors have been the target of pharmaceutical research, there are contract research organizations that provide testing services for many of them. Research efforts are still needed in some cases, such as with the AhR where distinguishing between effects that promote carcinogenesis from those that inhibit it cannot be readily determined through binding and transcriptional activation assays which is likely crucial for the identification of carcinogens (96).

Agents that modulate a ligand's synthesis, transport, distribution, biotransformation, and clearance can indirectly modulate receptor-mediated effects. We are only aware of one guideline assay investigating ligand synthesis (**Table 1**), and this is an area where many assays can be developed. The recent identification of the 10 KCs of endocrine disrupting chemicals also highlighted the need for additional assays to assess many aspects of endocrine disruption that may be useful in evaluating agents for this KC of carcinogens (i.e., KC8; ref. 97).

KC9: Causes immortalization

Cancer cells are immortal, and therefore have limitless replicative potential. Normal cells have a limited lifespan that has been described as the Hayflick limit, as measured *in vitro* by cell doublings of normal human fibroblasts. Several mechanisms can influence immortalization: cellular pathways that regulate stemness versus senescence, telomere length, as well as telomerase activity and Alternative Lengthening of Telomeres (ALT), break-induced telomere synthesis, and

mitotic DNA synthesis (MiDAS) at so-called common fragile sites and telomeres (98–100). Telomeres are the protective ends of chromosomes that are necessary to prevent chromosomal instability and are maintained through telomerase and other gene products. Cancers frequently have elevated telomerase activity and maintain or extend their telomeres through genetic and epigenetic mechanisms. Carcinogens have been shown to activate telomerase and/or extend telomeres (101–103).

Immortalization is associated with stemness, the ability of cells to self-replicate indefinitely. With the exception of normal stem cells, whose behavior relies on signals from stem cell niches (104), most normal cells differentiate and lose the capacity for self-replication (101–103). However, cancers generally appear to maintain a small subpopulation of their cells that are the cancer stem cells, which are required for unlimited replication. For example, the MYC oncogene, which is upregulated by some carcinogens, can promote the stemness of cancer stem cells (101–103). The opposite of immortalization and stemness is cellular senescence, a cellular program that results in terminal differentiation of cells that have undergone irreparable cellular stress, including DNA damage. Immortalization is regulated through many gene products including cell-cycle checkpoint inhibitors such as CDKN2A and DNA repair gene products such as TP53 (101–103). Carcinogens including human DNA and RNA viruses, such as human papillomaviruses, Epstein–Barr virus, Kaposi sarcoma–associated herpes virus, hepatitis B virus, hepatitis C virus, HIV, Merkel cell polyomavirus (MCPyV), and human T-lymphotropic virus type 1 (HTLV-1), are carcinogenic through effects on cellular immortalization and senescence (105). Similarly, chemical carcinogens including tobacco, PCBs, and asbestos have been shown to impede cellular responses to DNA damage that promote immortalization and inhibit senescence (106).

A carcinogen's influence on immortalization and senescence can be measured through a number of biochemical endpoints in cultured cells, including transformation assays, as well as more specific assays such as telomerase activity, telomere length, and regulation of certain genes in stem cells and cancer stem cells (e.g., *MYC, CDKN2A*, and *TP53*; refs. 101–103, 105, 106). Some of these endpoints can also be evaluated *in vivo* and in human biomarker studies to facilitate translation; however, more validation is needed in this area. In the future, the development of imaging-based reporters for telomerase, cell-cycle checkpoints, and senescence markers such as β-galactosidase activity may be useful for assessing carcinogenic activity.

KC10: Alters cell proliferation, cell death, or nutrient supply

Tumor size is regulated by cell proliferation (growth), cell loss by apoptosis (programmed cell death) or necrosis, and the vascular supply that provides oxygen and other nutrients. Cancer cells also often have different cellular energetics than normal cells, for example, using glycolysis for energy in aerobic conditions under the Warburg effect. Carcinogens may impact these processes by stimulating uncontrolled cell proliferation, angiogenesis to increase vascularity, and the evasion of apoptosis, rather than apoptosis induction. The resultant altered cell proliferation and/or cell-cycle control can contribute to carcinogenesis in three ways: (i) replicating cells may be predisposed to propagate unrepaired DNA damage and cancer-causing mutations, (ii) sustained replication may be an independent mechanistic event, and (iii) abnormal proliferation may allow transformed cells to evade usual checkpoints and to continue replication. Such scenarios foster evasion of apoptosis or other terminal programming, for example, autophagy (107).

