Mutation footprints of small-insertion-and-deletions from large cancer genomics data

Mo Liu, Arnoud Boot, Qi Zheng, Shenli Zhang, Runxi Shen, Nanhai Jiang, Willie Yu, Steven G. Rozen

**Abstract**

Somatic mutations resulting from various mutational processes are key drivers of tumorigenesis. Mutational signatures, which are distinctive patterns left by these processes, can be identified through experimental exposures or computational deconvolution of mutation catalogs. In this study, we analyzed over 7,000 whole genomes from the PCAWG (Pan-Cancer Analysis of Whole Genomes) and the HMF (Hartwig Medical Foundation) cohorts to create a comprehensive collection of ID (small insertions and deletions) mutational signatures using a hierarchical Dirichlet process-based approach. This analysis led to the identification of 15 novel signatures, in addition to the 23 currently cataloged in COSMIC. More specifically, we identified one novel signature, H\_ID29, associated with TOP1-TAM (Topoisomerase1 transcription-associated mutagenesis), using CRISPR/Cas9-induced knockout cells. Moreover, we identified three new dMMR (defective DNA mismatch repair) signatures—H\_ID33, H\_ID37, and H\_ID38—characterizing short deletions or insertions in repeat units within tumors exhibiting high mutation burdens. Notably, five ID signatures demonstrated significant gender bias. Our examination of signature contributions to cancer genes revealed that C\_ID3, associated with tobacco exposure, accounts for nearly 50% of IDs in LRP1B, which is implicated in lung carcinogenesis. This work establishes an expanded collection of ID signatures, validates a novel signature through functional modeling, elucidates distinct mutational processes, and offers insights into biological implications through extended sequence investigation and trait associations. This comprehensive characterization of ID signatures from over 7,000 genomes enhances our understanding of the mutational processes shaping cancer genomes.

**Introduction**

Somatic mutations are caused by various mutational processes and represent a driving force behind tumorigenesis and cancer development (Alexandrov et al. 2014). The mutations can arise from both endogenous and exogenous sources. These mutations can result from both endogenous sources, such as 5-methylcytosine (5mC) deamination or defective DNA repair mechanisms (Davies et al. 2017; Cooper et al. 2010; Grolleman et al. 2019), and exogenous sources, including exposure to chemical carcinogens in tobacco smoke or certain herbal medicines (Alexandrov et al. 2016; Ng et al. 2017). Mutational signature analysis provides insights into cancer etiology, prognosis, prevention, and mutational signatures can serve as biomarkers for mutagenic exposures (Boot et al. 2022; Davies et al. 2017; Dziubańska-Kusibab et al. 2020; Grolleman et al. 2019).

Mutational signatures are distinctive patterns of mutations left on genomes by specific mutagenic processes or exposures. They can be identified through two approaches: (1) exposing cultured cells, organoids, or experimental animals to suspected mutagens or perturbing DNA repair pathways and then sequencing the affected genomes (Boot et al. 2018; M. N. Huang et al. 2017; Kucab et al. 2019; Caipa Garcia et al. 2024; Riva et al. 2020); and/or (2) using machine learning to deconvolute large-scale somatic mutation data (Alexandrov, Kim, et al. 2020; Alexandrov et al. 2014; Nik-Zainal et al. 2012; Degasperi et al. 2022; Chen et al. 2024; Jin et al. 2024). For instance, data mining of liver cancer genomes detected several types of mutational signature due to the aristolochic acid exposure. These consisted of single-base-substitution (SBS), double-base-substitution (DBS), and insertion-and-deletion (ID/indels) signatures. These were further validated in cell-culture experiments (Chen et al. 2024).

While the characterization of mutational signatures has primarily concentrated on SBSs, ID signatures also offer valuable insights into mutagenic mechanisms. For instance, the tobacco smoking-associated mutational process not only includes C>A (SBS4) and CC>AA (DBS2) changes but also involves the removal of 1 bp C from polyC sequences of lengths 1-5, as indicated by ID3. However, the investigation of ID signatures has been comparatively neglected. To date, COSMIC v3.4 has collected 99 reference SBS signatures but only 23 ID signatures (<https://cancer.sanger.ac.uk/signatures/>).

<paragraph needs revisiting> The classification of ID mutational signatures typically involves the examination of several key features, including indel length, sequence context and indel type. Indel length refers to the number of nucleotides inserted or deleted, ranging from a single base pair to larger genomic fragments. Sequence context encompasses the nucleotide composition surrounding the indel site, which may provide insights into the underlying mutagenic mechanisms or sequence preferences. In this context, we do not consider complex indel events involving a combination of insertions and deletions. It considers insertions and deletions of single base pair of C or T, longer fragments from repeats or microhomologies, and result in 83 indel types (ID83).

