An APOBEC3A hypermutation signature is distinguishable from the signature of background mutagenesis by APOBEC3B in human cancers

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Elucidation of mutagenic processes shaping cancer genomes is a fundamental problem whose solution promises insights into new treatment, diagnostic and prevention strategies1. Single-strand DNA–specific APOBEC cytidine deaminase(s) are major source(s) of mutation in several cancer types2–4. Previous indirect evidence implicated APOBEC3B as the more likely major mutator deaminase, whereas the role of APOBEC3A is not established5,6. Using yeast models enabling the controlled generation of long single-strand genomic DNA substrates7, we show that the mutation signatures of APOBEC3A and APOBEC3B are statistically distinguishable. We then apply three complementary approaches to identify cancer samples with mutation signatures resembling either APOBEC. Strikingly, APOBEC3A-like samples have over tenfold more APOBEC-signature mutations than APOBEC3B-like samples. We propose that APOBEC3A-mediated mutagenesis is much more frequent because APOBEC3A itself is highly proficient at generating DNA breaks8–10, whose repair can trigger the formation of single-strand hypermutation substrates.

Recently, we and others have shown that some cancers have an abundance of apparently simultaneous, closely spaced mutations, variously referred to as ‘kataegis’ (ref. 11) and ‘mutation clusters’ (ref. 12). These characteristics of apparently simultaneous, closely spaced mutations, variously referred to as ‘kataegis’ (ref. 11) and ‘mutation clusters’ (ref. 12). These characteristics are consistent with the mutagenic properties of several APOBEC cytidine deaminases that target 5′-TC-3′ motifs in single-strand DNA (ssDNA)13–17. Analyses of cancer mutation data sets have implicated APOBEC3B (A3B) as the leading candidate5,6, with APOBEC3A (A3A) as another possible mutator4. Numerous recent reports have linked high APOBEC3B mRNA expression to various cancers, reflecting a widely held view that A3B is the likely major mutator1,4,9,18–22. In contrast, there is evidence that A3A also could be a mutator in cancers8–10,23,24. Consistent with this possibility, breast cancers from carriers of a germline APOBEC3B deletion allele, fusing the APOBEC3A transcript to the 3′ regulatory sequences of APOBEC3B, actually tend to have higher TC-signature mutation loads than cancers from non-carriers25. Such fusion transcripts are more stable, resulting in higher steady-state levels of A3A enzyme26.

A more conclusive way to distinguish between possible sources of mutagenesis in cancers is to match the mutation signatures extracted from statistical analysis of each cancer to the well-defined signature(s) of candidate mutagen(s)27,28. Thus, we collected large numbers of mutations induced by either A3A or A3B in a yeast reporter strain (deleted for the uracil glycosylase gene UNG1) that generates chromosomal ssDNA upon temperature shift7. Telomere uncapping in the presence of ssDNA-damaging mutagens results in selectable mutation clusters inactivating multiple reporter genes7,29. Crucially, resection of the complementary strand precludes excision-mediated repair, and the uracils resulting from cytidine deaminations gave rise to C→T transitions7. pLogo analysis30 of the mutations identified by whole-genome sequencing of yeast found almost diametrically opposite motif preferences for the APOBEC enzymes: A3A favored YTCA sites, whereas A3B favored RTCA sites (where Y is a pyrimidine base and R is a purine base; Fig. 1a–f and Supplementary Table 1). This finding was corroborated by our fold-enrichment methodology12 (Fig. 1g.h). Reanalysis of the mutation data from Taylor et al., generated by expressing A3A or A3B in a conventional yeast system31, yielded similar results (Supplementary Fig. 1). The motif preferences of APOBECs in yeast should be suitable models for the enzymes’ preferences in human cells, as the local sequence contexts flanking cytosines in the genomes of both species are quite similar, except for depletion of CpG motifs in human31.
pLogo analysis also showed that mutations at TCA motifs (a component of TCW motifs) were over-represented for both APOBECs, whereas mutations at TCT motifs were under-represented. Following on this, enrichment of TCA mutations in cancers should exceed enrichment for TCW mutations if TC mutations are caused by either A3A or A3B. We evaluated 15 cohorts of recently published cancer whole-genome sequencing samples. Five cancer types (six cohorts; Fig. 2) had high rates of APOBEC-signature mutagenesis: bladder (BLCA), breast (BRCA), head and neck (HNSC), lung adenocarcinoma (LUAD) and lung squamous cell (LUSC). In BLCA, BRCA and HNSC, high enrichment for TCA mutations was clearly evident. APOBEC mutagenesis was also detectable in LUAD and LUSC, as shown by high enrichment values for TCA motifs in C-coordinated mutation clusters, despite high genome-wide mutation loads from non-APOBEC sources. Data for cancer types with low levels of APOBEC mutagenesis are included in Supplementary Table 2.

