Using common variants to indicate cancer genes

Lucy F. Stead1, Helene Thygesen1, David R. Westhead2 and Pamela Rabbitts1

1 Leeds Institute of Cancer and Pathology, University of Leeds, St James’s University Hospital, Leeds, United Kingdom
2 Institute of Molecular and Cellular Biology, University of Leeds, Leeds, United Kingdom

The catalogue of tumour-specific somatic mutations (SMs) is growing rapidly owing to the advent of next-generation sequencing. Identifying those mutations responsible for the development and progression of the disease, so-called driver mutations, will increase our understanding of carcinogenesis and provide candidates for targeted therapeutics. The phenotypic consequence(s) of driver mutations cause them to be selected for within the tumour environment, such that many approaches aimed at distinguishing drivers are based on finding significantly somatically mutated genes. Currently, these methods are designed to analyse, or be specifically applied to, nonsynonymous mutations: those that alter an encoded protein. However, growing evidence suggests the involvement of noncoding transcripts in carcinogenesis, mutations in which may also be disease-driving. We wished to test the hypothesis that common DNA variation rates within humans can be used as a baseline from which to score the rate of SMs, irrespective of coding capacity. We preliminarily tested this by applying it to a dataset of 159,498 SMs and using the results to rank genes. This resulted in significant enrichment of known cancer genes, indicating that the approach has merit. As additional data from cancer sequencing studies are made publicly available, this approach can be refined and applied to specific cancer subtypes. We named this preliminary version of our approach PRISMAD (polymorphism rates indicate somatic mutations as drivers) and have made it publicly accessible, with scripts, via a link at www.precancer.leeds.ac.uk/software-and-datasets.

Cancer develops via the accumulation of somatic mutations (SMs), some of which confer a selective advantage to the tumour, enabling it to proliferate abnormally. Distinguishing such driver mutations, which highlight candidate genes for targeted therapeutics, from passenger mutations (nonpathological by-products of the underlying mutagenic process) is an important task. Two main approaches exist: (i) prioritise mutations predicted to detrimentally affect an encoded protein1 and (ii) identify genes repeatedly mutated within, or across, cancer subtypes.2 The latter results from the hypothesis that SMs in genes causally associated with cancer undergo positive selection in tumours, occurring more often than expected by chance. Scoring this requires determination of the background mutation rate (BMR), given the commonly hypermutated state of cancer genomes, from which to measure the significance of the mutation count in a given gene. Often the rate of synonymous SMs, scaled by the ratio of potential nonsynonymous-synonymous mutations, is used, under the assumption that synonymous mutations are selectively neutral (i.e., phenotypically silent).2 This is flawed: (i) it restricts analysis to protein-coding genes and (ii) the assumption of selective neutrality is increasingly hard to justify owing to the prevalence of functional noncoding transcripts. Nonprotein coding genes include microRNAs (miRNAs), long intergenic noncoding RNAs (lincRNAs) and pseudogenes, all shown to have causal associations with various cancers.3 The functionality of these transcripts results directly from nucleotide sequence, rather than encoded amino acids, based on binding other nucleotides or proteins in a sequence-specific manner.4 Genetic variation within noncoding transcripts will, therefore, alter their functionality with potential phenotypic consequences, but the notion of nonsynonymous and synonymous variation does not apply. Additionally, synonymous mutations in protein-coding genes can exert a phenotypic effect by altering the resulting mRNA’s ability to (i) interact with regulatory noncoding RNAs or (ii)
What's new?
Somatic mutations are important drivers of the cancerous process but identifying the key “driver” mutations remains a challenging question. The authors hypothesize that the variation level in healthy tissue represents a transcript's tolerance to mutation and that if the number of mutations in tumors exceeds this level, positive selection might have occurred that point this transcript as a major driver in carcinogenesis. They tested their program with a large dataset of somatic mutations and obtained a ranked list of genes significantly enriched in known cancer-associated genes. Their program called PRISMAD is publicly available and could help identify new driver mutations in various tumors.

Material and Methods
Genome annotation
Annotations for human reference genome GRCh37 were downloaded from GENCODE14 and transcript records merged, via a bespoke perl script, creating a single annotation per gene ID with nonredundant exons delineated.