In this study, we collected somatic mutation data from over 7,000 tumor genomes across two large pan-cancer datasets: PCAWG (Pan-Cancer Analysis of Whole Genomes) [Alexandrov, Ally, et al. 2020] and HMF (Hartwig Medical Foundation) [Priestley et al. 2019]. By systematically analyzing and classifying ID mutational signatures in these cancer genomes using a Hierarchical Dirichlet Process-based tool, we established a repertoire of 33 ID mutational signatures, including 15 novel signatures and several updated known signatures. We validated a novel ID mutational signature associated with TOP1-TAM (Topoisomerase 1-transcription-associated mutagenesis) within the context of RNASEH2B deficiency by investigating the genetic background and conducting in vitro experiments. Additionally, leveraging the higher rate of microsatellite instability (MSI) in the HMF dataset, we identified three novel ID signatures significantly associated with MSI status.

**Results**

***De novo* ID mutational signature discovery from large cohorts with mSigHdp.**

As Non-negative Matrix Factorization (NMF) is widely used for signature discovery analysis, the tool based on a non-parametric Bayesian approach demonstrates significant advantages. This approach allows for the automatic inference of optimal solutions and the sensitive and accurate extraction of mutational signatures from large cohorts. The development of Hierarchical Dirichlet Process (HDP) based extraction model mSigHdp allows a more sensitive and accurate extraction of ID signatures from large scales of genomics data (Liu et al. 2023). We performed *de novo* mutational signature analysis using mSigHdp on a total of 7,013 whole-genome sequencing (WGS) samples. This dataset comprises 2,780 genomes from the PCAWG dataset and 4,233 genomes from the HMF dataset. The extraction was performed in three ways: (1) all samples together, (2) samples with high tumor mutation burdens (TMBs, details in Method) and (3) analyzing each individual tumor type separately to identify tumor-type-specific rare signatures (Figure 1A).

We then consolidated highly similar signatures from all extractions and removed the ones that can be reconstructed by other signatures. Next, we compared our mSigHdp-extracted signatures to those in COSMIC v3.4, and categorized them into three groups: (1) previously reported signatures (matching COSMIC v3.4 with cosine similarity > 0.85), labeled "C\_IDX" (Figure 1B, Figure S1); (2) merged signatures combining multiple COSMIC v3.4 signatures; and (3) novel signatures not fitting the previous categories, labeled "H\_IDX" (Figure 1C). Our analysis concentrates on groups (1) and (3), omitting merged signatures as they are explicable by known signatures from (1). In total, we identified 33 ID mutational signatures.

**Previously report signatures**

Our analysis successfully reproduced 18 out of 23 COSMIC (v3.4) ID signatures. The remaining 5 signatures were either derived from whole-exome sequencing (WES) data (e.g., ID15 and ID16) or from studies not utilizing PCAWG or HMF data (e.g., ID20, ID21, ID22). In summary, mSigHdp's capability to identify nearly all COSMIC signatures underscores its reliability in mutational signature analysis..

Furthermore, several noteworthy differences were observed, and we believe that mSigHdp provides a more biologically reasonable analysis: (1) In contrast to the C\_ID9 identified in our extraction, the COSMIC ID9 signature exhibits a near-depletion of the INS:1:T:5+ motif. This discrepancy may arise from the prevalence of the INS:1:T:5+ peak in almost all tumors. Biologically, a mutagenic process removing a single thymine base from polyT sequences of lengths 1-4 would likely occur in longer polyT sequences as well. (2) The mSigHdp C\_ID5 signature incorporates elements from both COSMIC ID5 and ID8, despite a cosine similarity of 0.94 to COSMIC ID5. Our analysis revealed no tumor samples supporting COSMIC ID5 in isolation. We examined PCAWG tumors with reported ID5 activity from Alexandrov et al., finding that nearly all of these tumors (1282 out of 1295) also exhibited ID8 signals characterized by long deletions at single repeats or microhomology (Sup Table). These findings suggest that the mutational process represented by ID5 is also responsible for long deletions in these contexts. Additionally, we identified tumors that support C\_ID8 alone (Figure S2). Overall, our analysis indicates that C\_ID5 provides a more comprehensive view of genomic alterations rather than simply merging ID5 and ID8. Although the long deletion patterns are highly similar between ID5 and ID8, they exhibit distinct preferences in deletion length: ID5 primarily features long deletions less than 10 nt, with almost no deletions longer than 30 nt, while ID8 displays a more even distribution of deletions ranging from 5 to over 30 nt (Figure S2). (3) Compared to COSMIC ID17, we found that C\_ID17 signature enhanced the pattern of deletions at repeats and microhomologies, showing similarities to ID8 deletions. Boot et al. identified and validated an association between the TOP2A (Topoisomerase 2A) p.K743N mutation and ID17 (also known as ID\_TOP2A) using a yeast model. Our analysis revealed that our C\_ID17 signature demonstrates a closer resemblance to the ID\_TOP2A signature identified by Boot et al. than to COSMIC ID17 (Figure S2B, C, cosine similarity = 0.982).