Measures of cell proliferation include microscopic identification of mitotic cells on histology, measurement of S-phase cells by incorporation of ^{3}H-thymidine or bromodeoxyuridine, stains for proliferating cell nuclear antigen, and identification of the growth fraction with stains such as MIB1 for Ki-67. *In vitro* assays for cell proliferation can be challenging owing to the potential influence of paracrine actions and other homeostatic responses that may be lost in simple two-dimensional cell cultures or by use of transformed cell lines commonly used in the laboratory. Static measures of apoptosis include identification of apoptotic cells on histology (sometimes with TUNEL assay or Annexin-V), and expression of pro- and antiapoptotic genes. Evasion of apoptosis is determined as a change in apoptosis in response to a known therapeutic agent, for example, tamoxifen, compared with controls after exposure to a chemical of interest (108, 109). The vascularity of a tumor can be assessed by visual observation of blood vessel density, sometimes employing stains for capillary basement membrane.

Discussion

Our primary goal was to clearly describe each KC, based on the latest scientific developments and from experience in applying them in cancer hazard identification. In demonstrating the applicability of the KCs to identify measurable, proximal effects of carcinogens rather than endpoints that may be a consequence of tumorigenesis, this exercise showed that the KCs are clearly distinct from the Hallmarks. It also showed the applicability of KCs to a broad range of agents, not just environmental chemicals but also drugs/xenobiotics, defined mixtures, and complex exposures as well as viruses, fibers, and engineered nanomaterials.

A second goal was to describe some of the endpoints that best define each KC and the current and emerging assays and *in vivo* biomarkers that can be used to measure these endpoints. This exercise revealed that the KCs not only vary in complexity, but also in the number of endpoints and available assays and useful model systems. Representative assays and biomarkers listed in **Tables 1** and **2** were compiled from literature sources to illustrate the types of endpoints that reflect each KC and current approaches for measuring them. The assays and biomarkers presented in the Tables are by no means an exhaustive list and were compiled from literature sources. They represent a range of states of validation and experience in applying them. It would be valuable to understand the sensitivities and specificities for each of these as well as domains of applicability to utilize them most effectively. This could perhaps be accomplished through development and testing of a panel of known carcinogens and noncarcinogens covering a wide range of mechanisms across these assay panels.

A third goal was to make recommendations for future assay development and validation to improve how chemical agents (e.g., xenobiotics, therapeutics) can be systematically evaluated for cancer hazard *in vitro* and *in vivo*. In this regard, the compilation of assays revealed specific challenges for certain KCs. For KC7 (is immunosuppressive) *in vivo* approaches remain paramount, but existing rodent and other experimental tumor models have limitations for detecting cancer hazards associated with immunomodulators. New methods are needed to evaluate systemic immune status based on systems immunology approaches, leveraging methods such as mass cytometry to interrogate multiple cell types and functions concomitantly (e.g., evidence of hematotoxicity affecting leukocytes, impact on cytotoxic T cells and/or NK cells). For some of these endpoints, assays still need to be optimized, and there remains a very limited

understanding of how changes in such endpoints relate to cancer hazard. For KC6 (induces chronic inflammation), chronic *in vivo* exposures (in experimental animals or in humans) also remain more pertinent than *in vitro* cocultures, followed by more simplistic *in vitro* studies. Development and validation of *in vivo* alternatives, for example, the colonic organoid–based cell transformation assay (see **Table 1**), could be prioritized to fill these gaps.