Population data
A bespoke script (available online) accessed Ensembl69, via its perl programming interface, and extracted the total number of basepairs, and the number of commonly polymorphic loci, within each exon. A commonly polymorphic locus is a variant position sequenced in germline samples at least 20 times with a minor allele frequency of 5–50% (see Supporting Information for justification of the chosen allele frequency). The rate (commonly polymorphic alleles per kilobase) is calculated and output per gene. Ensembl69 contained information from dbSNP137, including all data from the 1000 Genomes Project phase 1 and HapMap phase 3.

Somatic mutations
We use SM to mean a tumour-specific substitution or indel involving less than 500 bp. Genomic coordinates were converted, wherever necessary, to GRCh37 using the University of California, Santa Cruz (UCSC) liftOver tool. SMs were downloaded from catalogue of somatic mutations in cancer (COSMIC) via Biomart or extracted from Supporting Information in additional publications (with no study overlap). All COSMIC SMs were validated from primary tumours and identified using whole genome sequencing. All manually extracted data were from whole genome or exome sequencing studies only. Supporting Information Table 1 outlines all references for the SMs collated. A bespoke perl script (available online) was used to ascertain the SM rate using our amended genome annotation files. Analysis was restricted to exon regions.

Statistical analysis
CP and SM rates were analysed in R. Attempts to ascertain the best way to amalgamate the CP rate and SM rate information into a single metric are given in Supporting Information. Functional analysis was performed using the DAVID Bioinformatics Resources, release 6.7. Statistical tests were performed in R.

Comparison with other programmes
The Supporting Information contains information on a comparison between PRISMAD (polymorphism rates indicate somatic mutations as drivers) and another programme that is applicable to noncoding regions.

miRNA folding predictions
The fasta sequence for the wild-type and mutant miRNA precursor, hsa-mir-99b, were input to RNAfold. Resulting predictions are those according to the minimum free energy and partition function. Free energy values were output for
The median CP rate ing to a higher SM rate in cancer-associated genes compared with noncancer-associated genes. This is similar to the notion that driver genes are more likely to be phenotypically detrimental (owing to effects at the RNA as well as protein level) and are, thus, causally associated with cancer. We attempted several statistical modelling approaches (Supporting Information), concluding that the best results were obtained using created a metric called the rate difference (RD), obtained by subtracting the CP rate from the SM rate:

\[
\text{RD} = \text{SM rate} - \text{CP rate}.
\]

The median RD for cancer genes was \(-0.83\) variants/kb and for noncancer genes \(-1.17\) variants/kb. The RD is significantly higher for cancer genes and the effect is greater than that of both SM rate and CP rate in isolation (Wilcoxon, \(p = 1.04 \times 10^{-14}\)).

**Using RD to rank genes, genome-wide**

Our approach is applicable genome-wide as it uses the CP rate, which can be ascertained for any given genomic region, as a baseline for interpreting SM rates. We calculated the RD for each of 20,036 protein-coding genes, 6,296 lincRNAs, 3,110 miRNAs, 786 lncRNAs and 13,004 pseudogenes (Table 1 and Supporting Information Tables 2 and 3). The top 1,000 protein-coding genes (ca. 5%), ranked by descending RD, included significantly more known cancer genes than expected by chance (\(\chi^2, p = 0.00028\)), whereas the top 1,000 ranked by descending SM rate did not (\(\chi^2, p = 0.38\)). This indicates that RD is a more powerful predictor than SM rate alone. This enrichment was not observed if the datasets were separated into synonymous and nonsynonymous variants (\(\chi^2, p > 0.01\), Supporting Information).

Functional analysis of the top 1,000 protein-coding genes according to RD revealed significant enrichment in the pathways of cadherin signalling (PANTHER P00012, adjusted \(p < 0.05\)) in which 21 members were highlighted (Supporting Information Table 4), and Wnt signalling (PANTHER P000057, adjusted \(p < 0.05\)), with 34 members highlighted (Supporting Information Table 5).