**Signature activity**

We evaluated the activity of our 33 mSigHdp signatures using mSigAct, a tool incorporating statistical analysis for the presence of a given signature (Jiang, Wu, and Rozen 2024). Tumors with high TMB often exhibit large amounts of 1 bp T deletions and/or insertions in polyT sequences (DEL:T:1:5+ and/or INS:T:1:5+). These predominant peaks can obscure other signals, affecting the accuracy of signature assignment analysis. To address this, we propose a novel approach for analyzing signature assignments in high TMB tumors: first, we removed DEL:T:1:5+ and INS:T:1:5+ mutations to enhance the visibility of other peaks, resulting in ID81 catalogs/signatures. Next, these ID81 catalogs were reconstructed from the ID81 signatures. After this reconstruction, DEL:T:1:5+ and INS:T:1:5+ mutations were reintroduced, and the signature assignment analysis was performed by comparing the original and reconstructed catalogs with C\_ID1 and C\_ID2. This method allows for the extraction of more detailed information in MSI tumors that may be obscured by the presence of DEL:T:1:5+ and INS:T:1:5+.

Consistent with previous studies, C\_ID1, C\_ID2, C\_ID5, and C\_ID8 were detected across most cancer types, with C\_ID3 showing a strong presence in lung cancers and C\_ID13 prominently observed in skin cancers. The novel signatures identified by mSigHdp were generally active in fewer cancer types compared to COSMIC signatures, with the exception of H\_ID24 and H\_ID25, which were widespread across various cancers (Figure 2). We analyzed the correlations between our ID signature activities and the SBS signature activities from Degasperi et al. in PCAWG and HMF samples (Figure S3). Our analysis confirmed strong correlations among C\_ID3, SBS4, and SBS92, all linked to tobacco-induced lung cancer (Spearman correlation coefficients: 0.74 between C\_ID3 and SBS4, 0.56 between C\_ID3 and SBS92, Figure 3A). Additionally, a strong correlation was observed between C\_ID13 and SBS7a, both associated with UV exposure (Spearman correlation coefficient: 0.73, Figure 3A).

Highly correlated genes were clustered into several interesting modules: we identified a module of four signatures related to cell replication: SBS1 (5mC deamination during cell replication), SBS18 (linked to reactive oxygen species), C\_ID1 and C\_ID2 (replication slippage) (Figure 3B). A dHR module was identified consisting of SBS3, SBS8 and C\_ID6: SBS3 and C\_ID6 were classified related to defective HR DNA damage repair, which suggests the potential etiology of SBS8 (Figure 3C). A correlation module was also noted, including C\_ID14, SBS35, SBS88, and SBS93 (Figure 3D). SBS88, and SBS93 are frequently observed in gastrointestinal (GI) tracts, while SBS35 is associated with platinum treatment, suggesting a possible etiology for C\_ID14 related to platinum treatment in GI tract cancers. Notably, we identified a dMMR (defective DNA mismatch repair) module comprising five signatures: SBS44, C\_ID7, H\_ID33, H\_ID37, and H\_ID38 (Figure 3E). Interestingly, only 1 out of 7 dMMR SBS signatures was strongly associated with indels, indicating a distinct mutational process underlying SBS44 compared to the other SBS signatures.

**Novel Signatures**

**MSI signatures**

Leveraging the higher proportion of microsatellite instability (MSI) tumors in the Hartwig Medical Foundation (HMF) dataset, we identified additional MSI-associated ID signatures beyond COSMIC ID7: H\_ID33, H\_ID37 and H\_ID38 (Figure 4A). The 4 MSI associated ID signatures show strong enrichment of presence and high activity in MSI tumors compared to MSS tumors (Sup Table). COSMIC v3.4 lists seven SBS signatures associated with mismatch repair (MMR) deficiency: SBS6, SBS14, SBS15, SBS20, SBS21, SBS26, and SBS44. These signatures often co-occur and show overlapping peaks. For instance, SBS44 and SBS20 have nearly identical C>A mutation patterns, while SBS6 and SBS15 share a predominant CCG>CTG peak. We observed similar patterns in ID signatures, with H\_ID33, H\_ID37, and C\_ID7 all showing >1bp deletions at repeat sequences, but they preferentially characterize different ID types (Figure 4A). These four signatures have significantly higher activities in MSI tumors compared to MSS tumors (Figure 4B). In addition, these MSI signatures account for over 50% of indels in MSI tumors but are less prevalent in microsatellite stable (MSS) tumors (Figure 4C).

C\_ID7 is characterized primarily by 1 bp deletions of C or T from long C or T sequences, while H\_ID33 mainly represents TT deletions from 4-5 TT repeats. H\_ID37 is primarily associated with TTT deletions from 3 TTT repeats (Figure 4D). In contrast to these deletion patterns, H\_ID38 predominantly describes insertions, including 1 bp and 2 bp insertions at long repeats. It consists of two main scenarios: when a sample predominantly features insertions, these primarily involve TT repeats (e.g., CPCT02030532T, DRUP0105003T in Figure 4D); when a sample has a more balanced amounts of deletions and insertions, a wider variety of dinucleotide insertions is observed (e.g., SP94933, SP102133, CPCT02450014T, WIDE01010606T in Figure 4D). Notably, some MSS tumors exhibit a high ratio of MSI signature activity, likely due to their strong MSI characteristics, such as high indels and single-base substitution (SBS) mutation loads, despite being classified as MSS. Furthermore, we investigated the potential of the MSI signature activity ratio as a biomarker for detecting MSI status. An area under the receiver operating characteristic curve (AUROC) analysis was conducted to compare the MSI ratio with pre-labeled MSI status, resulting in an AUROC of 0.81, indicating strong predictive capability (Figure 4E).

