Mis-annotated multi nucleotide variants in public cancer genomics datasets can lead to inaccurate mutation calls with significant implications

Sujaya Srinivasan[1†], Natallia Kalinava[1†], Rafael Aldana [2], Zhipan Li [2], Sjoerd van Hagen[3], Sander Y.A. Rodenburg[3], Megan Wind-Rotolo[4], Ariella S. Sasson[1], Hao Tang[1], Xiaozhong Qian [4,5], Stefan Kirov[1*]

[1] Informatics & Predictive Sciences, Bristol Myers Squibb, Princeton, NJ 08648 USA

[2] Sentieon Inc, Mountain View, CA, USA.

[3] The Hyve, Arthur van Schendelstraat 650, 3511 MJ Utrecht, The Netherlands.

[4] Translational Medicine, Bristol Myers Squibb, Princeton, NJ 08648 USA

[5] Translational Sciences, Daichi Sankyo, Basking Ridge NJ, USA

[†] These authors contributed equally to this work

* Corresponding author: Stefan Kirov, stefan.kirov@bms.com

Abstract

Background

Next generation sequencing is widely used in cancer to profile tumors and detect variants. Most somatic variant callers used in these pipelines identify variants at the lowest possible granularity – single nucleotide variants (SNVs). As a result, multiple adjacent SNVs are called individually instead of as a multi-nucleotide variant (MNV). The problem with this level of granularity is that the amino acid change from the individual SNVs within a codon could be different from the amino acid change based on the MNV that results from combining the SNVs. Most variant annotation tools do not account for this, leading to incorrect conclusions about the downstream effects of the variants.

Method

Here, we used Variant Call Files (VCFs) from the TCGA Mutect2 caller, and developed a solution to merge SNVs to MNVs. Our custom script takes the phasing information from the SNV VCFs and based on a gene model, determines if SNVs are at the same codon and need to be merged into a MNV prior to variant annotation.

Results

We analyzed 10,383 VCFs from TCGA and found 12,141 MNVs that were incorrectly annotated. Strikingly, the analysis of seven commonly mutated genes from 178 studies from cBioPortal revealed that MNVs were consistently missed in 20 of these studies, while they were correctly annotated in 15 more recent studies. The best and most common example of MNVs was found at the BRAF V600 locus, where several public datasets reported separate BRAF V600E and BRAF V600M variants, instead of a single merged V600K variant.

Conclusion

While some datasets merged MNVs correctly, many public datasets have not been corrected for this problem. As a best practice for variant calling, we recommend that MNVs be accounted for in NGS processing pipelines, thus improving analyses on the impact of somatic variants in cancer genomics.

Background

Next generation sequencing is commonly used in cancer to determine the underlying genomic features of the tumor[1]. Pipelines that convert the raw sequencing data into useful knowledge include sequence alignment, variant calling and annotation tools. Single nucleotide variants and indels are the most common type of variants called by most variant callers, and these variants are prevalent in many important cancer genes. Most popular variant callers like Mutect2[2], VarScan2[3], VarDict[4], strelka2[5] and the Sentieon[6] suite of tools call variants at the most granular level of single nucleotide variants (SNVs) and indels. Missense and nonsense variants produce amino acid changes that could result in a protein that is either non-functional, or has a different or impaired function. Accurate annotation, of the amino acid changes that occur due to the SNVs and indels, is therefore critical to understanding the functional consequences of these variants.

A multi-nucleotide variant (MNV) is defined as two or more variants within the same codon on the same haplotype (see Figure 1). Variant callers commonly detect SNVs and small indels, but most callers and downstream variant annotation tools fail to consider whether nearby variants are part of the same haplotype. If multiple nearby variants happen to be within a single codon, the amino acid change could be different from the individual amino acid changes resulting from the SNVs. Many variant callers, such as Strelka, VarScan

61 and VarDict, do not include haplotype or phase information with the variant calls. Some of
62 the more recent variant callers such as Mutect2, Sentieon TNScope and Sentieon
63 TNHaplotyper include phase information to indicate if nearby variants are in phase (i.e. part
64 of the same haplotype) when there is enough evidence from the reads supporting the
65 variants.

