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

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**Introduction**

Somatic mutations, arising from various mutational processes, representing a driving force behind tumorigenesis and cancer development (Alexandrov et al., 2014). Mutations can arise from both endogenous and exogenous sources. The endogenous causes include the deamination of 5-methylcytosince (5mC) or defective DNA repair mechanisms (Cooper et al., 2010; Davies et al., 2017; Grolleman et al., 2019), while the exogeneous causes include the exposures to chemical carcinogens from tobacco smoking or the inappropriate use of certain Chinese herbal medicines (e.g., aristolochic acid, (Alexandrov et al., 2016; Ng et al., 2017). Broadly, mutational signature analysis offers insights into cancer etiology, prognosis, and prevention, as well as biomarkers for mutagenic exposures (Boot et al., 2020; Davies et al., 2017; Dziubańska-Kusibab et al., 2020; Grolleman et al., 2019).

Mutational signatures describe distinctive patterns left by the specific mutagenic processes or exposure leave on genomes, and they can be discovered by two approaches: (1) exposing cultured cells to suspected mutagen or gene editing and sequence the cell genomes (Boot et al., 2018; Huang et al., 2017; Kucab et al., 2019); and/or (2) using machine learning approaches to deconvolute from large scale of somatic mutations, (Alexandrov et al., 2014, 2020; Degasperi et al., 2022; Nik-Zainal et al., 2012). For example, datamining from liver cancer genomics data found the SBS, DBS and ID signature of aristolochic acid exposure, with further validation in in-vitro experimental system (Chen et al., 2024). Collectively, SBS (single-base-substitution), DBS (doublet-base-substitution) and ID (small-insertions-and-deletions, i.e., indels) signatures comprehensively describe the genetic footprints of mutational processes.

While the characterization of mutational signatures has traditionally focused on single nucleotide variants (SNVs), the analysis of ID signatures has gained traction due to their unique properties. To date, COSMIC v3.4 now has collected 99 SBS signatures while only 20 DBS signatures and 23 ID signatures (https://cancer.sanger.ac.uk/signatures/). A recent study reported the most comprehensive collection by adding 40 novel SBS signatures and 18 novel DBS signatures from 18,640 genomes of three big data cohorts (Degasperi et al., 2022). This shows the need of a comprehensive analysis of ID signature extraction from large cohorts.

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 >7000 tumor genomes from two large pan-cancer cohorts: PCAWG [Pan-Cancer Analysis of Whole Genomes (Aaltonen et al., 2020)] and HMF [Hartwig Medical Foundation (Priestley et al., 2019)]. By systematically analyzing and classifying indels mutational signatures in these cancer genomes using a Hierarchical Dirichlet Process based tool, we established a repertoire of 31 indel mutational signatures including 14 novel signatures and some minor update of known signatures. By investigating the genetic background and validation in the in-vitro experimental system, we validate a novel indel mutational signature caused by RNASEH2B deficiency. In addition, taking the advantage of higher MSI (microsatellite instability) rate in HMF cohort, we found 2 new ID signatures significantly associated with defective DNA mismatch repair deficiency.

**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 indel signatures from large scales of genomics data(Liu et al., 2023). We performed de-novo mutational signature analysis using mSigHdp on a total of 7013 whole-genome sequencing (WGS) samples. This dataset comprises 2780 genomes from the PCAWG cohort and 4233 genomes from the HMF cohort. The extraction was performed in two ways: (1) aggregating all samples together and (2) 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 catogrized 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. In total, we identified 31 ID mutational signatures.

**Previously report signatures**

Our analysis successfully reproduced 16 out of 23 COSMIC (v3.4) ID signatures. The remaining 7 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), potentially exhibiting different mutational backgrounds. Notably, the signal associated with ID10 was recapitulated in H\_ID25 (Figure S1), likely due to insufficient samples strongly supporting its presence, suggesting that ID10 extraction lacks robust biological support. In summary, the ability of mSigHdp to identify nearly all COSMIC signatures demonstrates 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) mSigHdp C\_ID5 signature incorporates elements of both COSMIC ID5 and ID8, despite having a 0.94 cosine similarity to COSMIC ID5. This can be attributed to the co-occurrence of these signatures in tumors and their shared correlation with aging. We found no tumor samples supporting COSMIC ID5 alone, justifying the merger of these signatures. In our analysis, while neither C\_ID5 nor C\_ID8 showed strong individual correlations, their sum strongly correlated with patient age. (3) Compared to COSMIC ID17, mSigHdp 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\_ID7 signature demonstrates a closer resemblance to the ID\_TOP2A signature identified by Boot et al. than to COSMIC ID17 (Figure S2).