We next subcategorized samples enriched for mutations at TCA sites into A3A-like and A3B-like subsets by comparing YTCA enrichment with RTCA enrichment (Fig. 3). Samples with a non-random ratio of YTCA to RTCA mutations (Online Methods) were
Figure 2  Enrichment for mutations at various target motifs among all genome samples and sample-by-sample comparison of genome-wide enrichment at TCA versus TCW motifs within six cohorts of cancer types with high levels of APOBEC mutagenesis. (a–f) Enrichment for mutations at TC, TCW, TCA, RTCA, and YTCA motifs is shown for BLCA (a), BRCA (b), HNSC (d), LUAD (e) and LUSC (f) cohorts from The Cancer Genome Atlas (TCGA), as well as a BRCA cohort from ICGC (c). High genome-wide non-APOBEC mutation loads obscured the presence of APOBEC mutagenesis in the lung cancers. Nevertheless, the APOBEC-signature enrichment values in C-coordinated clusters for LUAD (e) and LUSC (f) are similar to those for other cancer types (a–d), confirming that examination of such clusters is the most sensitive means to detect APOBEC mutagenesis. (g–l) Sample-by-sample comparison of enrichment for mutations at TCA versus TCW motifs for BLCA (g), BRCA (h), BRCA ICGC (i), HNSC (j), LUAD (k) and LUSC (l) cohorts. Samples are binned by quartile of TCW enrichment. \( \chi^2 \) tests for trend (P values shown in Fig. 3) indicated significant skewing toward A3A-like signatures as the enrichment for mutations at TCA sites increased. The results for other cohorts are given in Supplementary Figure 4. We estimated the minimum number of TCA mutations attributable to an APOBEC enzyme in each sample (Fig. 3g and Supplementary Table 3), finding that the overall median value for A3A-like mutations (1,480) was over 11-fold greater than the median for A3B-like mutations (133). Thus, A3A is a much more prolific mutator than A3B.

To verify these findings, we compared the proportions of mutations at each NTCA site in cancers and each yeast model, using root-mean-square deviation (r.m.s. deviation) calculations (Online Methods) and generated corresponding pLogos for the BRCA International Cancer Genomics Consortium (ICGC) cohort (Fig. 4). The results for five other whole-genome sequencing cohorts with high levels of APOBEC mutagenesis are given in Supplementary Figure 5. NTCA and pLogo analyses concurred with YTCA versus RTCA results: samples from quartiles with lower TCA enrichment usually had an A3B-like mutation pattern (smaller r.m.s. deviation versus the A3B model), transitioning to an A3A-like mutation pattern (smaller r.m.s. deviation versus the A3A model) for samples from the upper quartiles.