66 Commonly used variant annotation tools, such as SnpEff[7], ANNOVAR[8], & Ensembl
67 Variant Effect Predictor (VEP)[9], annotate variants individually without considering haplotype
68 information or combining nearby in-phase variants to MNVs. There are some tools such as
69 bcftools csq[10] (haplotype aware consequence caller) that have tried to address this problem,
70 but the software expects phased VCFs as input with phasing information in the genotype
71 (GT) field in a specific and seldom used format. MAC [11](Multi-nucleotide Variant Annotation
72 Corrector) requires both the VCF and the corresponding Binary Alignment Map (BAM) file in
73 order to correct for MNVs, corresponding to adjacent SNVs. MACARON (Multi-bAse Codon
74 Association variant ReannotatiON)[12] is another tool that uses both the VCF and the BAM to
75 re-annotate VCFs with corrected MNVs from multiple SNVs within a codon.

76 There are several important cancer genes that are known to have hotspot regions with
77 many variants. A few examples are BRAF at the V600 locus, and KRAS at G12 and G13 loci.
78 Sometimes these variants are part of the same haplotype, and therefore should be
79 annotated as MNVs, but most pipelines annotate them as multiple SNVs. This could lead to
80 incorrect functional predictions for the effect of the variants.

81 In this paper, we consider some common public cancer genomics datasets to
82 understand if MNVs are accounted for, and propose a method to merge SNVs into MNVs.
83

Results

TCGA results

We downloaded 10,383 Mutect2 VCF files processed with the human reference genome (GRCh38) from The Cancer Genome Atlas (TCGA). The downloaded VCFs comprise 33 cancer types or indications.

We post-processed the TCGA mutect2 VCFs using a custom developed MNV merge script. This script takes the SNVs that are in phase and within the same codon and merges them into MNV. We excluded repeat regions and major histocompatibility complex (MHC) regions for this analysis, and only characterized the instances of merged SNVs. Indels were not considered at this time. We found that across all files, there were a total of 12,141 MNVs that were originally annotated as multiple SNVs, and of these 6,357 had a completely novel protein effect, i.e the new protein effect was different from the SNVs' protein effects (Table 1, Fig. 2). The most frequent novel MNV events were new missense events (5,413). Nonsense events, both stop gain (254) and rescue of nonsense (517), had the most impact on the interpretation of protein function. This shows that annotating MNVs correctly can significantly alter downstream analysis results.

Skin Cutaneous Melanoma (SKCM) and lung cancers: lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) had the highest percentage of samples with MNVs (Fig. 3a). We also found the highest median number of SNVs and MNVs in SKCM, LUAD and LUSC (Fig. 3b and c). This is expected because of the high Tumor Mutation Burden (TMB) in these indications. Breast cancer (BRCA), the indication with the largest number of samples in this dataset (1,040 samples), is known to have a low TMB[13], and our results are consistent with this.

107 While most genes had only one or two MNVs, we found 22 genes that had 10 or more
108 MNVs (Fig. 3d). Many of these genes are known for hotspot mutations, so this finding is not
109 that surprising. The most consistent MNVs were in the BRAF gene: 43 out of 46 MNVs were
110 at the V600 locus, all with a novel missense outcome. Furthermore, a single BRAF V600M
111 never occurred alone, but always co-occurred in phase with another variant V600G or
112 V600E, leading to the novel mutations V600R and V600K respectively.
113
114 **cBioPortal results**
115 We analyzed mutation annotation files (MAF) from cBioPortal[14,15]
116 (http://www.cbioportal.org) from all non-redundant studies (178) for 7 cancer genes (BRAF,
117 KRAS, NRAS, PTEN, BRCA1, BRCA2, MUC16). Since cBioPortal MAFs do not have phasing
118 information, we used counts for the variant reads and variant allele frequencies, as proxies
119 for phase. If the variant allele frequencies of two variants within a codon was approximately
120 the same, we inferred that they co-occurred on the same read (Fig. 4).
121 Some common hotspot regions of cancer genes, like BRAF V600 and KRAS G12 loci, were
122 particularly affected by not merging the SNVs into MNVs. While some studies did call the
123 MNVs correctly, there were 20 studies, including several TCGA studies, that did not. Table 1
124 shows the most common mis-annotated MNVs among the seven genes that we studied. The
125 most frequently mis-annotated MNV was at the BRAF V600 locus, with a total of 61 MNVs
126 (V600K and V600R). The KRAS G12 locus had 14 MNVs with the most common being co-
127 occurring G12V and G12C SNVs which should have been annotated as G12F.
128 In our analysis of all BRAF V600 variants from cBioPortal, we found only two occurrences
129 of V600M alone, with no other variant. Since we did not have the full set of variant calls