**A novel ID-TOP1 signature**

We identified a novel mutational signature, H\_ID29, characterized by 1-3 bp deletions from two repeats or microhomology, with strong support from both PCAWG and HMF samples (Figure 5A, B). Notably, two PCAWG samples displayed significant H\_ID29 activity: a skin melanoma genome (SP103894) contained 3,772 H\_ID29 mutations, while a breast cancer genome (SP5559) had 949 H\_ID29 mutations. Analyzing additional samples allowed for the detection of rare signatures within the PCAWG datasets.

Upon re-examining the rnh201Δ *Saccharomyces cerevisiae* genomes, we observed 2 bp deletion patterns similar to those of H\_ID29, although deletions within microhomology were depleted (Williams et al. 2019; Conover et al. 2015, Figures S4D). We established an RNASEH2B deficiency model using the CRISPR/Cas9 system in the HEK293T cell line, and whole genome sequencing revealed patterns consistent with H\_ID29 (Figure 5C, D). The primary peak predominantly represents the deletion of CT from 5’-CTCT-3’ (or AG from 5’-AGAG-3’), as indicated by the extended sequence analysis of RNASEH2B-KO cell lines and the five genomes exhibiting the highest H\_ID29 activity (Figure 5E, F). The weights of each nucleotide on each position suggest a preference of NTNT sequences at deletion sites for both H\_ID29 and C\_ID4 (Figure 5G).

Our extended sequence analysis reveals differences in sequence context: H\_ID29 tends to delete CT/TC within tandem repeats, whereas a common NTNT motif is found in microhomologies (Figure 6A). The results from tumors with high H\_ID29 activities demonstrate a highly similar patterns in deletion sequences when compared to our RNASEH2B null HEK293T cells and the published Rnaseh2b KO mouse tumors and RNase H2 null RPE1 cells (Figure 6B-D). In contrast, C\_ID4 displays a more balanced preference for deleting CT and TT within tandem repeats, with a common CTNTN motif present in microhomologies (Figure 6E).

Collectively, our analysis presents H\_ID29 as a novel mutational signature identified through de novo extraction from cancer genomic data, suggesting its association with TOP1-dependent deletions in RNASEH2A and/or RNASEH2B deficient cells. Previous work by Reijns et al. developed RNASEH2A-deficient mammalian cell lines and Rnaseh2b-KO mouse intestinal cancer models, revealing the enrichment of 2 bp deletions from tandem repeats or microhomology (Reijns et al. 2022, Figure S4B, C). Our findings indicate that H\_ID29 more closely resembles the mutational spectra from these knockout models than ID4, with average cosine similarities of 0.945 in mouse models, 0.965 in human cell line models, and 0.947 in yeast models, compared to C\_ID4’s average cosine similarities of 0.690, 0.721, and 0.798 (Figure S4 B-D). (ID29 doesn’t have 3-4bp deletions). Thus, H\_ID29 provides a more accurate representation of the genomic footprints associated with TOP1-TAM (transcription-associated mutagenesis) during the cleavage of embedded ribonucleotides in the absence of RNASEH2A and/or RNASEH2B (S. N. Huang, Ghosh, and Pommier 2015; Sparks and Burgers 2015; Chon et al. 2009).

**Extended sequence context characterization of novel signatures**

We observed that some signatures share dominant peaks, prompting an investigation into whether they represent distinct mutational processes. To explore this, we examined the extended sequence contexts of samples with high activity for these signatures to better understand the preferential sequence context of the indels.

Both H\_ID24 and C\_ID9 display a similar pattern of 1 bp C deletions (DEL:C:1:0). However, analysis of their extended sequence contexts revealed that H\_ID24 preferentially deletes C from 5'TTTCX3', while C\_ID9 favors deletion from 5'XCTTT3' (Figure 7A). These findings suggest that H\_ID24 and C\_ID9 originate from distinct mutational processes: H\_ID24 preferentially removes cytosine 3' of poly-T sequences, whereas C\_ID9 removes cytosine 5' of poly-T sequences. Additionally, DEL:C:1:0 is prominent in H\_ID32, where the extended sequence surrounding DEL:C:1:0 shows a balanced ratio of A and T.

Furthermore, both H\_ID27 and C\_ID14 exhibit high levels of INS:C:1:0, with extended sequence analysis indicating that the INS:C:1:0 of these signatures preferentially occurs within poly-G sequences (Figure 7B). Several HMF samples strongly support the presence of H\_ID27, leading us to propose that H\_ID27 is a variant form of C\_ID14, characterized by a lower proportion of INS:T:1:5+ (Figure S7).