Recent publications have reported that carriers of APOBEC3B germline deletions are at higher risk for breast cancer\(^{34,35}\) and tumors from these patients have higher levels of APOBEC-signature mutagenesis\(^{25}\). Thus, we investigated possible relationships between APOBEC3B germline copy number variation (CNV) and the prevalence of A3A- or A3B-like mutation signatures. By all three analyses, samples with APOBEC3B deletion from the BRCA ICGC cohort had predominantly A3A-like signatures (Fig. 5a). In contrast, samples with wild-type APOBEC3B showed a roughly equal split between A3A-like (Fig. 5b) and A3B-like (Fig. 5c) signatures. Fisher’s exact tests \( (P = 0.0024 \text{ by Y} \text{TCA versus RTCA analysis and } P = 0.0277 \text{ by NTCA analysis) confirmed significant skewing toward A3A-like signatures among APOBEC3B deletion samples. Similar results were obtained when the other cohorts with high levels of APOBEC mutagenesis were evaluated (Supplementary Fig. 6). Our results (summarized in Fig. 6) strongly suggest that, in general, A3A is the predominant mutagenic deaminase in cancers. In cancers, APOBEC signatures were clearly detectable because the abasic sites from uracil excision in ssDNA were not repaired. Instead, they were
likely bypassed by error-prone translesional DNA polymerases to create mutations (ref. 36 and references therein). Our approach relies on the supposition that, with respect to the motif preferences of APOBECs, cytosines in yeast ssDNA are suitable models for cytosines in the ssDNA of human cancers. Because the molecular machinery of DNA transactions is not identical for the two species, we do not rule out the possibility that APOBEC motif preferences might be at least somewhat different between yeast and human. As sequencing technologies mature, it should become feasible to put this question to a rigorous test, by analyzing APOBEC motif preferences at thousands of mutated cytosines in human tissue culture models and comparing to our results in yeast.

The finding that A3A-signature mutagenesis is more prominent in cancers might seem surprising, as APOBEC3B mRNA abundance tends to be higher than that of APOBEC3A in cancer samples (Supplementary Fig. 3). However, A3A is a much more potent inducer of DNA damage, likely via strand breakage as demonstrated by staining for γH2AX (a marker for double-strand breaks) and/or comet assay8–10,23. This is also consistent with observations that APOBEC-signature mutations and clusters frequently colocalize with rearrangement breakpoints in cancers11,12,37.
We propose that A3A-signature mutagenesis is more prominent, at least in part because A3A itself can trigger homology-directed repair–mediated generation of ssDNA substrates (by end resection or break-induced replication) much more readily than A3B can.

As clinical cancer genetics progresses toward genomic analysis of each cancer sample, we have recently integrated sample-specific APOBEC-signature mutation analysis into a standard platform for the analysis of large cancer genome data sets. Analyses to distinguish between A3A-like and A3B-like signatures will be incorporated into future pipeline updates, as this might prove important when weighing treatment options, given the substantially higher genotoxic and mutagenic potential of A3A. Moreover, early detection of APOBEC-signature mutation enrichment, for example, in cell-free circulating DNA, could have important diagnostic or prognostic value, especially for individuals at higher risk, such as APOBEC3B deletion carriers.

When detected in a tumor sample, a high prevalence of APOBEC mutagenesis might be exploited for therapeutic purposes. It has been suggested that hypermutation could enhance the effectiveness of immune stimulation therapy to treat cancer, by generating tumor-specific neoantigens (proteins with new epitopes) that might trigger targeted destruction by the immune system. There are two immune therapies for bladder cancer, mainly by A3A, could contribute substantially to the success of immune therapies. Likewise, other cancers with high levels of A3A-like APOBEC mutagenesis could be promising candidates for similar immune stimulation treatments.

URLs. Broad Genome Data Analysis Center (GDAC) Firehose, http://gdac.broadinstitute.org/; Broad GDAC Firehose standard data run (15 February 2014), http://gdac.broadinstitute.org/runs/stddata__2014_02_15/; database of Genotypes and Phenotypes.

Figure 5 Relationship between APOBEC3B germline copy number and mutation signatures in the BRCA ICGC cohort. Samples passed the same filtering criteria as in Figure 4. (a) Samples with APOBEC3B deletion (one homozygous, denoted by an arrowhead; remainder heterozygous) skew toward A3A-like signatures. (b) A3A-like (b) and A3B-like (c) subsets of samples with wild-type (WT) APOBEC3B copy number.