130 from this dataset, it was not possible for us to determine if these two occurrences were
131 actually V600M, or if they co-occurred with another variant that was filtered out for quality
132 reasons, or due to the fact that it was a synonymous variant. There were 64 other samples
133 that had a BRAF V600M variant, but those samples also had either a V600G or V600E variant
134 (Supplementary Table 1). When we examined all studies, including duplicate samples from
135 studies that were submitted at different times, we found that there were conflicting entries
136 for some samples. The SNVs from the earlier submissions were replaced by MNVs in later
137 submissions, indicating that pipelines had probably been updated to correct for MNVs.
138 Some examples of these are the corrections for the KRAS G12 variants and the BRAF V600
139 variants (Supplementary table 1). One important example of a corrected MNV was a V600D,
140 which consists of a synonymous variant along with a V600E. These variants would be
141 completely missed in our analysis from cBioPortal, since synonymous variants are filtered
142 out. They would only appear if MNVs were correctly handled.
143
144 **Double base mutation patterns**
145 Somatic variants in cancer genomes have specific patterns, known as Mutational
146 Signatures[16] associated with underlying processes that characterize the specific etiology of
147 the cancer. The Doublet Base Substitution (DBS) Signatures published in Mutational
148 Signatures v3[17] show the two base-pair signatures that are characteristic of certain cancer
149 types.
150 We analyzed the TCGA data after it had been corrected for MNVs, and identified the
151 most common double-base mutation patterns in Fig. 5a. We found that the CC to TT change
152 was prominent in melanoma samples (Fig. 5b). This is consistent with the reported

153 signature, DBS 1, which is a characteristic of UV related damage. Lung cancer (LUAD and
154 LUSC) samples predominantly showed CC to AA change (Fig. 5b), which was consistent with
155 the DBS 2 signature, indicating exposure to tobacco smoking[17]. This shows that the detected
156 MNVs are consistent with the expected mutational signatures, and by analyzing MNVs, we
157 can detect underlying patterns that would be missed otherwise.
158

159 **Discussion**

160 We analyzed VCFs from TCGA as well as MAF files from cBioPortal, and found that there
161 were over 12,000 MNVs that were characterized as SNVs in TCGA. Many of these MNVs are
162 in important cancer genes, such as BRAF and KRAS. From a functional perspective, it is
163 important to annotate these variants correctly, so that the effects of the variants can be
164 properly evaluated and interpreted. For example, we did not find a single occurrence of a
165 BRAF V600M alone in any of the studies, it was always in phase with a V600E or V600G.
166 At the same time a number of publications reference V600M[18–28] ; COSMIC database at
167 the time we reviewed the data lists 31 occurrences of V600M. The methods for detecting
168 the mutation are extremely diverse, ranging from Restriction Fragment Length
169 Polymorphism(RFLP) and direct Sanger sequencing to MassArray/Sequenom platform. We
170 cannot evaluate to what extent these methods have the ability to detect MNVs as this is
171 beyond the scope of this study, but it likely these errors are more broadly occurring.
172 Other studies identified double V600M-V600E or V600M-V600G mutants that are
173 possibly MNVs, as the detection method does not allow for phasing information to be
174 known (typically Sanger sequencing)[29,30]. In this specific case, the correct identification of
175 the amino acid change may have serious consequences. A number of BRAF inhibitors are

176 approved for either V600E or V600E/K[31,32], but treatment options may differ for other rare
177 mutations, including V600M. For example, there is preclinical data suggesting that BRAF
178 kinase activity may not be altered in V600M/A unlike V600E/K/D[33]. Retrospective analysis
179 points to V600K carriers having a worse prognosis[34] and worse PFS response to existing
180 BRAF inhibitors[35].

