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Experimental and pan-cancer genome analyses reveal widespread contribution of acrylamide exposure to carcinogenesis in humans

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Humans are frequently exposed to acrylamide, a probable human carcinogen found in commonplace sources such as most heated starchy foods or tobacco smoke. Prior evidence has shown that acrylamide causes cancer in rodents, yet epidemiological studies conducted to date are limited and, thus far, have yielded inconclusive data on association of human cancers with acrylamide exposure. In this study, we experimentally identify a novel and unique mutational signature imprinted by acrylamide through the effects of its reactive metabolite glycidamide. We next show that the glycidamide mutational signature is found in a full one-third of approximately 1600 tumor genomes corresponding to 19 human tumor types from 14 organs. The highest enrichment of the glycidamide signature was observed in the cancers of the lung (88% of the interrogated tumors), liver (73%), kidney (>70%), bile duct (57%), cervix (50%), and, to a lesser extent, additional cancer types. Overall, our study reveals an unexpectedly extensive contribution of acrylamide-associated mutagenesis to human cancers.

[Supplemental material is available for this article.]

Cancer can be caused by lifestyle factors, environmental or occupational exposures involving chemicals, their complex mixtures, and physical and biological agents. Many human carcinogens show shared key characteristics (Smith et al. 2016), and different carcinogens may have a spectrum of such characteristics and operate through distinct mechanisms to produce genetic alterations. Recognizable somatic alteration patterns characterize carcinogens that are mutagenic. Single-base substitution (SBS) mutational signatures can be expressed in simple mathematical terms that enable them to be extracted from thousands of cancer genomes (Alexandrov et al. 2013a, 2018). Several of the identified mutational signatures have been attributed to specific external exposures or endogenous factors through epidemiological and/or experimental studies (Alexandrov et al. 2018). The majority of the signatures represent main of unknown origin, and additional, yet unrecognized, signatures are likely to be extracted from rapidly accumulating cancer genome data. Well-controlled experimental exposure systems can help identify the causes of the orphan mutational signatures and define new carcinogen-generated patterns (for review, see Hollstein et al. 2017; Zhivagui et al. 2017).

Various diet-related and iatrogenic exposures contribute to human cancer burden, involving, for instance, food contaminants ( aflatoxin B1 [AFB1]) or alternative medicines (aristolochic acid [AA]) with well-documented mutagenic properties; AFB1 induces predominantly C:G > A:T and AA generates T:A > A:T transversions. These characteristic mutations, arising in preferred sequence contexts, allowed unequivocal association of exposure to AFB1 or AA with specific subtypes of hepatobiliary or urological cancers (Poon et al. 2013; Meier et al. 2014; Scelo et al. 2014; Jelaković...
et al. 2015; Hoang et al. 2016; Chawanthayatham et al. 2017; Huang et al. 2017; Ng et al. 2017; Zhang et al. 2017).

Among dietary compounds with carcinogenic potential, acrylamide (ACR) is of interest because of its ubiquitous presence. Important sources of exposure to ACR include tobacco smoke (Mojska et al. 2016), coffee (Takatsuki et al. 2003), and a spectrum of occupational settings (IARC 1994). ACR forms in carbohydrate-rich foods (e.g., potatoes and cereals) heated at high temperatures, because of Maillard reactions involving reducing sugars and the amino acid asparagine (Tareke et al. 2002). There is sufficient evidence that ACR is carcinogenic in rodents (Beland et al. 2013, 2015), and it was classified by the International Agency for Research on Cancer (IARC) as a probable carcinogen (Group 2A) (IARC 1994). The associations of dietary ACR exposure with renal, endometrial, and ovarian cancers have been explored in epidemiological studies (Hogervorst et al. 2008; Vrik-Baker et al. 2014; Pelucchi et al. 2015). However, accurate ACR exposure assessment by questionnaires has been difficult, whereas more direct measures of molecular markers, such as hemoglobin adduct levels, may not yield conclusive findings on past exposures (Olesen et al. 2008; Wilson et al. 2009; Xie et al. 2013; Obón-Santacana et al. 2016a, b,c).

Thus, innovative well-controlled exposure model systems can improve our understanding of the ACR exposure-associated effects and risk.

Oxidation of ACR by cytochrome P450 produces the highly reactive electrophilic epoxide glycidamide (GA) (Segerbäck et al. 1995; Sumner et al. 1999; Ghanayem et al. 2005). The Hras mutation loads in neoplasms of mice exposed to ACR or GA were higher upon exposure to GA (Von Tunegn et al. 2012), and more mutations in the cII reporter gene of Big Blue mouse embryonic fibroblasts were obtained by GA treatment in comparison to ACR (Besaratinia and Pfeifer 2003, 2004). In vivo and in vitro reporter gene mutagenesis studies showed an increased association of ACR and GA exposure with T:A > C:G transitions and T:A > A:T and C:G > G:C transversions (Besaratinia and Pfeifer 2003, 2004; Von Tunegn et al. 2009, 2012; Ishii et al. 2015; Manjanatha et al. 2015). In addition, GA exposure induces C:G > A:T transversions (Besaratinia and Pfeifer 2004). However, these ACR- and GA-specific patterns were based on limited mutation counts and do not allow translating adequately the reported mutation types into genome-wide patterns.