H\_ID32 primarily consists of 1 bp C/T insertions and deletions in TA-rich sequences, while H\_ID26 describes T insertion sequences with a higher number of A bases (Figure 7C, D). Although H\_ID27 and H\_ID28 both display 1 bp C insertions (INS:C:1:0), they represent two distinct processes: H\_ID27 preferentially inserts a cytosine 3' of poly-A sequences, while H\_ID28 inserts a cytosine or guanine 3' of poly-G sequences. Based on these observations, we conclude that H\_ID27 and H\_ID28 arise from two distinct mutational processes rather than an over-splitting of a single process. Additionally, the primary mutation types in H\_ID28 exhibit a similar pattern in extended sequence context analysis; specifically, the insertion of repeats, along with 1 bp C and 1 bp T, tends to occur 3' of poly-G sequences (Figure 7B, E)

**Gender comparison**

It is intriguing to investigate whether mutational processes, as represented by mutational signatures, exhibit gender-specific patterns. We firstly exclude the cancer types with strong gender characteristic including prostate cancer (only in males), uterus cancer, breast cancer and ovary cancer (only in females). To assess gender-specific prevalence of mutational signatures, we employed Fisher's Exact Test. From a total of 5,000 patients with available gender data, we identified 7 signatures demonstrating significant gender-specific associations: 5 signatures (C\_ID3, C\_ID5, C\_ID8, C\_ID13 and H\_ID25) showed a significant prevalence in male patients. Conversely, 3 signatures (C\_ID2, C\_ID9, C\_ID12) were more commonly observed in female patients (Figure 8A). Some of the observations can be explained biologically, for example, C\_ID3 is associated with tobacco smoking which has a higher proportion of male patients.

**Signature attributions to cancer genes**

We examined the contribution of mutational signatures to indels in cancer genes, focusing on 581 Tier 1 genes from the Cancer Gene Census (Sondka et al. 2018). We excluded DEL:1:T:5+ and INS:1:T:5+ from our analysis, as these indels are primarily contributed by C\_ID1 and C\_ID2, and single-base thymine insertions/deletions in poly-T regions rarely have significant biological impacts. The genes most frequently affected by insertions were CAMTA1, ERBB4, FHIT, FOXP1, LPP, LRP1B, NRG1, PRDM16, PTPRT, and RUNX1. Several signatures with known causes contribute to these insertions, including DNA replication slippage, defective MMR, defective HR DNA damage repair, and UV exposure. Deletions most frequently affected CAMTA1, CUX1, ERBB4, FHIT, FOXP1, GPHN, LPP, LRP1B, NRG1, and PRDM16 (Figure 8B). These deletions are primarily caused by DNA replication slippage and defective MMR. Notably, the tobacco smoking signature (C\_ID3) contributes to nearly 50% of cytosine-deletions and thymine-insertions in LRP1B. Previous research has linked LRP1B mutations to lung cancer pathogenesis (Ding et al. 2008). Our analysis potentially uncovers the mutational processes responsible for LRP1B mutations.

**Discussion**

Using a novel nonparametric Bayesian approach, we analyzed over 7,000 whole-genome sequencing (WGS) tumor samples encompassing 25 cancer types from the Pan-Cancer Analysis of Whole Genomes (PCAWG) and Hartwig Medical Foundation (HMF) cohorts. As the first study using >7000 genomes for ID signature analysis, our study established a comprehensive collection of 32 ID mutational signatures. We identified one indel signature associated with RNaseH2B deficiency, validating this finding via CRISPR/Cas9 system. Additionally, we found three ID signatures strongly linked to microsatellite instability (MSI) status, which implement the understanding of indel footprints left my defective MMR mechanism. We also performed an extended sequence context analysis to understand more information behind the formation of mutational signatures.

We attempted signature extraction using SigProfilerExtractor, an NMF-based model recognized for its strong performance in signature analysis (Figure S5, Islam et al. 2022). However, this method proved ineffective for our large cohort, yielding an optimal solution of K=12 but failing to identify either novel signatures or previously established COSMIC signatures. Similarly, we employed the minimum-volume NMF model, MuSiCal, across all genomes, which resulted in an optimal K=13 (Figure S6, Jin et al. 2024). This limitation is likely due to the challenges Non-negative Matrix Factorization faces in managing the high data sparsity associated with indels. In contrast, using mSigHdp, we identified 30 mutational signatures in the extraction of all genomes, with 24 included in the finalized collection. Our study highlights the effectiveness of mSigHdp for mining large datasets and demonstrates its ability to reveal novel signatures in highly sparse, low-count data.

As sequencing technology advances, numerous national cancer research initiatives are underway. Mutational signatures have proven valuable in predicting cancer treatment efficacy and tracing disease etiology. By integrating more data into mutational signature analysis, we anticipate discovering additional signatures that characterize genomic mutational processes. Furthermore, we expect the development of mutational signatures as clinical biomarkers to enhance cancer diagnosis and treatment strategies.

**Materials and methods**

Data source

We considered two large pan-cancer whole genome cohorts: the PCAWG cohort which comprises 2780 whole-genome–sequenced samples; and the HMF cohort, comprising 3417 whole-genome–sequenced tumor samples. The mutational spectra used for mutational signature extraction were provided in Table S1. Variant calls for 2,780 WGS samples from the ICGC/TCGA (International Cancer Genome Consortium/The Cancer Genome Atlas) Pan-Cancer Analysis of Whole Genomes Consortium and clinical traits were obtained from the ICGC data portal (<https://dcc.icgc.org/releases/current/Projects/>, now the repository is retired, the data was downloaded on 9 May, 2024). Variant calls for 3417 WGS samples from the HMF cohort were obtained from xxxx. Clinical traits such as cancer type, age and gender of the HMF genomes were found from supplementary files of Priestley et al., 2019. These data was also provided in Table S2. The COSMIC Cancer Gene Census was used to identify known cancer driver genes (Sondka et al., 2018, downloaded from <https://cancer.sanger.ac.uk/cosmic/census?tier=1> on 9 Jun, 2024).