Another important issue that emerged is the interrelationship of KCs and the impact on testing strategies and interpretation. For instance, multiple KCs consider seemingly similar effects on genome integrity. However, this complementarity makes it possible to functionally distinguish among genotoxic mechanisms, providing a more biologically accurate understanding of how the agent may cause cancer. Thus, each of the implicated KCs can be seen to have an important, but distinct role. At the other extreme, KC5 (induces oxidative stress) does not have a stand-alone role in causal identification of carcinogens, and is best seen in the context of its impact on other KCs [such as KC2 (is genotoxic)]. Endpoints and markers such as oxidative damage to DNA and inflammation that specifically probe KC5 (induces oxidative stress) in concert with KC2 (is genotoxic) and KC6 (induces chronic inflammation), may be informative for interpretation. Similarly, for KC8 (modulates receptor-mediated effects), which broadly encompasses a range of different ligands and receptors with varying relation to cancer causation, related efforts on the KCs of endocrine disrupting chemicals (97) may aid interpretation. As multiple KCs may be additive or synergistic, further work is merited to understand how to best integrate endpoints and biomarkers impacted by multiple KCs, as well as to define minimal set(s) of KCs that could by themselves characterize certain carcinogens. Such efforts could, in concert with *in silico* predictions, inform the order of testing and attendant conclusions.

This exercise also raised questions about whether the KCs as currently defined capture all the pertinent mechanisms of carcinogens. For instance, carcinogens may impact the tumor microenvironment, enhance invasiveness, and promote metastasis, but there are very few specific examples. These impacts may be investigated using organoids or models on chips and as such may currently be encompassed in KC10 (alters cell proliferation, cell death, or nutrient supply), but further focus on these critical aspects of carcinogen action may be warranted.

Despite the identified challenges, this compilation of assays and biomarkers is useful in various ways. For one, it can inform priority setting for funding and development of new assays, and to fill the identified gaps. The updated definitions and lists of associated assays will also advance application of the KCs in cancer hazard identification, as the basis for improving searches for existing mechanistic data relevant to the KCs, and in prioritizing the findings informative for evaluation. This compilation can also be used to design and conduct a battery of tests to probe carcinogenic activity. This could address all KCs for a complete assessment or target-specific assays based on agent properties, predictive modeling (e.g., computational test for electrophilicity), or outstanding research gaps. Ultimately the choice of assay and extent of testing would be dependent on the application of the data, from screening and prioritization of agents for further testing, to exploring specific hypotheses, supporting product registration, or performing hazard identification and risk assessment. In some cases, standard assays are already available (e.g., with guidance from the Organization for Economic Co-operation and Development, OECD) and amenable to study *in vitro*. In others, there is a possibility for investigation in populations exposed to carcinogens, such as in occupational settings, to provide data especially relevant in hazard identification exercises (110). It would be especially useful if the mutational signatures of tumors from individuals exposed to specific carcinogens could be identified. These *in vivo* biomarkers and signatures would then be of great value in the translation of *in vitro* hazards and assessment of dose–response in experimental animals or humans.

To support these future applications, a number of short- and long-term steps can be envisioned. In addition to the outstanding assay development needs for various KCs, as noted above in the sections on KC5 (induces oxidative stress), KC6 (induces chronic inflammation), and KC7 (is immunosuppressive), assay validation work is relevant for several KCs. For instance, error-corrected NGS systems for detecting rare somatic mutations that approach the background level of mutations in humans are especially relevant for KC2 (is genotoxic; ref. 33). It would be beneficial to develop standard lists of carcinogens and noncarcinogens to support assay qualification and validation, as well as future efforts toward evidence-based KC grouping and weighting. Publicly available resources such as open-source bioinformatic tools, detailed compilations of assays, and the associated best practices for study design, conduct, and reporting could also be useful. Further work could outline the priorities for such assay development, validation, and guidance for reporting and interpretation. In all, these future efforts will help to improve current methods for hazard screening and testing, and thereby advance carcinogen identification, a first step in cancer prevention.

Disclosure of Potential Conflicts of Interest

M.T. Smith is an expert witness or consultant at various law firms. D.W. Felsher reports receiving consulting fees received from attorneys in cases involving exposure to asbestos, TCEs, PCBs, and other chemical agents and materials. No potential conflicts of interest were disclosed by the other authors.

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