Massively parallel sequencing allows studying a large number of mutations in a single sample, thus significantly enhancing the power of mutation analysis in experimental models. Analogously to human cancer genome projects, genome-scale mutational signatures can be extracted from highly controlled carcinogen exposure experiments using mammalian cell clonal expansion model (Olivier et al. 2014; Nik-Zainal et al. 2015; Huang et al. 2017). By integrating massively parallel sequencing and DNA adduct analysis in a mammalian cell clonal expansion model (Olivier et al. 2014; Nik-Zainal et al. 2015; Huskova et al. 2017) and by computational interrogation of the Pan-Cancer Analysis of Whole Genomes (PCAWG) data, we aimed to systematically investigate the mutational signatures of ACR and GA and to determine the contribution of ACR/GA to human carcinogenesis.

Results

Human TP53 mutations generated by ACR or GA treatment

Primary Hupki MEF cultures from three different embryos (Prim_1, Prim_2, and Prim_3) exposed to ACR or GA at the predetermined cytotoxic and genotoxic conditions yielded multiple immortalized clones (Methods) (Supplemental Fig. S1) suitable for massively parallel sequencing (Olivier et al. 2014). Sanger sequencing of TP53 in the clones derived from ACR exposure (ACR clones) and GA exposure (GA clones) and spontaneous immortalization (Spont), showed that ACR clones obtained from the Prim_2 MEFs showed loss of heterozygosity in the TP53 codon 72 involving a loss of the proline allele (ACR_1 clone), and also loss of the arginine allele resulting in a hemizygous ACR_2 clone (Table 1). No TP53 mutations were observed in the Spont clones. The detection of TP53 mutations in three out of seven ACR clones and in one out of five GA clones (Table 1) provided a sound rationale for extended sequencing at the exome scale.

Analysis of mutation spectra

Whole-exome sequencing (WES) of all Spont as well as exposed clones revealed that the total number of acquired SBS did not differ markedly between the ACR and Spont clones. The Spont clones harbored on average 190 (median = 151, range = 141–277) SBSs, whereas the ACR clones had on average 208 (median = 173, range = 151–262) SBSs. In contrast, the total number of SBSs was considerably increased in the GA clones, with an average of 485 SBSs (median = 448, range = 370–592) (Supplemental Tables S1, S2). This finding reveals stronger mutagenic properties of GA in the MEFs.

Principal component analysis (PCA) performed on the resulting SBS spectra unambiguously separated the GA clones from other experimental conditions (Fig. 1A). The ACR-exposed samples showed a diffuse pattern across the six SBS classes, whereas the Spont clones showed an enrichment of C:G > G:C SBSs in the 5′-GCG-3′ context, also present across the exposed cultures (Supplemental Fig. S2). This background mutation type appears related to the culture conditions used for the MEF immortalization assay, and its consistent formation has been observed previously (Olivier et al. 2014; Nik-Zainal et al. 2015). No significant transversion strand bias (TSB) was observed for any mutation class in the Spont or ACR clones (Supplemental Fig. S3). In the clones derived from the ACR-treated primary MEF cultures, we observed an enrichment of T:A > A:T and C:G > A:T transversions and T:A > C:G transitions (Supplemental Fig. S2B), marked by significant TSB (Supplemental Fig. S3). The GA-associated clones showed lower numbers (25 per clone) of small insertions/deletions (indels) in comparison to the ACR (44 per clone) or Spont clones (39 per clone) (see Supplemental Tables S1, S3). Thus, higher SBS counts owing to GA treatment may selectively promote the senescence bypass and the selection, with a decreased functional contribution of indels, whereas an inverse scenario is plausible for the Spont and ACR clones, consistent with a previous report based on the Big Blue mouse embryonic fibroblasts and cII transgene (Besaratinia and Pfeifer 2005).