Figure 6 Summary of data analyses and conclusions. (a,b) Sets of mutations induced by either A3A (a) or A3B (b) in yeast are successively more enriched at TC, TCW and TCA motifs. (a) For A3A, mutations at YTCA sites are more enriched than mutations at RTCA sites. (b) In contrast, for A3B, mutations at RTCA sites are more enriched than mutations at YTCA sites. (c) In YTCA versus RTCA enrichment analysis, of 243 cancer genome samples with statistically significant TCA mutagenesis, 101 (41.6%) have A3A-like signatures and 63 (25.9%) have A3B-like signatures. The remaining 79 (32.5%) samples are indeterminate. (d) In NTCA proportion analysis, 124 cancer samples (51.0%) have A3A-like signatures, 75 (30.9%) have A3B-like signatures and 44 (18.1%) are indeterminate. (e) In A3B-like cancer samples, background A3B mutagenesis results in low overall TCA enrichment with higher RTCA (especially ATCA) enrichment. (f) In A3A-like cancer samples, background A3B mutagenesis is dwarfed by A3A mutagenic activity, leading to high TCA enrichment with even higher YTCA (especially CTCA) enrichment.
METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. ySR127 reference genome (GenBank), CP011547–CP011563; Illumina reads (SRA), SRP056805.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank T.A. Kunelk, S.A. Lujan and D.V. Zaykin for critical reading of the manuscript. This work was supported by US National Institutes of Health Intramural Research Program Project Z1AES103266 to D.A.G. and US National Institutes of Health grants U24CA143845 to G.G., R01GM052319 to P.A.M., 1P01CA120964 to D.J.K., R00ES022633 to S.A.R. and K99ES024424 to K.C.

AUTHOR CONTRIBUTIONS

K.C. and D.A.G. designed the study. K.C., S.A.R., J.F.S., N.S., E.P.M. and P.A.M. contributed reagents, materials or analysis tools. K.C. and D.A.G. designed the study. K.C., S.A.R., J.F.S., N.S., E.P.M. and P.A.M. contributed reagents, materials or analysis tools. K.C. and D.A.G. wrote the manuscript, with contributions from S.A.R., N.S., D.J.K. and G.G.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Construction of integrated A3A- and A3B-expressing yeast strains. Human APOBEC3A and APOBEC3B ORFs with appended 5′ ClaI and 3′ StuI restriction sites were codon optimized for expression in yeast and purchased from DNA 2.0 as inserts in the pJ201 vector. Each ORF was released from the vector backbone by ClaI and StuI double digestion and ligated into the multiple-cloning site of a tetracycline-regulatable pCM252-derived vector, to create plasmids pSR435 (encoding A3A) and pSR440 (encoding A3B) with hph (hygromycin resistance) as the selectable marker instead of TRP1. A fragment of each plasmid containing the APOBEC ORF, the tetracycline-regulated promoter and the hph marker was amplified by PCR with primers (see Supplementary Table 4 for primer sequences) to add flanking sequences with homology to either side of the LEU2 gene on yeast chromosome III.

Purified PCR products were transformed into a yeast host strain descended from CG379 (ref. 49), with the following genotype: MATα his7-2 leu2-3,112 trpl-1 289 cdcl13-1 ung1::NAT. CAN1, URI3 and ADE2 were deleted from their native loci and reintroduced into a closely spaced triple-reporter gene array near the de novo telomere on the left arm of chromosome VII within the LYS2 gene previously moved from its normal locus to the subtelomeric location7. Transformants with an APOBEC-hph cassette stably integrated into the LEU2 target locus (by homologous recombination) were selected by replica plating on hygromycin plates and verified by diagnostic replicates on single-colony isolates, followed by DNA sequencing of the insert.

Mutagenesis by A3A and A3B in yeast. Yeast were inoculated into 5 ml of YPDA medium (1% yeast extract, 2% peptone, 2% dextrose and 0.01% adenine sulfate, filter sterilized) and grown at 23 °C for 72 h. Yeast were then diluted tenfold into 5 ml of fresh YPDA with 20 μg/ml doxycycline hyclate (Sigma-Aldrich) and shifted to 37 °C for 6 h. Cells were washed in 5 ml of PBS and held at 37 °C for 42 h more. Appropriate dilutions were plated onto synthetic complete plates to verify viability and onto arginine-dropout plates with 60 mg/ml canavanine sulfate and 20 mg/ml adenine sulfate to identify Can′Ade′ double mutants, i.e., colonies with mutation clusters.