**Mutational signature extraction**

We used mSigHdp (v 2.1.2) for de novo mutational signature extraction analysis. when applying to all samples de novo mutational signatures were extracted using the cancer type to construct the hierarchy; when applying to genomes of each cancer type and high TMB genomes, the de novo mutational signatures were extracted with 2-layer HDP mixture models. In both scenario, we used the following parameters: seedNumber=1234, burnin=1000, bunin.multiplier=20, post.n = 200, post.space = 100, num.child.process=20, gamma.alpha=1, gamma.beta=50.

For SigProfilerExtractor, *de novo* mutational signatures were extracted from each mutational matrix using SigProfilerExtractor and default parameters (v1.1.24). NMF was performed with finding solutions between k = 10 and k = 30 signatures; each factorization was repeated 100 times. We ran MuSiCal with the following parameters: min\_n\_components=9, max\_n\_components=33, method=“mvnmf”, n\_replicates=100, max\_iter=10000, min\_iter=1000.

Match mSigHdp signatures into COSMIC reference signatures

The mSigHdp signatures were matched to previously identified COSMIC signatures (v3.4). We compared all de novo signatures to COSMIC signatures and categorized them into three groups: (1) known signature: if a mSigHdp signature has a cosine similarity of ≥ 0.9 with a COSMIC signature; (2) merged signatures: if a mSigHdp signatures can be reconstructed by at most 4 COSMIC signatures with a reconstructed similarity of ≥ 0.9; (3) novel signatures: the signatures do not fit into the known signatures or the merged signatures.

Signature attribution analysis

The 33 ID signature activities were attributed to each sample using a two-step approach: first, we used find\_best\_reconstruction\_QP function of SigTools R package (v1.0.7) to which provides a fast signature attribution analysis with quadratic programming optimization; second, we used the PresenceAttributeSigActivity function and default parameters in mSigAct R package (v3.0.1) to further refined the result from the previous step.

Cell line culture and RNaseH2B CRIPSR

Need help here

MSI/MSS status and high/low TMB status

For PCAWG genomes, the MSI status was evaluated by the PCAWG working group and obtained from the synapse repository (<https://www.synapse.org/#!Synapse:syn8016399>, the data was downloaded on May 2022). For HMF genomes, the MSI status was downloaded from the supplementary data of Priestley et al., 2019. The genomes with >14,000 IDs and >15,000 SBSs were labelled as high TMB tumors. The thresholds were selected based on the minimum number of mutations of the pre-defined MSI tumors.

Gender enrichment by Fisher’s exact test

To evaluate the presence of mutational signatures in male and female, we used Fisher's Exact Test to determine the statistical significance of signature enrichment by gender. We quantified the frequency of the presence of each signature (exposure > 0) in both groups and applied the test to assess associations. A p-value threshold of 0.05 was established to indicate significant enrichment.

Extended sequence context

To analyze a specific signature and indel type of interest, we first identified the 5 genomes with the highest contribution of the corresponding signature activity. From these genomes, we extracted all indels of the relevant type. We then examined the nucleotide sequence within a 21-base pair window centered on each indel site (±10 nucleotides from the indel position). For each position within this window, we calculated the frequency of each nucleotide (A, T, C, and G). The logo was plotted based on the frequency matrix by seqLogo function of seqLogo R package (version 1.71.0)

**Acknowledgement**

**Funding**

Reference

Alexandrov, Ludmil B., Adrian Ally, Kathryn Alsop, Eva G. Alvarez, Fernanda Amary, Samirkumar B. Amin, Brice Aminou, et al. 2020. ‘Pan-Cancer Analysis of Whole Genomes’. *Nature* 578 (7793): 82–93. https://doi.org/10.1038/s41586-020-1969-6.

Alexandrov, Ludmil B., Young Seok Ju, Kerstin Haase, Peter Van Loo, Iñigo Martincorena, Serena Nik-Zainal, Yasushi Totoki, et al. 2016. ‘Mutational Signatures Associated with Tobacco Smoking in Human Cancer’. *Science* 354 (6312): 618–22. https://doi.org/10.1126/science.aag0299.

Alexandrov, Ludmil B., Jaegil Kim, Nicholas J. Haradhvala, Mi Ni Huang, Alvin Wei Tian Ng, Yang Wu, Arnoud Boot, et al. 2020. ‘The Repertoire of Mutational Signatures in Human Cancer’. *Nature* 578 (7793): 94–101. https://doi.org/10.1038/s41586-020-1943-3.

Alexandrov, Ludmil B, Serena Nik-zainal, David C Wedge, and Samuel A J R Aparicio. 2014. ‘Signatures of Mutational Processes in Human Cancer’ 500 (7463): 415–21. https://doi.org/10.1038/nature12477.Signatures.