181 From a cancer biology perspective, it is also curious to understand how these MNVs
182 evolve. The V600K/R for example are not driven by UV damage as V600K originates from
183 GT->AA and V600R originates from GT->AG, whereas UV signature is associated with C->T
184 events[36]. Studies on germ-line MNVs have shown that this type of events tends to be more
185 pathological than SNVs and associated mostly with APOBEC and DNA polymerase zeta[37].
186 Another potential mechanism would argue that two independent SNVs happen to occur by
187 chance in the same codon, and that the resulting MNV clone gains an advantage and
188 eventually displaces the original SNV clone from the tumor population. However, we should
189 be able to at least occasionally detect the founding clone mutation in the same tumor
190 specimen, evidence of which we have not seen to date.

191 There have been many large-scale efforts to characterize MNVs within a germline
192 context, most recently with gnomAD[38]. However, one of the potential issues we did not
193 address in this paper is when germline variants are part of the same haplotype with a
194 proximal somatic variant as part of the same codon. There is not much evidence that this is
195 a widespread problem[39], but it would be important to assess the effect of it.

196 In our analysis of the various cBioPortal studies, we observed that several studies after
197 2017 from larger academic hospitals and institutions had corrected for MNVs, indicating
198 that the problem was recognized and fixed in some of these pipelines. We also found that

199 the ICGC PCAWG[40] effort and the AACR Genie[41] project called MNVs correctly. However,
200 there are still several smaller academic and commercial labs that may not have fixed this
201 issue, and our analysis shows the need for the MNV merge step to be incorporated into
202 variant-calling pipelines as a standard best practice. Needless to say, clinical assays should
203 be assessed not only on the correct characterization of BRAF V600 mutants, but also the
204 precise amino acid change associated with it.
205

206 ## Methods

207 **MNV Merging for TCGA VCFs**

208 We downloaded 10,383 TCGA VCFs processed using the Mutect2 variant caller on the
209 GRCh38 reference genome from the Cancer Genomics cloud. When nearby variants are part
210 of the same haplotype (in phase), Mutect2 adds tags to indicate this – PGT is the phased
211 genotype of the variant, and PID is an ID that is shared between variants of the same
212 haplotype; this information is then used by a python script to merge SNVs to MNVs.

213 We downloaded Refseq transcripts BED file from the UCSC table browser
214 (https://genome.ucsc.edu) and pre-processed it into a codon file that had the positions of
215 each codon defined. The MNV merge script then used this codon file to determine whether
216 to merge SNVs, based on whether they are part of the same haplotype and codon.

217 The python script (merge_mnp.py) takes the input VCF, reference genome, pre-
218 processed codons text file and a parameter that specifies if indels should be considered. For
219 the purposes of this study, we did not consider indels. The python script identifies SNVs that
220 are both in phase and within the same codon into a new MNV. The new MNV has a PASS in
221 the filter field, while the original SNVs have a MERGED in the filter field to represent that

222 they have been superseded by the MNV. All code can be found on GitHub at
223 https://github.com/Sentieon/sentieon-scripts.
224 The VCFs that have the merged MNVs were annotated using SnpEff. Annotations from
225 gnomAD v2.1.1 [42], dbSNP[43] version 146 and COSMIC[44] version 84 were added to the VCFs,
226 and both "PASS" and "MERGED" variants were retained in order to be able to trace the
227 MNVs and the original SNVs. The repeat masker GRCh38 annotations were used to mask the
228 repetitive regions and were excluded from the MNV analysis. The highly variable MHC
229 region at chromosome 6 position 28510120 - 33480578 was also excluded from the MNV
230 analysis.
231 **cBioPortal**
232 We downloaded Mutation Annotation Files (MAF) from the cBioPortal
233 (https://www.cbioportal.org/) by choosing "Curated list of non-redundant studies" for 7
234 genes – BRAF, KRAS, NRAS, PTEN, BRCA1, BRCA2, MUC16. To identify variants that were
235 part of the same haplotype and at the same codon position, we looked for those instances
236 where there were multiple variants from the same sample at the same codon position, and
237 had the same Variant Allele Frequency (VAF). This indicated that it was highly likely that the
238 variants appeared together on most reads
239 In addition, we queried the public cBioPortal API (https://www.cbioportal.org/api/),
240 retrieving the complete collection of mutation data for all loaded studies. We then filtered
241 mutations for few selected mutation hotspots, i.e. BRAF V600, KRAS G12, and NRAS Q61,
242 and subsequently determined which variant calls occurred in each sample at these hotspots.
243 Samples occurring in multiple studies were combined, but we kept track of the cases where
244 samples had different variant calls between studies.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for Publication