Whole-genome sequencing of yeast. Yeast colonies with mutation clusters were streaked onto YPDA. A single-colony isolate from each streak was verified for Can, Ura, Ade and respiratory competency phenotypes by replica plating. Genomic DNA was purified from isolates of interest using a QIAcube robot, according to the manufacturer’s instructions (Qiagen). Paired-end reads of 100 nt were obtained from a HiSeq 2000 sequencer (Illumina). Reads were mapped to the ySR127 reference genome, and mutations were identified using the fixed-ploidy caller in CLC Genomics Workbench 7.5 (Qiagen). To minimize the possibility of analyzing mutations that were accumulated during routine passaging and growth in culture, only unique mutations were included in mutation signature analyses. Illumina reads were uploaded to the NCBI Sequence Read Archive (SRA).

Cancer and other yeast sequencing data. Cancer genome and exome data sets were obtained from publications25,32 or from the dbGaP TCGA controlled-access Data Portal. hg19 was the human genome reference for our analyses. Cancer mutation catalogs were filtered to remove calls that overlapped with entries in dbSNP or the UCSC Genome Browser simpleRepeat track. Data for multiple myeloma genomes were from ref. 12. Additional yeast data were obtained from ref. 31 and reanalyzed, as described in detail below and previously in ref. 4, using the sacCer3 reference genome. Only mutations from the ung1 background were analyzed, as these were the closest equivalents to our yeast data.

APOBEC mRNA abundance and APOBEC3B germline copy number data. APOBEC RNA sequencing (RNA-seq) data for 5,868 tumor and 834 normal samples across 17 cancer types (bladder, breast, cervical, colorectal, glioblastoma multiforme, head and neck, kidney chromophobe and renal clear cell, acute myeloid leukemia, lower-grade glioma, lung adenocarcinoma and squamous cell carcinoma, ovarian, prostate, melanoma, thyroid and uterine corpus endometrial) were downloaded from the Broad GDAC Firehose standard data run of 15 February 2014. Segmented copy number data for 7,191 tumor-normal pairs from these same cancer types were also downloaded. In total, 5,526 samples had both RNA-seq and copy number data. These data were available for 17 bladder, 95 breast, 25 head and neck, 44 lung adenocarcinoma and 44 lung squamous cell genomic samples (225 in total), which enabled 2 types of correlation analyses: between mRNA abundance and minimum TCA mutation load and between mutation signature and APOBEC3B copy number. APOBEC3B copy number data for the ICGC breast cancer cohort were obtained from ref. 25.

APOBEC3B copy number annotation. Examination of the segmented copy number data showed that most APOBEC3B germline deletion events were localized on chromosome 22 between 39,363,650 and 39,375,350 bp. Some samples had a short deletion within or multiple discontinuous segmentation events overlapping this region. This necessitated binning of the region into twelve 1-kb windows and identifying all segmental CNV events overlapping any window. The cutoffs for classification were determined by examination of the histogram of inferred APOBEC3B copy number values (Supplementary Fig. 6): APOBEC3B copy number ≤ 0.7, homozygous deletion (homo.del); 0.7 < APOBEC3B copy number ≤ 1.69, heterozygous deletion (het.del); 1.69 < APOBEC3B copy number ≤ 2.29, wild type; APOBEC3B copy number > 2.29, amplification (amp). Of the samples assessed, 7,061 each had a unique segmental CNV. Among the remaining 130 samples that had more than one segmental CNV, classification was based on the segmental copy number farthest removed from the wild-type value of 2. Copy number call totals were as follows: 99 homo.del (13.88%), 998 het.del (13.88%), 5,699 wild type (79.25%) and 395 amp (5.49%).