Boot, Arnoud, Mi Ni Huang, Alvin W.T. Ng, Szu Chi Ho, Jing Quan Lim, Yoshiiku Kawakami, Kazuaki Chayama, Bin Tean Teh, Hidewaki Nakagawa, and Steven G. Rozen. 2018. ‘In-Depth Characterization of the Cisplatin Mutational Signature in Human Cell Lines and in Esophageal and Liver Tumors’. *Genome Research* 28 (5): 654–65. https://doi.org/10.1101/gr.230219.117.

Boot, Arnoud, Mo Liu, Nicole Stantial, Viraj Shah, Willie Yu, Karin C. Nitiss, John L. Nitiss, Sue Jinks-Robertson, and Steven G. Rozen. 2022. ‘Recurrent Mutations in Topoisomerase IIα Cause a Previously Undescribed Mutator Phenotype in Human Cancers’. *Proceedings of the National Academy of Sciences* 119 (4): e2114024119. https://doi.org/10.1073/pnas.2114024119.

Caipa Garcia, Angela L., Jill E. Kucab, Halh Al-Serori, Rebekah S. S. Beck, Madjda Bellamri, Robert J. Turesky, John D. Groopman, et al. 2024. ‘Tissue Organoid Cultures Metabolize Dietary Carcinogens Proficiently and Are Effective Models for DNA Adduct Formation’. *Chemical Research in Toxicology* 37 (2): 234–47. https://doi.org/10.1021/acs.chemrestox.3c00255.

Chen, Lei, Chong Zhang, Ruidong Xue, Mo Liu, Jian Bai, Jinxia Bao, Yin Wang, et al. 2024. ‘Deep Whole-Genome Analysis of 494 Hepatocellular Carcinomas’. *Nature*, March. https://doi.org/10.1038/s41586-024-07054-3.

Chon, Hyongi, Alex Vassilev, Melvin L. Depamphilis, Yingming Zhao, Junmei Zhang, Peter M. Burgers, Robert J. Crouch, and Susana M. Cerritelli. 2009. ‘Contributions of the Two Accessory Subunits, RNASEH2B and RNASEH2C, to the Activity and Properties of the Human RNase H2 Complex’. *Nucleic Acids Research* 37 (1): 96–110. https://doi.org/10.1093/nar/gkn913.

Conover, Hailey N, Scott A Lujan, Mary J Chapman, Deborah A Cornelio, Rabab Sharif, Jessica S Williams, Alan B Clark, Francheska Camilo, Thomas A Kunkel, and Juan Lucas Argueso. 2015. ‘Stimulation of Chromosomal Rearrangements by Ribonucleotides’. *Genetics* 201 (3): 951–61. https://doi.org/10.1534/genetics.115.181149.

Cooper, David N, Matthew Mort, Peter D Stenson, Edward V Ball, and Nadia A Chuzhanova. 2010. ‘Methylation-Mediated Deamination of 5-Methylcytosine Appears to Give Rise to Mutations Causing Human Inherited Disease in CpNpG Trinucleotides, as Well as in CpG Dinucleotides’. http://www.hgmd.org.

Davies, Helen, Dominik Glodzik, Sandro Morganella, Lucy R. Yates, Johan Staaf, Xueqing Zou, Manasa Ramakrishna, et al. 2017. ‘HRDetect Is a Predictor of BRCA1 and BRCA2 Deficiency Based on Mutational Signatures’. *Nature Medicine* 23 (4): 517–25. https://doi.org/10.1038/nm.4292.

Degasperi, Andrea, Xueqing Zou, Tauanne Dias Amarante, Andrea Martinez-Martinez, Gene Ching Chiek Koh, João M.L. Dias, Laura Heskin, et al. 2022. ‘Substitution Mutational Signatures in Whole-Genome–Sequenced Cancers in the UK Population’. *Science* 376 (6591). https://doi.org/10.1126/science.abl9283.

Ding, Li, Gad Getz, David A. Wheeler, Elaine R. Mardis, Michael D. McLellan, Kristian Cibulskis, Carrie Sougnez, et al. 2008. ‘Somatic Mutations Affect Key Pathways in Lung Adenocarcinoma’. *Nature* 455 (7216): 1069–75. https://doi.org/10.1038/nature07423.

Dziubańska-Kusibab, Paulina J., Hilmar Berger, Federica Battistini, Britta A.M. Bouwman, Amina Iftekhar, Riku Katainen, Tatiana Cajuso, et al. 2020. ‘Colibactin DNA-Damage Signature Indicates Mutational Impact in Colorectal Cancer’. *Nature Medicine* 26 (7): 1063–69. https://doi.org/10.1038/s41591-020-0908-2.

Grolleman, Judith E., Richarda M. de Voer, Fadwa A. Elsayed, Maartje Nielsen, Robbert D.A. Weren, Claire Palles, Marjolijn J.L. Ligtenberg, et al. 2019. ‘Mutational Signature Analysis Reveals NTHL1 Deficiency to Cause a Multi-Tumor Phenotype’. *Cancer Cell* 35 (2): 256-266.e5. https://doi.org/10.1016/j.ccell.2018.12.011.