The top-ranking noncoding transcripts mostly lacked a single exonic CP, with only 76 containing more than one SM (Supporting Information Table 3). Literature searches revealed a dearth of information regarding the functionality of the top-ranking noncoding transcripts according to RD except in the case of MIR99B. This is a miRNA with an RD of 14.5 variant/kb owing to an SM identified in a gastric tumour.\(^\text{14}\) The MIR99B gene produces two mature miRNAs (hsa-miR-99b-3p and hsa-miR-99b-5p); dysregulation of both has been associated with carcinogenesis.\(^\text{15}\) Mutations within miRNAs can have causal associations with cancer.\(^\text{16}\) The SM, NC_000019.9.g.52195904A, highlighted by our approach resides within a predicted base-paired portion of the hsa-miR-99b precursor hairpin from which the two mature miRNAs are excised (Fig. 1a). The mutation is predicted to alter precursor folding in such a way that removes local base pairing and causes a predicted reduction in folding stability by 1.24 kcal/mol program (Fig. 1b). This altered configuration and change in stability could alter the processing of the hairpin, required to excise the mature miRNAs.
Discussion

Machine learning methods that identify disease-causing nonsynonymous mutations reveal that evolutionary conservation is an, if not the most, important predictor variable. This is because genetic variants that detrimentally alter the function of an encoded protein undergo negative selection throughout evolution. It follows that genetic variation that detrimentally alters the noncoding function of a transcript will also undergo negative selection. We tested the hypothesis that the number of commonly occurring germline polymorphisms within a genomic region (protein coding and/or noncoding) can be used as a BMR from which to score SM rates in tumours and identify potential cancer-driving genes. In support of this theory, exonic SNP density (number of polymorphic loci) is one of the most informative predictive features in a machine-learning tool to predict cancer-driving mutations. We developed a parsimonious method for ranking genes/genomic regions using the RD [Eq. (1)]. Our method is not restricted to protein-coding regions and makes no prior assumptions regarding which mutations are phenotypically silent.

General cancer pathways

The top ranked genes highlighted by PRISMAD were enriched for Wnt signalling and cadherin signalling pathways. Wnt signalling is involved in cell–cell communication and its study is becoming increasingly widespread in cancer research. Similarly, the role of cadherins in various types of cancer continues to be an area of active research. The elucidation of cancer-related pathways by our approach further indicates its merit.

Application to noncoding genes

Attempts to investigate noncoding SMs thus far have been on the level of specific mutations within single samples, without reproducibility, or have been anecdotal. In those cases, though, it has been stressed that it is likely that some drivers will lie within noncoding regions. We applied our method to several types of noncoding genes implicated in carcinogenesis: lincRNAs, miRNAs, IncRNAs and pseudogenes. We revealed few CPs in many of these genes. This is expected given that interest in noncoding regions, and the ability to sequence them to the required depth, has only increased in the last decade, meaning there is a dearth of information on variation rates therein. The 1000 genomes pilot constituted whole genome sequencing, but thereafter the project focused on protein-coding regions. We believe that although economically understandable, negation of noncoding regions may be detrimental to cancer research. Many whole genomes have been sequenced, with SM data deposited in relevant databases. Similar deposition of genome-wide germline variants into dbSNP would facilitate the creation and use of approaches such as ours.

Our approach is to highlight some noncoding genes as potentially harbouring driver SMs, but a lack of functional information makes these difficult to verify. Rather, we hope that validating our approach in protein-coding genes suggests the noncoding genes highlighted are worthy of prioritisation or, at least, when additional noncoding germline variation is present in online databases, ours is an approach worth applying. We highlighted one known cancer-associated miRNA gene, MIR99B, using PRISMAD, and predicted how the SM identified within it may result in altered processing and expression of two mature miRNAs.

Many methods exist to specifically identify nonsynonymous cancer-driving mutations. Our approach can be used alongside these, potentially highlighting distinct genes, but we do not propose our method replace them if the goal is to highlight nonsynonymous variants.

It has recently been shown that additional factors, i.e., gene expression level and stage of replication, affect the number of tumour-specific SMs that a gene acquires, irrespective of involvement in carcinogenesis. This is thought to result from DNA repair and replication effects: genes expressed at...
low levels are less exposed to transcription-coupled repair, and those replicating late succumb to error owing to a reduction in the concentration of free nucleotides within the cell. These factors will equally affect mutation rates in noncancer cells, though to a lesser degree because these cells are not aberrantly proliferating. Our original hypothesis better incorporates these recent findings: an RD calculated from commonly polymorphic sites specifically within matched normal tissue (which will include germline and nondiseased tissue SMs) to the tumour in question will factor in the aforementioned biases, assuming that expression profiles and replication timing of such cells are similar to the cancer cells that originated from them. Unfortunately, there is currently insufficient publicly available appropriate sequencing data to test this extended hypothesis, but it provides an avenue for future research.

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