Mutation cluster analysis. Mutation cluster analysis was performed as described previously4,12. Mutations spaced ≤10 bases apart were treated as a single mutagenic event, as low-fidelity translesional DNA synthesis polymerases often synthesize a short tract of T to a lesion bypass and misincorporate bases at high frequencies50,51. Groups of closely spaced mutations were identified, such that any pair of adjacent mutations within each group was separated by less than 10 kb. To identify clusters that were unlikely to have formed by the random distribution of mutations within a genome, we computed a P value for each group. Let x be the number of bases spanned by a group (from the first mutation to the last), k be the number of mutations in a group, π be the number of total mutations divided by the number of total bases in a genome and j be an indexing parameter. Then, by the negative binomial distribution12, the cluster P value is as follows:

\[
p = \sum_{j=0}^{x} \binom{k-j-2}{j} (1-\pi)^j \pi^{k-1}
\]

\[\pi=\text{was computed using all mutations (i.e., including those filtered out for presence in dbSNP and the simpleRepeat track), as this could only increase the P values. Each group with } P \leq 1 \times 10^{-4}\text{ was considered a bona fide mutation cluster. A recursive approach was applied, i.e., all clusters passing } P\text{-value filtering were identified, even if a cluster represented a subset within a larger group that did not pass the } P\text{-value filter. Clusters composed of only mutations that originated from cytosines along the same DNA strand were classified as being } C\text{-coordinated. Mutations not found in a cluster were classified as scattered.}

Mutation signature analyses. The overall structure of the signature analysis involving complementary approaches to identify, statistically evaluate and compare mutation signatures is outlined in Figure 6 and detailed below.

Enrichment calculations. For all analyses, substitutions at C-G base pairs were treated as mutations at cytosines. Enrichment quantifies how frequently C→G or C→T mutations occur in a specific sequence context in comparison to C→G or C→T mutations at cytosines overall. C→A substitutions were excluded because such mutations are rare owing to abasic site bypass32,36 and to avoid confounding overlap with frequent G→T substitutions in some cancers32. To compute enrichment for mutations at TCA sites, let MUT_TCA be the number of TCA→TGA or TCA→TTA mutations and COP_TCA be the number of occurrences of TCA (and its reverse complement, TGA) contexts in the set of 41-mers centered on each mutation in a sample. Similarly, let MUT_C be the
number of C→G or C→T mutations and ConC be the number of cytosines or guanines in the set of 41-mers centered on each mutation in a sample. Then, the enrichment for mutations at TCA sites is:

\[
E_{\text{TCA}} = \frac{\text{Mut}_{\text{TCA}}/\text{Con}_{\text{TCA}}}{\text{Mut}_{\text{C}}/\text{Con}_{\text{C}}}
\]

Enrichments for the other contexts, TC→TGW, RTCA, YTCA, and each NTCA site, were calculated analogously.

Identification of samples significantly mutated by APOBEC(s). Statistical over-representation of APOBEC mutagenesis in each sample was evaluated by one-sided Fisher’s exact test. Taking TCA as an example, the test computed the p value for a comparison between the MutTCA/(MutC – MutTCA) ratio and the ConTCA/(ConC – ConTCA) ratio, based on the prediction that the former ratio exceeds the latter. All samples not matching this prediction were assigned p = 1. Benjamini-Hochberg (BH) p-value correction for multiple testing was applied using the p.adjust() function in the R statistical computing package. Samples with adjusted q value < 0.05 were considered significant.

Estimating the number of mutations created by APOBEC(s). A minimum estimate for the number of TCA mutations created by APOBEC(s) was computed as:

\[
\text{Min}_{\text{TCA}} = \text{Mut}_{\text{TCA}} \times \frac{E_{\text{TCA}} - 1}{E_{\text{TCA}}}
\]

Because enrichment = 1 implies that TCA mutations are neither more nor less frequent (when corrected for motif abundance) than mutations at cytosines in general, this minimum estimate reports the number of TCA mutations in excess of enrichment = 1. It is only this excess that should be attributed to mutagenesis by an APOBEC enzyme. Samples with Fisher’s exact test q > 0.05 for mutation enrichment at TCA sites were assigned MinTCA = 0.