Variant allele frequency (VAF) analysis performed for GA clones detected a large proportion of acquired mutations manifesting at VAF between 25% and 75% (Supplemental Fig. S4C). Upon grouping of substitutions into bins of high (67%–100%), medium (34%–66%), and low (0%–33%) VAF, the predominant GA-specific mutation types (T:A > A:T, T:A > C:G, and C:G > A:T) started manifesting at high VAF and became increasingly enriched in the medium and low VAF intervals. The background 5′-N[J]G[T]′-3′ SBS, corresponding to COSMIC signature 17 arising in cultured mouse cells including MEFs (Behjati et al. 2014; Nik-Zainal et al. 2015; Milholland et al. 2017), displayed minor, although not statistically
Mutational signature of acrylamide in human cancer

Table 1. Summary of cell lines, treatment conditions, and TP53 mutation status

| Sample ID | Embryo | Exposure | Conc. (mM) | Exposure duration (h) | Coding DNA changea | Genomic DNA changeb | aa change | Codon 72 (rs1042522)c |
|-----------|--------|----------|------------|-----------------------|-------------------|---------------------|-----------|-----------------------|
| Prim_1    | E210   | -        | -          | -                     | -                 | -                   | Pro/Pro   | -                     |
| Prim_2    | E213   | -        | -          | -                     | -                 | -                   | Arg/Pro   | -                     |
| Prim_3    | E214   | -        | -          | -                     | c.881delA         | g.7577057delT       | p.E294fs  | Arg/-                |
| Spon_1    | E213   | -        | -          | -                     | -                 | -                   | Pro/Pro   | -                     |
| Spon_2    | E214   | -        | -          | -                     | c.818G>c-T        | g.7577120C>A        | p.R273L   | Pro/Pro               |
| Spon_3    | E214   | -        | -          | -                     | c.740A>T; c.839G>c | g.7577541T>A; g.7577099C>G | p.N247I; p.R280T | Pro/Pro |
| ACR_S9_1  | E213   | ACR      | 5          | 24                    | -                 | -                   | -         | -                     |
| ACR_S9_2  | E213   | ACR      | 5          | 24                    | c.309-310CC>TA    | g.7579377-7579378G>G>TA | [p.Y103Y; p.Q104K] | Pro/Pro |
| ACR_1     | E213   | ACR      | 10         | 24                    | c.881delA         | g.7577057delT       | p.E294fs  | Arg/-                |
| ACR_2     | E213   | ACR      | 10         | 24                    | c.818G>c-T        | g.7577120C>A        | p.R273L   | Pro/Pro               |
| ACR_3     | E214   | ACR      | 10         | 24                    | c.740A>T; c.839G>c | g.7577541T>A; g.7577099C>G | p.N247I; p.R280T | Pro/Pro |
| ACR_4     | E214   | ACR      | 10         | 24                    | -                 | -                   | Pro/Pro   | -                     |
| ACR_5     | E214   | ACR      | 10         | 24                    | -                 | -                   | Pro/Pro   | -                     |
| GA_1      | E210   | GA       | 3          | 2                     | -                 | -                   | Pro/Pro   | -                     |
| GA_2      | E210   | GA       | 3          | 2                     | c.309-310CC>TA    | g.7579377-7579378G>G>TA | [p.Y103Y; p.Q104K] | Pro/Pro |
| GA_3      | E210   | GA       | 3          | 2                     | -                 | -                   | Pro/Pro   | -                     |
| GA_4      | E214   | GA       | 3          | 2                     | -                 | -                   | Pro/Pro   | -                     |
| GA_5      | E214   | GA       | 3          | 2                     | -                 | -                   | Pro/Pro   | -                     |

(5′)-3′ (TP53) human TP53 gene; (Prim) primary cells; (Spon) spontaneously immortalized clones; (ACR) acrylamide-exposure derived clones; (GA) glycida-mide-exposure derived clones. Each exposure condition was carried out in two biological replicates (embryos). (S9) human S9 fraction; (Pro) proline; (Arg) arginine; (Arg/-) or (Pro/-) loss of allele; (fs) frameshift; (aa) amino acid.

aNM_000546.4 coding sequence.

bhg19 genomic coordinates.

cHuman polymorphic site (rs1042522).

significant, lower-VAF enrichment (P = 0.25, assessed by χ2 test) (Supplemental Fig. S5). These observations suggest early effects of the GA exposure, reproducible contribution of the induced mutations to senescence bypass, and their clonal propagation during the immortalization stage.