**Signature activity**

We evaluated the activity of our 31 mSigHdp signatures using mSigAct, a tool that outperforms others in signature attribution analysis (Jiang et al., 2024). Consistent with previous studies, C\_ID1, C\_ID2, C\_ID5, and C\_ID8 were observed across most cancer types, with C\_ID3 showing a strong presence in lung cancers. The novel signatures identified by mSigHdp were generally active in fewer cancer types compared to COSMIC signatures, except H\_ID24 and H\_ID25 which were widespread across cancer types (Figure 2). We analyzed correlations between our ID signature assignments and the SBS signature assignments by Degasperi et al (Degasperi et al., 2022). in PCAWG and HMF samples. Our analysis confirmed strong correlations between C\_ID3, SBS4, and SBS92 exposures, all linked to tobacco-induced lung cancer (Spearman correlation coefficients: 0.74 between C\_ID3 and SBS4, 0.62 between C\_ID3 and SBS92, Figure 3A & B). We observed a strong correlation between C\_ID13 and SBS7a, both associated with UV exposure (Spearman correlation coefficient: 0.85, Figure 3A). C\_ID9 (unknown etiology based on COSMIC v3.4) was identified in a module of homologous recombination (HR) deficiency signatures, including C\_ID6, SBS3, and SBS8. This suggests a potential association between C\_ID9 and HR deficiency (Figure 3C). We identified a correlation module including C\_ID14, SBS93, SBS17, SBS88, and SBS35 (Figure 3D). SBS17, SBS88, and SBS93 are frequently observed in gastrointestinal (GI) tracts, while SBS35 is associated with platinum treatment. This correlation suggests a possible etiology for C\_ID14: platinum treatment in GI tract cancers.

**Novel Signatures**

**RNaseH2B deficiency preferentially removes 2bp nucleotides on genome**

A novel signature consisting of 1-3bp deletions from 2 repeats or microhomology was found in samples from HMF (Figure 4A). The major peak exclusively describes the deletion of CT from 5’-CTCT-3’ (or AG from 5’-AGAG-3’), suggested by the extended sequence context of 5 samples with the highest H\_ID29 activity, and two of these samples are from PCAWG cohort (Figure 4B). We found the samples with mutations on RNASEH2B gene have higher H\_ID29 activity compared to the samples without mutant RNASEH2B. The inclusion of more samples enables the discovery of rare signatures from PCAWG datasets. Interestingly, in addition to HMF samples, we also observed two PCAWG samples with mutant RNASEH2B carrying high H\_ID29: a skin melanoma genome SP103894 was detected with 3772 H\_ID29 mutations, and a breast cancer genome SP5559 was detected with 949 H\_ID29 mutations (Figure 4D).

We observe a trend of samples with more RNASEH2B mutations tend to have higher H\_ID29 activity, we therefore hypothesized that the signature is a result of RNASEH2B deficiency. RNaseH2 is a key mammalian genome surveillance enzyme required for ribonucleotide removal. It is composed of a single catalytic subunit (A) and two non-catalytic subunits (B and C) and specifically degrades the RNA of RNA:DNA hybrids (Chon et al., 2009).

To validate our hypothesis, we established the RNASEH2B deficiency model by CRISPR/Cas9 system in HEK293T cell line. The whole genome sequencing shows highly similar patterns. We found the ID\_RNASEH2B KO pattern in the RNaseH2B deficient HEK293T genomes, but not in either RNaseH2A KO (Figure 4C&E).

(need more details here, e.g., what position of RNASEH2B, what cell line?)

**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 and H\_ID37 (Figure 5A). 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. These MSI signatures show a strong binomial distribution of exposures in tumors: they have high activity in MSI tumors, while extremely low activity in MSS tumors (Figure 5B). C\_ID7 characterizes mainly 1bp C or T deletions from long C or T sequences, H\_ID33 mainly characterizes TT deletions from 4-5 TT repeats, while H\_ID37 mainly characterizes TTT deletions from 3 TTT repeats (Figure 5C). We also found some MSS samples with strong MSI signatures activity, suggesting the potential of MSI signatures as a MSI status biomarker.