Not applicable

Availability of data and materials

TCGA data is available from the Genomic Data commons at https://portal.gdc.cancer.gov/. Data from cBioPortal is available at https://www.cbioportal.org/.

Financial & competing interests disclosure

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Author contributions

SS and NK identified, processed and analyzed the datasets, and wrote the manuscript. SK identified the problem presented in the manuscript and SK, SS and NK conceived the

idea. RA and ZL developed the scripts and code. SH and SR helped with data retrieval and analysis from cBioPortal. MWR, AS, HT and XQ contributed to the analysis of the results and downstream implications. All authors discussed results and contributed to the final manuscript.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Figures and Tables legends

Fig. 1: *Schematic presentation of MNV and SNV events. (a)* Two *SNV*s co-occurring on the same read indicate *they are part of the same haplotype and should be annotated as MNV. (b)* Two adjacent SNVs *are on different reads and should be annotated as individual SNVs.*

Fig. 2: *Novel MNV effects in TCGA data. (a) Categories and examples of the MNV novel annotation effects as a result of combination of two SNVs. (b) Number of MNVs for novel effects in TCGA data.*

Fig. 3*: MNV summary in TCGA dataset. (a) Distribution of TCGA samples by indication. The bars indicate the percent of samples that had MNV(s). (b) Boxplot of the SNV count per indication. (c) Boxplot of the MNV count per indication. Indications are ordered the same as the SNV count. (d) Distribution of novel and original MNV for genes with total MNV ≥ 10.*

Fig. 4: *Variant allele frequencies of variants present on the same codon in cBioPortal. The high correlation between the VAs of the variants indicates that they were present on the same reads.*

Fig. 5: *Double-base mutation patterns found in the TCGA data based on the MNV corrections. (a) Frequency of double-base mutation patterns found in all indications of TCGA results. The reverse complement was accounted according to the double-base signatures described in Alexandrov et al, 2020. (b) Double-base mutation patterns plotted for the selected indications: melanoma and lung carcinoma. Lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) were combined into one panel for lung carcinoma.*

Table 1: *Most commonly mis-annotated MNVs in cBioPortal among the 7 genes that were studied*

Supplementary Table 1: *Table of all samples from cBioPortal that have a variant at the BRAF V600 and G469, KRAS G12 and NRAF Q61 loci. The common samples that have conflicting annotations between studies are indicated by separating with a ";"*