Mutational signature of GA

Three distinct mutational signatures were extracted from all MEF clones, termed signatures A, B, and C. Signatures A and C were enriched in the Spont and ACR clones, whereas the more robust signature B was selectively enriched in the GA clones (Fig. 1B; Supplemental Fig. S6). The TSB analysis in the GA clones revealed significant enrichment of the prominent mutation types C:G > A:T, T:A > A:T, and T:A > C:G (using the pyrimidine-based mutation class convention) on the transcribed strand (~0.05, χ2 test), consistent with the less efficient transcription-coupled nucleotide excision repair because of adduct formation on purines (Fig. 1C; Supplemental Fig. S3). In signature C and to a lesser extent in signatures A and B, we observed an admixture of a pattern identical to the COSMIC signature 17 (T:A > G:C in the 5′-NTT-3′ trinucleotide context), present in human cancers (notably esophageal and gastric adenocarcinomas) but also seen in AFB1-driven mouse liver cancers (Huang et al. 2017), in murine small cell lung carcinoma initiated by loss of Trp53 and Rb1 (McFadden et al. 2014), and in primary MEF-derived clones (Olivier et al. 2014; Nik-Zainal et al. 2015). This signature has been linked to cell culture conditions (Behjati et al. 2014; Milholland et al. 2017) and may be linked to oxidative stress effects on the free dGTP pool (Tomkova et al. 2018). To further refine the putative GA mutational signature from signature B, we used extended-input nonnegative matrix factorization (NMF) by combining the MEF clone data with signature 17 (see Methods) (Supplemental Methods; Supplemental Figs. S6, S7). This considerably reduced the number of signatures to senescence bypass, and their clonal propagation during the immortalization stage.

Quantitative DNA adduct analysis supports the GA mutational signature

Following metabolic activation, ACR induces GA-DNA adducts at the N7 and N3 positions of guanine and adenine, respectively. Analysis using liquid chromatography–tandem mass spectrometry (LC-MS/MS) revealed the absence of these adducts in the untreated samples, as well as in MEFs exposed to ACR in the absence of S9 fraction (with levels below the limit of detection [LOD]). This suggests a lack of Cyp2e1 activity normally required for the metabolism of ACR to GA in the MEFs. Upon addition of human S9 fraction, N7-(2-carbamoyl-2-hydroxyethyl)-guanine (N7-GA-Gua) levels increased to 11 adducts/108 nucleotides (twice the LOD levels), suggesting limited metabolic activation of ACR despite the enzymatic activity of the S9 fraction (Fig. 1E,F). In contrast, cells exposed to GA showed high DNA adduct levels, with N7-GA-Gua and N3-(2-carbamoyl-2-hydroxyethyl)-adenine (N3-GA-Adenine) observed at 49,000 adducts/108 nucleotides and 350 adducts/108 nucleotides, respectively, after subtracting the trace amounts of contamination from the internal standard (Fig. 1E,F). These observed DNA adducts provide a possible mechanistic basis for the mutation types, the TSB, and the mutational signature arising upon treatment with GA, the reactive metabolite of ACR.
Comparison of the GA signature with PCAWG mutational signatures

We next performed cosine similarity comparison of the putative GA signature with the recently updated PCAWG SBS mutational signatures (Alexandrov et al. 2018) and with known T:A > A:T-rich experimental signatures (Fig. 2A; Supplemental Figs. S7, S9). The highest cosine similarity value (84%) corresponded to PCAWG SBS25 (Fig. 2A). However, unlike the GA signature, neither SBS25 nor any other signatures show TSB for the three
Figure 2. Comparison of GA signature to known signatures. (A) Cosine similarity matrix comparing GA mutational signature with the human PCAWG data (SBS3, -4, -5, -8, -22, -25, -35, -39, and -40) and other A > T-rich mutational signatures from experimental exposure assays using specific carcinogens (7,12-dimethylbenz[a]anthracene [DMBA], urethane, and aristolochic acid [AA]). (B) Comparison of PCAWG SBS4 with two experimentally derived signatures: B[a]P_exp = benzo[a]pyrene mutational signature extracted from HMECs; GA_exp = GA mutational signature extracted from MEF cells. Cosine similarity between the T > N (adenine) components of SBS4 and GA signature is shown on the right. (C) Transcription strand bias analysis for the six mutation types underlying the signatures in panel B. For each mutation type (using the pyrimidine convention), the number of mutations occurring on the transcribed (T) and nontranscribed (N) strand is shown on the left y-axis. The significance is expressed as –log10(P-value) indicated on the right y-axis. (***) P < 10^{-8}, (**) P < 10^{-6}, (*) P < 10^{-2}.

Discussion
ACR and GA exposures induce an almost identical set of tumors in both mice and rats, providing a substantial argument for a GA-mediated tumorigenic effect of ACR (Beland et al. 2015). This is supported by further mechanistic studies showing that lung tissue from mice exposed to ACR and GA displays comparable DNA adduct patterns, as well as similar mutation frequencies in the cII transgene (Manjanatha et al. 2015). Similar observations were
made in the context of in vitro mutagenicity of ACR in human and mouse cells, suggesting the key role for the epoxide metabolite GA to form premutagenic DNA adducts (Besaratinia and Pfeifer 2004). Thus, in keeping with the established ACR/GA carcinogenicity in rodents (IARC 1994; Olstørn et al. 2007; Von Tungeln et al. 2012; Beland et al. 2015), our findings provide new information on the characteristic mutagenic effects of GA and their contribution to tumor development.