**Gender comparison**

We investigated whether mutational processes, as represented by mutational signatures, exhibit gender-specific patterns. 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 10 signatures demonstrating significant gender-specific associations: 6 signatures (C\_ID19, C\_ID4, C\_ID8, C\_ID17, H\_ID24, C\_ID6, and C\_ID9) showed a significant prevalence in female patients. Conversely, 4 signatures (C\_ID12, C\_ID14, C\_ID13, and C\_ID3) were more commonly observed in male patients (Figure 5D). Some of the observations can be explained biologically, C\_ID6 is associated with BRCAness in breast cancers which are often found in female patients, while C\_ID3 is associated with tobacco smoking which has a higher proportion of male patients.

**Extended sequence context characterization of novel signatures**

We observed that some signatures share dominant peaks, prompting us to investigate whether they represent distinct mutational processes. To address this, we examined the extended sequence context of samples with high activity for these signatures to understand the preferential sequence context of the indels. H\_ID24 and C\_ID9 both exhibit a similar 1bp C deletion pattern (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 C deletion from 5'XCTTT3' (Figure 6A). These findings suggest that H\_ID24 and C\_ID9 stem from distinct mutational processes: H\_ID24 preferentially removes cytosine 3' of poly-T sequences, while C\_ID9 removes cytosine 5' of poly-T sequences. H\_ID27 and part of H\_ID28 both describe 1bp C insertion (INS:C:1:0), but they characterize two distinct processes: H\_ID27 preferentially inserts a cytosine 3' of poly-A sequences, while H\_ID28 inserts a cytosine 3' of poly-C sequences. Based on these observations, we conclude that H\_ID27 and H\_ID28 result from two distinct mutational processes rather than an over-splitting of a single process.

**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. 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 mismatch repair (MMR), defective homologous recombination (HR) DNA damage repair, and UV exposure (Figure 6C). Deletions most frequently affected CAMTA1, CUX1, ERBB4, FHIT, FOXP1, GPHN, LPP, LRP1B, NRG1, and PRDM16 (Figure 6C). 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. 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 31 insertion-deletion (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 (2 new ID signatures, H\_ID33 and H\_ID37) strongly linked to microsatellite instability (MSI) status, which implement the understanding of indel footprints left my defective MMR mechanism. This study, for the first time, performs an extended sequence context analysis to understand more information behind the formation of mutational signatures.

We attempted signature extraction using SigProfilerExtractor which is a NMF based extraction model with the outstanding performance in signature extraction analysis (Islam et al., 2022). However, we found it ineffective for this large cohort (optimal solution K=12): it was not able to identify novel signatures and previously identified COSMIC signatures. This is probably due to the limitations of Non-negative Matrix Factorization in handling the high data sparsity characteristic of indels (Supplementary figure). Jin et al. identified 25 indel signatures using MuSiCal, a minimum-volume NMF model (Jin et al., 2024). Our study confirmed two MuSiCal signatures using a different approach (Figure SX). Moreover, our study added more novel signatures because of integrating HMF data. Our study demonstrates the efficacy of mSigHdp in mining large datasets and reveals how this alternative approach can uncover novel signatures in highly sparse, low-count data.

In addition to HEK293T, we also generated RNASEH2B deficiency genotype in HeLa cell lines. But we didn’t observe H\_ID29, whereas we found H\_ID29 in HeLa RNASEH2A KO clones. This suggests that some additional mechanisms were added on the formation of H\_ID29, e.g., DNA repair deficiency. It is known that HEK293T has defective mismatch repair, which may also play a role in the formation of H\_ID29.

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).

**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 samples of each cancer type, 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=100.

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.

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 ≥ 0.85 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 ≥ 0.85; (3) novel signatures: the signatures do not fit into the known signatures or the merged signatures.

Signature attribution analysis

The 31 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

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>). For HMF genomes, the MSI status was downloaded from the supplementary data of Priestley et al., 2019.

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 10 genomes with the highest exposure to the corresponding signature. 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).

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