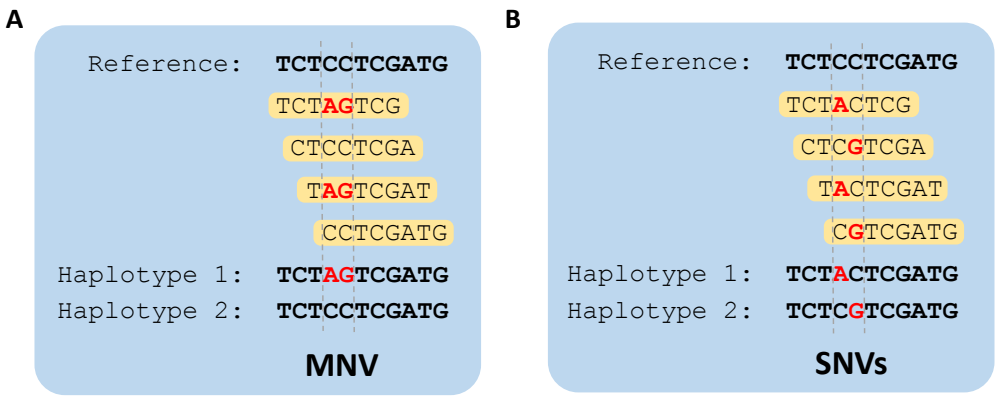

Figure 1: Schematic presentation of MNV and SNV events. (A) The 2 SNVs co-occurring on the same reads indicates they are part of the same haplotype and should be annotated as MNV. (B) The 2 SNVs in this case are adjacent but on different reads, and should be annotated as individual SNVs.

Gene	SNVs	MNV	Count
BRAF	V600M + V600E	V600K	52
BRAF	V600M + V600G	V600R	9
BRAF	G469V + G469*	G469L	2
KRAS	G12V + G12C	G12F	8
KRAS	G12A + G12C	G12S	2
KRAS	G12V + G12S	G12I	2
KRAS	G12V + G12R	G12L	2
NRAS	Q61R + Q61K	Q61R	5

Table 1: Most commonly mis-annotated MNVs in cBioPortal among the 7 genes that were studied

A

Category	SNVs effects	MNV effect
Novel missense	missense A, missense B	missense C
Rescue of nonsense	stop gained, missense A	missense B
Gain of nonsense	missense A, missense B	stop gain
Novel splice	splice A, splice B	splice C
Rescue of missense	missense A, missense B	synonymous
Other	stop lost, missense A	missense B

B

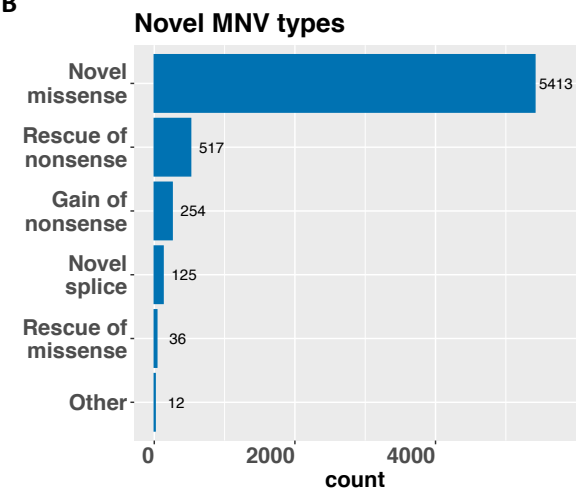

Figure 2: Novel MNV effects in TCGA data. (A) Categories and examples of the MNV novel annotation effect as a result of combination of two SNVs. (B) Number of MNVs for novel effects in TCGA data.