The observation that ACR itself is not efficiently metabolized by MEFs is consistent with similar differences reported by previous animal carcinogenicity studies. In neonatal B6C3F1 mice, GA, but not ACR, induces hepatocellular carcinomas, likely because of the inability of neonatal mice to efficiently metabolize ACR (Von Tungeln et al. 2012). Moreover, unlike ACR, GA induces tumors in the small intestine in a dose-dependent manner upon perinatal exposure (Olstørn et al. 2007). Similar differences between GA and ACR mutagenicity, possibly because of limited metabolization of ACR, were observed in vitro (Besaratinia and Pfeifer 2004). We addressed the lack of ACR activation by the addition of human S9 fraction, yet the assessment of DNA adducts suggested limited metabolic activation of ACR with adduct levels substantially lower compared with the direct GA exposure. This may explain the mutagenicity differences observed between GA and ACR. A consistent minor contribution of the GA mutational signature was detected in the majority of ACR clones, whereas it was mostly absent in the Spont clones, suggesting subtle metabolic activation of ACR in the MEFs resulting in low levels of GA. However, a robust mutational signature in the experimental setting was generated exclusively by exposing the cells directly to GA.

Figure 3. Identification of experimental GA signature in the human cancer PCAWG data sets. (A) Scatter plots of the experimental GA_exp and B[a]P_exp mutational signature assignments by mSigAct show reconstruction of tobacco-smoking mutagenicity signature SB54 assignments in cancer types with SB54 present. (Lung.AdenoCA) Lung adenocarcinoma, (Lung.SCC) lung squamous cell carcinoma, (Liver.HCC) liver hepatocellular carcinoma (Head.SCC) head squamous cell carcinoma. The combination of GA_exp and B[a]P_exp mutation counts reconstructed SB54 mutation counts in Lung.AdenoCA and Lung.SCC and, to an extent, in Head.SCC. In liver HCCs, GA counts alone partially reconstructed SB54 mutation counts and indicate GA_exp-positive and B[a]P_exp-negative tumors (third row, right scatter plot). The lines in GA versus B[a]P scatter plots have a slope of 0.3, reflecting the 3:1 ratio of B[a]P:GA mutation counts that reconstruct SB54. (B) Summary of GA mutation assignment analysis of 1584 individual tumors of 19 cancer types from the PCAWG data sets. Assignments were performed using mSigAct (positivity was determined by the signature.presence.test tool at FDR < 0.05) with the PCAWG annotations of signature present in each subtype, in addition to the GA and B[a]P signatures. The tumor types manifesting or lacking SB54 signature of tobacco smoking are labeled accordingly in the column SB54. Asterisk denotes borderline SB54 presence in PCAWG Billiard.AdenoCA (two of 173, 1.16%) and Eso.AdenoCA (two of 347, 0.06%). Proportion indicates percentage of GA-positive tumors within each listed cancer type. (C) The dot plot shows the proportion of mutations assigned to GA signature among other identified signatures (see Supplemental Material) in individual tumors of cancer types not showing the direct effects of tobacco smoking (i.e., lacking signature SB54). Red horizontal lines denote median values (y-axis, 1 = 100%).
2005), underlines the relationship between DNA adduct profiles and the mutational signature of GA. N3-GA-Ade and N7-GA-Gua are depurinating adducts resulting in apurinic/apyrimidinic sites. During replication, these lead to misincorporation of deoxyadenosine, leading to the respective T>A and C>G > A:T transversions observed in the GA signature. The T>A>C>G transitions enriched in the GA signature correspond to the miscoding N1-GA-Ade adduct, the most commonly identified adenine adduct in vitro (Randall et al. 1987; da Costa et al. 2003; Besaratinia and Pfeifer 2005; Ishii et al. 2015). The levels of the guanine adduct were especially high in the GA-exposed MEF cells, whereas the associated C>G > A:T transversions in the resulting postmenopausal clones were less represented. This could reflect differences in DNA repair efficiency concerning the individual guanine and adenine adduct species or the fact that the resulting clones are derived from single cells that selectively immortalized but do not accurately represent the bulk exposed primary cell population in which the GA-DNA adduct levels were measured after exposure. It is also plausible that the excessive and possibly highly cytotoxic N7-GA-Gua adduct burden leads to negative selection of a large number of affected cells.

The established animal models (Beland et al. 2013, 2015) of ACR- and GA-mediated tumorigenesis provide a suitable starting point for a comparison of the mutational signatures obtained from the mouse and in vitro. Next, genome-scale sequencing of human tumors and adduct analysis of normal tissues collected in well-designed molecular epidemiological studies focusing on ACN intake are warranted to provide further evidence that the GA signature mutations identified in various cancer types indeed correlate with the exposure to ACR.