Huang, Mi Ni, Willie Yu, Wei Wei Teoh, Maude Ardin, Apinya Jusakul, Alvin Wei Tian Ng, Arnoud Boot, et al. 2017. ‘Genome-Scale Mutational Signatures of Aflatoxin in Cells, Mice, and Human Tumors’. *Genome Research* 27 (9): 1475–86. https://doi.org/10.1101/gr.220038.116.

Huang, Shar-yin Naomi, Sanchari Ghosh, and Yves Pommier. 2015. ‘Topoisomerase I Alone Is Sufficient to Produce Short DNA Deletions and Can Also Reverse Nicks at Ribonucleotide Sites’. *Journal of Biological Chemistry* 290 (22): 14068–76. https://doi.org/10.1074/jbc.M115.653345.

Jiang, Nanhai, Yang Wu, and Steven G Rozen. 2024. ‘A New Approach to the Challenging Problem of Mutational Signature Attribution’. *bioRxiv*. https://doi.org/10.1101/2024.05.20.594967.

Jin, Hu, Doga C. Gulhan, Benedikt Geiger, Daniel Ben-Isvy, David Geng, Viktor Ljungström, and Peter J. Park. 2024. ‘Accurate and Sensitive Mutational Signature Analysis with MuSiCal’. *Nature Genetics* 56 (3): 541–52. https://doi.org/10.1038/s41588-024-01659-0.

Kucab, Jill E., Xueqing Zou, Sandro Morganella, Madeleine Joel, A. Scott Nanda, Eszter Nagy, Celine Gomez, et al. 2019. ‘A Compendium of Mutational Signatures of Environmental Agents’. *Cell* 177 (4): 821-836.e16. https://doi.org/10.1016/j.cell.2019.03.001.

Liu, Mo, Yang Wu, Nanhai Jiang, Arnoud Boot, and Steven G Rozen. 2023. ‘mSigHdp: Hierarchical Dirichlet Process Mixture Modeling for Mutational Signature Discovery’. *NAR Genomics and Bioinformatics* 5 (1): lqad005. https://doi.org/10.1093/nargab/lqad005.

Ng, Alvin W T, Song Ling Poon, Mi Ni Huang, Jing Quan Lim, Arnoud Boot, Willie Yu, Yuka Suzuki, et al. 2017. ‘Aristolochic Acids and Their Derivatives Are Widely Implicated in Liver Cancers in Taiwan and throughout Asia’. https://www.science.org.

Nik-Zainal, Serena, Ludmil B. Alexandrov, David C. Wedge, Peter Van Loo, Christopher D. Greenman, Keiran Raine, David Jones, et al. 2012. ‘Mutational Processes Molding the Genomes of 21 Breast Cancers’. *Cell* 149 (5): 979–93. https://doi.org/10.1016/j.cell.2012.04.024.

Priestley, Peter, Jonathan Baber, Martijn P. Lolkema, Neeltje Steeghs, Ewart de Bruijn, Charles Shale, Korneel Duyvesteyn, et al. 2019. ‘Pan-Cancer Whole-Genome Analyses of Metastatic Solid Tumours’. *Nature* 575 (7781): 210–16. https://doi.org/10.1038/s41586-019-1689-y.

Reijns, Martin A. M., David A. Parry, Thomas C. Williams, Ferran Nadeu, Rebecca L. Hindshaw, Diana O. Rios Szwed, Michael D. Nicholson, et al. 2022. ‘Signatures of TOP1 Transcription-Associated Mutagenesis in Cancer and Germline’. *Nature* 602 (7898): 623–31. https://doi.org/10.1038/s41586-022-04403-y.

Riva, Laura, Arun R. Pandiri, Yun Rose Li, Alastair Droop, James Hewinson, Michael A. Quail, Vivek Iyer, et al. 2020. ‘The Mutational Signature Profile of Known and Suspected Human Carcinogens in Mice’. *Nature Genetics* 52 (11): 1189–97. https://doi.org/10.1038/s41588-020-0692-4.

Sondka, Zbyslaw, Sally Bamford, Charlotte G. Cole, Sari A. Ward, Ian Dunham, and Simon A. Forbes. 2018. ‘The COSMIC Cancer Gene Census: Describing Genetic Dysfunction across All Human Cancers’. *Nature Reviews Cancer* 18 (11): 696–705. https://doi.org/10.1038/s41568-018-0060-1.

Sparks, Justin L, and Peter M Burgers. 2015. ‘Error‐free and Mutagenic Processing of Topoisomerase 1‐provoked Damage at Genomic Ribonucleotides’. *The EMBO Journal* 34 (9): 1259–69. https://doi.org/10.15252/embj.201490868.

Williams, Jessica S., Scott A. Lujan, Zhi-Xiong Zhou, Adam B. Burkholder, Alan B. Clark, David C. Fargo, and Thomas A. Kunkel. 2019. ‘Genome-Wide Mutagenesis Resulting from Topoisomerase 1-Processing of Unrepaired Ribonucleotides in DNA’. *DNA Repair* 84 (December):102641. https://doi.org/10.1016/j.dnarep.2019.102641.