YTCA versus RTCA enrichment analysis. The \(\chi^2\) test for goodness of fit was used to identify samples that had a ratio of YTCA to RTCA mutations that differed statistically from random, by comparing observed and expected mutation counts. The expected number of YTCA mutations, given the null hypothesis of random mutagenesis, simply scales with the fraction of YTCA motifs:

\[
\text{Exp}_{\text{YTCA}} = \text{Mut}_{\text{TCA}} \times \frac{\text{Con}_{\text{YTCA}}}{\text{Con}_{\text{TCA}}}
\]

The expected number of RTCA mutations was computed analogously. P values were corrected by the BH method, with q value < 0.05 considered significant. Samples in each cohort were filtered first for significant enrichment of TCA mutagenesis and then for a significant difference from a random distribution of YTCA versus RTCA mutations. Samples passing only the first filter were plotted in the relevant figures as unfilled, gray-bordered circles, and samples passing both filters were plotted as colored circles and included in the \(\chi^2\) tests for trend toward A3A-like signatures with increasing TCA enrichment.

NTCA proportion analysis. Similarly, the \(\chi^2\) test for goodness of fit was used to identify samples that had a proportion of observed ATCA/CTCA/GTCA:TTCA mutations that differed statistically from random. The expected number of mutations at each NTCA motif is:

\[
\text{Exp}_{\text{NTCA}} = \text{Mut}_{\text{TCA}} \times \frac{\text{Con}_{\text{NTCA}}}{\text{Con}_{\text{TCA}}}
\]

P values from comparing observed and expected mutation counts were corrected by the BH method, with q value < 0.05 considered significant. Only samples passing filtering for both significant enrichment of TCA mutagenesis and non-randomness of NTCA proportion were included in r.m.s. deviation (also called root-mean-square error) comparisons. r.m.s. deviation is used commonly to quantify the similarity between two corresponding sets of quantities, for example, the three-dimensional spatial coordinates of the α carbon atoms in one protein structure versus another.

r.m.s. deviation was used to quantify the difference between the normalized enrichment observed in each sample for mutations at each NTCA motif versus the corresponding normalized enrichment values in each yeast model. Taking ATCA as an example, the normalized enrichment is:

\[
\text{NE}_{\text{ATCA}} = \frac{\text{E}_{\text{ATCA}}}{\text{E}_{\text{ATCA}} + \text{E}_{\text{CTCA}} + \text{E}_{\text{GTCA}} + \text{E}_{\text{TTCA}}}
\]

Let \(y_{\text{NE,NTCA}}\) be the normalized enrichment for mutations at NTCA observed in a yeast model. Then, the r.m.s. deviation of a cancer sample versus a yeast model is:

\[
\text{r.m.s. deviation} = \sqrt{\frac{1}{4} \sum \left(\text{NE}_{\text{NTCA}} - y_{\text{NE,NTCA}}\right)^2}
\]

Samples with r.m.s. deviation versus A3A < r.m.s. deviation versus A3B were considered to have A3A-like signatures, whereas those with r.m.s. deviation versus A3B < r.m.s. deviation versus A3A were considered to have A3B-like signatures.

pLogo analysis. pLogos identify nucleotides statistically over- or under-represented in a ‘foreground’ set of sequences, relative to abundances in a ‘background’ set. pLogos were generated using all C→T substitutions from yeast data and all C→G and C→T substitutions from cancer samples. Each element in the set of foreground sequences comprised the two bases immediately 5’ to a mutation, the mutated base itself (always C) and one base immediately 3’ to the mutation. The corresponding background was the set of 41-mers each centered on a mutation included in the foreground. The deaminated cytosine was set to position 0. Nucleotides above the horizontal axis in pLogo plots were over-represented, whereas those below the axis were under-represented. The height of each nucleotide denotes the magnitude of over- or under-representation. Red lines represent cutoffs for P = 0.05. In rare cases, the number of bases in the background set was apparently greater than what could be accommodated by the pLogo online tool, so the set of C→G or C→T substitutions was analyzed separately from the set of G→C or G→A mutations. As such pairs of pLogos were always very similar, we report those generated from C→G or C→T substitutions only.

Additional statistical analyses. Additional statistical analyses, including Kolmogorov-Smirnov tests, Spearman’s correlation, \(\chi^2\) tests with Yates correction, and \(\chi^2\) tests for trend, were performed using GraphPad Prism 6 software.

Code availability. The APOBEC mutagenesis pattern was analyzed similarly to the analysis incorporated into the Broad Institute’s TCGA GDAC Firehose pipeline. The R code is available upon request.

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