The GA signature has not been identified among the currently known computationally extracted PCAWG signatures (Fig. 2A; Alexandrov et al. 2018). Here we show that a new pattern can be identified in a large subset of pan-cancer tumors when experimentally modeled signatures are combined with sophisticated computational signature reconstruction methods while considering the extended features, such as TSB supported by premutagenic adduct analysis. Such integrated approaches can thus lead to future identification of yet unrecognized carcinogen signatures that may be eluding the solely computation-based analyses of the pan-cancer data.

The quest for understanding the contribution of ACR to cancer development is reflected by recent accumulation of mechanistic data on the compound’s mutagenicity and carcinogenicity in experimental models. The possibly carcinogenic effects of ACR in humans were recommended for re-evaluation by the Advisory Group to the Monographs Program of the International Agency for Research on Cancer (Straif et al. 2014). Our findings related to the reconstruction of signature SBS4 by the experimental signatures of GA and B[a]P, together with the detection of the GA signature in lung and liver cancer, are relevant given the established high content of ACR in tobacco smoke. Compared with the GA effects, experimental B[a]P exposure generates very few T>N (adenine) mutations. However, we cannot exclude a possibility that in the human tissues directly exposed to tobacco smoke the adenine residues can be targeted by carcinogens such as B[a]P derivatives or nitrosamines.

A subset of 184 liver tumor samples identified in this study harbored the GA signature but no features of the B[a]P signature or SBS4 (Fig. 3B; Supplemental Material). Furthermore, we found 217 GA-positive, SBS4-negative tumors of additional 15 cancer types (Fig. 3B,C). The numerous GA-positive, SBS4-negative tumors are of particular interest as they likely reflect dietary and/or occupational exposures to ACR unrelated to tobacco smoking. Overall, our findings offer new insights into the thus-far tenuous association of ACR with human carcinogenesis.

### Methods

#### Source and authentication of primary cells

Primary human-p53 knock-in (Hupki) MEFs were isolated from 13.5-d-old *Trp53<sup>Lox/Lox</sup>* mouse embryos from the Central Animal Laboratory of the Deutsches Krebsforschungszentrum as described previously (Liu et al. 2004). The mice had been tested for specific pathogen-free (SPF) status. The derived primary cells were genotyped for the human TP53 codon 72 polymorphism (Table 1) to authenticate the embryo of origin. Cells from three different embryos (E210, E213, and E214) were used for the exposure experiments (Table 1). All subsequent cell cultures were routinely tested at all stages for the absence of mycoplasma.

#### Cell culture, exposure, and immortalization

The primary MEF cells were expanded in advanced DMEM supplemented with 15% fetal calf serum, 1% penicillin/streptomycin, 1% pyruvate, 1% glucose, and 0.1% β-mercaptoethanol. The cells were then seeded in six-well plates and, at passage 2, were exposed for 24 h to 5 mM ACR (A4058, Sigma-Aldrich) in the presence of 2% human S9 fraction (Life Technologies) complemented with NADPH (Sigma-Aldrich) or the absence of S9 to 10 mM ACR or 3 mM GA (04704, Sigma-Aldrich), or to vehicle (PBS). Exposed and untreated control primary cells were cultured until they by-passed senescence and immortalized clonal cell populations could be isolated (Todaro and Green 1963). The HMEC cultures used in this study for WGS were generated from primary HMECs (passage 4) exposed to B[a]P and propagated in M87A medium to passage 13, as described previously (Stampfer and Bartley 1985, 1988; Garbe et al. 2009; Severson et al. 2014).

#### MTT assay for cell metabolic activity and viability

Cells were seeded in 96-well plates and treated as indicated. Cell viability was measured 48 h after treatment cessation using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Plates were incubated for 4 h at 37°C, and absorbance was measured at 492 nm using the Apollo 11 LB913 plate reader. The MTT assay was performed in triplicate for each experimental condition.