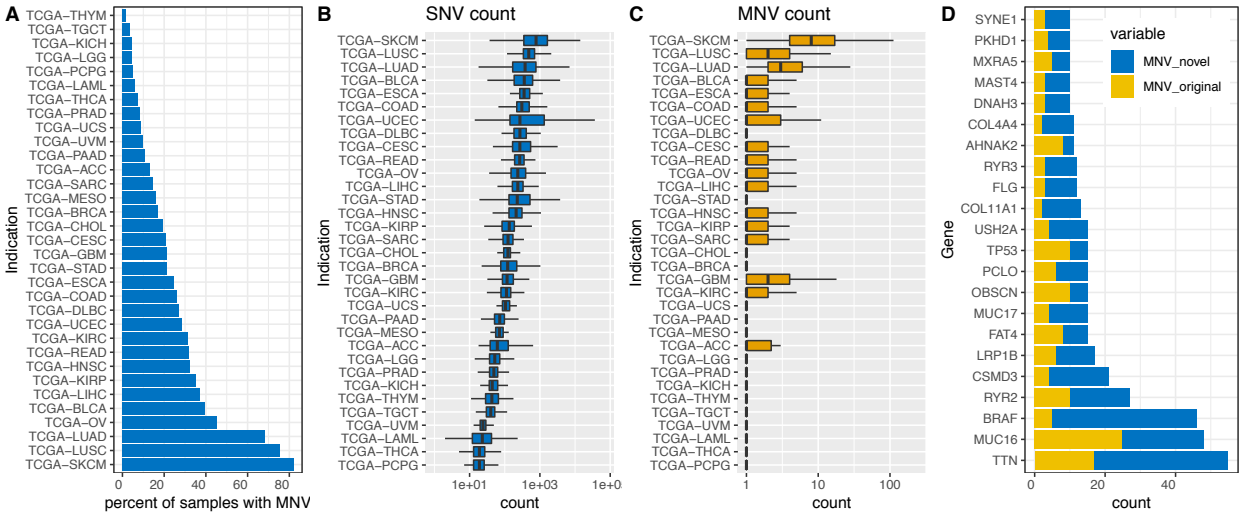

Figure 3: MNV summary in TCGA dataset. (A) Distribution of TCGA samples by indication. The bars indicate the percent of samples that had MNV(s). (B) Boxplot of the SNV count per indication. (C) Boxplot of the MNV count per indication. Indications are ordered the same as the SNV count. (D) Distribution of novel and original MNV for genes with total MNV ≥ 10.

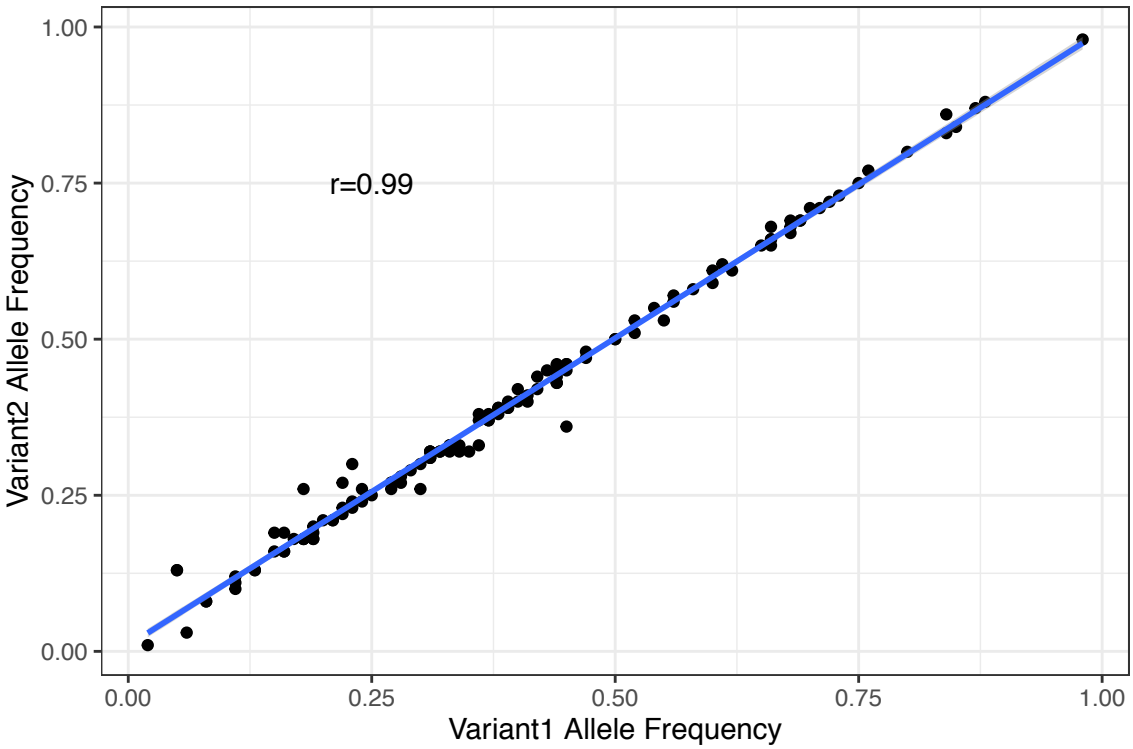

Figure 4: Variant allele frequencies of variants present on the same codon in cBioPortal. The high correlation between the VAFs of the variants indicates that they were present on the same reads.