#### Phospho-H2AFX immunofluorescence

Immunofluorescence staining of phosphorylated histone H2AFX (γH2AFX) was performed using phospho-histone H2A.X (Ser139) (20E3) Rabbit monoclonal antibody (9718, Cell Signaling Technology). Briefly, primary MEFs were seeded on coverslips in 12-well plates and, the following day, treated as indicated in duplicate for 24 h. Four hours after treatment cessation, the cells were fixed with 4% formaldehyde for 15 min at room temperature. Following blocking in 5% normal goat serum (31872, Life Technologies) for 60 min, they were incubated with the γH2AFX-antibody (1:500 in 1% BSA) overnight at 4°C. Subsequent incubation with a fluorescein-conjugated secondary antibody (4412, Cell Signaling Technology) was performed for 60 min at room temperature. Coverslips were mounted in Vectashield mounting medium with DAPI (Eurobio). Immunofluorescence images were captured using a Nikon Eclipse Ti.
DNA adduct analysis
GA-DNA adducts (N7-GA-Gua and N3-GA-Ade) were quantified by LC-MS/MS with stable isotope dilution as previously described (da Costa et al. 2003). The DNA was isolated from the cells using standard digestion with Proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. The DNA was subsequently treated with RNase A and T1, extracted with phenol-chloroform, and reprecipitated with ethanol. N7-GA-Gua and N3-GA-Ade were released by neutral thermal hydrolysis for 15 min, using Eppendorf Thermomixer R (Eppendorf North America) set to 99°C. The samples were filtered through Amicon 3K molecular weight cutoff filters (Merck Millipore) to separate the adducts from the intact DNA. The LC-MS/MS used for quantification consisted of an Acuity UPLC system (Waters) and a Xevo TQ-S triple quadrupole mass spectrometer (Waters). The following MRM transitions were monitored with a cone voltage of 50 V and a collision energy of 20 eV: N3-GA-Ade, m/z 223→178; [15NS]N3-GA-Ade (internal standard), m/z 228→183; N7-GA-Gua, m/z 239→152; and [15NS]N7-GA-Gua (internal standard), m/z 244→157 (da Costa et al. 2003).

TP53 genotyping
Exons 4 to 8 of the knocked-in human TP53 gene (NC_000017.11) were sequenced using standard protocols. Sanger sequencing of PCR products was performed at BIOFidal, using the Applied Biosystems 3730xl genetic analyzer. The amplicon and sequencing primers are listed in the Supplemental Methods. Sequences were analyzed using the CodonCode Aligner version 7.1 software.

Library preparation and WES
Refer to the online Supplemental Methods for details on the standard procedures for library preparation and WES, sequencing data preprocessing, read alignment, and the calling of the SBS and indel variants in the MEF and HMEC cell lines.

Bioinformatics and extraction of experimental mutational signatures
Refer to the Supplemental Methods for detailed information on PCA, assessment of sequencing-related artifacts and damage, and computation of the TSB and its significance. The TSB was considered statistically significant at $P \leq 0.05$. To analyze the mutation spectra and treatment-specific mutational signatures, filtered mutations were classified into 96 types corresponding to the six possible base substitutions (C→G, A→T, C→G, G→C, C→G, T→A, T→A, T→A, T→C, T→C, G→C, and the 16 combinations of flanking nucleotides immediately 5’ and 3’ of the mutated base). Mutation patterns were then deconvolved into mutational signatures using NMF (Brunet et al. 2004; Alexandrov et al. 2013b) embedded in the MutSpec suite (Ardin et al. 2016). For details on estimates of the optimal number of signatures to extract, see the Supplemental Methods. The reconstruction error calculation evaluated the accuracy with which the deciphered mutational signatures describe the original mutation spectra of each sample by applying Pearson’s correlation and cosine similarity (Supplemental Fig. S10).

Identification of the experimental signatures in PCAWG data
We used the mutational signature activity (mSigAct v0.10.0) software (Ng et al. 2017) to test for the presence of the experimental mutational signatures of GA and B[a]P in the human primary tumor data from PCAWG study. mSigAct conducts a statistical test for optimal reconstructions of the observed human tumor mutational spectrum with and without the GA mutational signature, in addition to a set of other mutational signatures from the PCAWG study. The 192-class strand-biased versions of the GA and B[a]P mutational signatures (Supplemental Fig. S8; Supplemental Table S4) were used to detect tumors with the experimentally defined signatures present, at high stringency achieved also by incorporating the same TSB information in the 192-class reconstructions of each tumor. To generate a 192-class reconstructed spectrum, the assignment of mutation counts for each 192-class signature is determined by mSigAct and multiplied with the 192-class versions of the PCAWG, GA, and B[a]P mutational signatures. The 192-class versions of each signature and spectrum is equivalent to the 96-class versions when the mutation counts on each strand are summed and then represented in the pyrimidine mutations (C→A, C→G, G→T, T→A, T→C, T→G). Specifically, B[a]P was added to cancer types with tobacco-smoking SBS4 and the 192-class signatures was used in these cancers to reconstruct SBS4.
For other signatures and cancers without evidence of SBS4 present, only GA was used to reconstruct the tumor spectra. This was followed by computing the likelihood ratio test between the original spectrum and the reconstructed tumor. A total of 1673 tumor samples from the PCAWG repository from 20 cancer subtypes were interrogated. We excluded hypermutated and recently identified AA signature–containing tumors (Ng et al. 2017) as the presence of strong T>A signature adversely affected the reconstruction process. A set of active mutational signatures were obtained from the PCAWG annotations of each cancer subtype, with flat signatures (SB5, SB8) removed to improve the sparsity of the mutation assignments. Final assignments of mutations to each mutational signature were performed by using the 96-class mutational signatures. Further fine-tuning was conducted using parameters for a negative binomial model, and the FDR was adjusted for mutational signature presence (FDR < 0.05).

The proportion matrices of the strand-biased and NMF versions of the experimental GA signature, the GA signature normalized to the human genome trinucleotide frequency to allow for human PCAWG data screening, and the strand-biased and NMF versions of the whole-genome B[a]P signature are available in Supplemental Table S4. The statistics underlying the assignment of GA_exp to PCAWG cancer data sets (for the individual BioSample accession numbers, refer to Supplemental Tables S12, S13). The WES data reported here are a two-year drinking water exposure.

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data access

Aligned WES reads from the primary MEF cells and clones arising from ACR- and GA-treated cultures and immortalized spontaneously, as well as Sanger sequencing files, have been submitted to the NCBI BioProject database (BioProject; https://www.ncbi.nlm.nih.gov/bioproject) under accession number PRJNA238303 (for the individual BioSample accession numbers, refer to Supplemental Tables S12, S13). The WES data reported here are a new extension of the BioProject PRJNA238303 dedicated to systematic identification of mutational signatures of carcinogenic agents (Olivier et al. 2014).

FPR and FDR estimation for GA signature detection in synthetic tumors

To determine how often false positives arise when detecting the GA signature with mSigAct and to accurately estimate the FDR of the detection of GA signature, we performed a deeper validation analysis. We generated 2000 synthetic tumors with signatures from the PCAWG-7 data set and assignments sampled from the assignments to each signature in the PCAWG-7 data set, which represented the tumor types in which we found GA signature present, with similar signatures and mutation burdens associated with each signature. The synthetic tumors had the same frequency of observing a particular signature for a cancer type, similar to the PCAWG-7 tumors. One hundred tumors per 20 tumor types (included in the main analysis and listed in Supplemental Table S9) have been generated, with 1015 of the tumors harboring GA signature and 985 of GA_exp to PCAWG cancer data sets (Supplemental Table S8), per cancer type distribution (Supplemental Table S7). The proportion matrices of the strand-biased and NMF versions of the whole-genome B[a]P signature are available in Supplemental Table S4. The statistics underlying the assignment of GA_exp to PCAWG cancer data sets (P-values for “signature.presence.test” and cosine similarity between the reconstruction and spectra) are summarized in Supplemental Table S8.

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Author contributions: M.Z., M.K., and J.Z. drafted the manuscript and prepared figures. M.I.C. and F.A.B. performed DNA ad-duct analyses. M.Z., M.P., S.V., and M.K. performed laboratory studies under the supervision of M.R.S., J.M., A.H., M.H., and J.Z.; M.Z., A.W.T.N, M.A., C.R., V.C., A.R., L.B., M.O., and S.R.G performed computational analyses and prepared relevant display items. M.Z., A.W.T.N., A.H., K.G., J.M., M.O., F.A.B., M.K., and J.Z. edited the manuscript, and M.Z., K.G., M.H., M.K., and J.Z. designed the study.

DNA adduct reactions of the whole-genome B[a]P signature are available in Supplemental Table S4. The statistics underlying the assignment of GA signature, we established the true-positive

decoding, with 1015 of the tumors harboring GA signature and 985 of GA_exp to PCAWG cancer data sets (Supplemental Table S8), per cancer type distribution (Supplemental Table S7). The proportion matrices of the strand-biased and NMF versions of the whole-genome B[a]P signature are available in Supplemental Table S4. The statistics underlying the assignment of GA_exp to PCAWG cancer data sets (P-values for “signature.presence.test” and cosine similarity between the reconstruction and spectra) are summarized in Supplemental Table S8.

For other signatures and cancers without evidence of SBS4 present, only GA was used to reconstruct the tumor spectra. This was followed by computing the likelihood ratio test between the original spectrum and the reconstructed tumor. A total of 1673 tumor samples from the PCAWG repository from 20 cancer subtypes were interrogated. We excluded hypermutated and recently identified AA signature–containing tumors (Ng et al. 2017) as the presence of strong T>A signature adversely affected the reconstruction process. A set of active mutational signatures were obtained from the PCAWG annotations of each cancer subtype, with flat signatures (SB5, SB8) removed to improve the sparsity of the mutation assignments. Final assignments of mutations to each mutational signature were performed by using the 96-class mutational signatures. Further fine-tuning was conducted using parameters for a negative binomial model, and the FDR was adjusted for mutation-
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