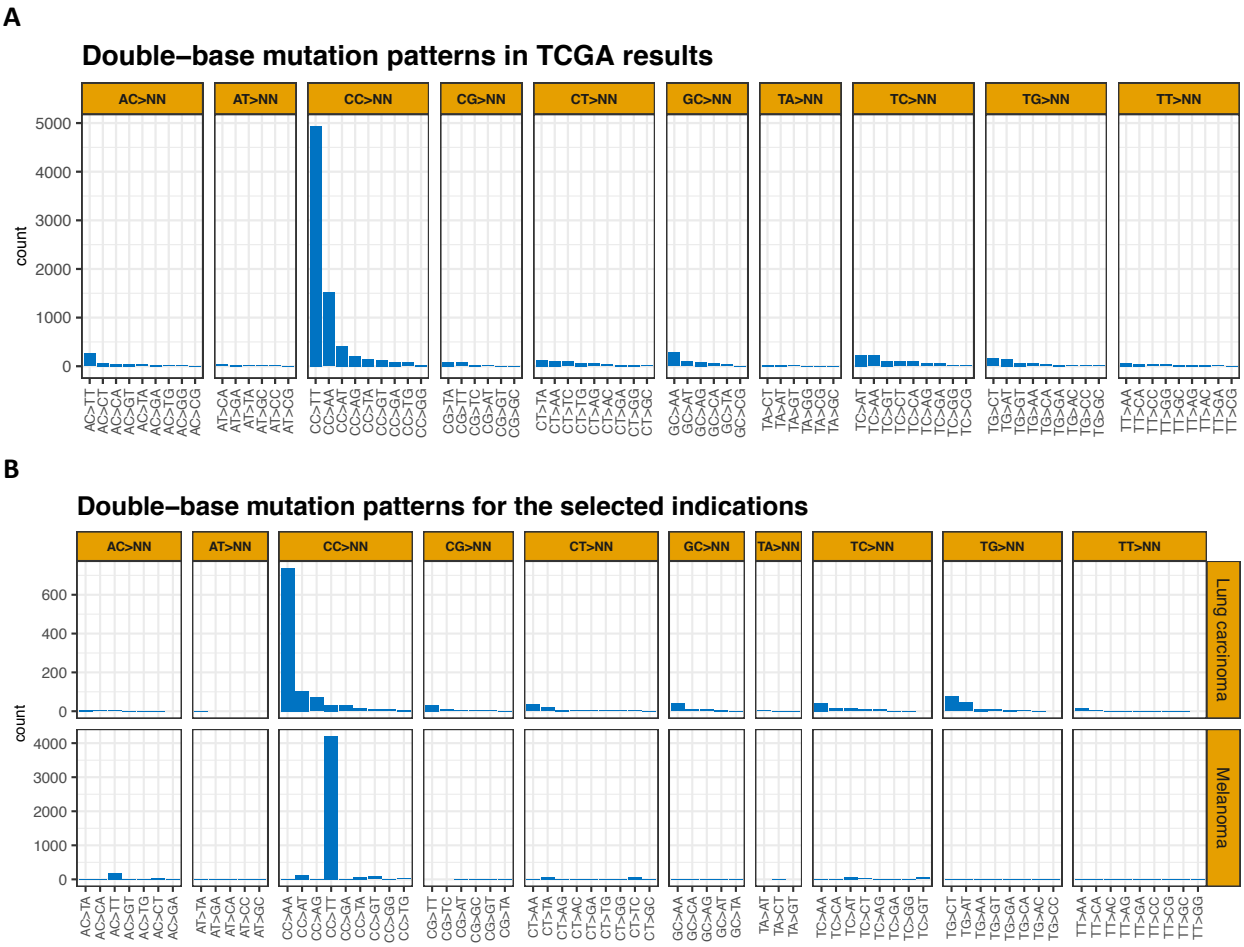

Figure 5: Double-base mutation patterns found in the TCGA data based on the MNV corrections. (A) Frequency of double-base mutation patterns found in all indications of TCGA results. The reverse complement was accounted according to the double-base signatures described in Alexandrov et al, 2020. (B) Double-base mutation patterns plotted for the selected indications: melanoma and lung carcinoma. Lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) were combined into one panel for lung carcinoma.