Estimating the Predictive Power of Silent Mutations on Cancer Classification and Prognosis

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Abstract

In recent years it has been shown that silent mutations, in and out of the coding region, can affect gene expression and may be related to tumorigenesis and cancer cell fitness. However, the predictive ability of these mutations for cancer type diagnosis and prognosis has not been evaluated yet. In the current study, based on the analysis of 9,915 cancer genomes and approximately three million mutations, we provide a comprehensive quantitative evaluation of the predictive power of various types of silent and non-silent mutations over cancer classification and prognosis. The results indicate that silent-mutation models outperform the equivalent null models in classifying all examined cancer types and in estimating the probability of survival 10 years after the initial diagnosis. Additionally, combining both non-silent and silent mutations achieved the best classification results for 68% of the cancer types and the best survival estimation results for up to nine years after the diagnosis. Thus, Silent mutations hold considerable predictive power over both cancer classification and prognosis, most likely due to their effect on gene expression. It is highly advised that silent mutations are integrated in cancer research in order to unravel the full genomic landscape of cancer and its ramifications on cancer fitness.

1. Introduction

The rapid developments of New Generation Sequencing (NGS) technologies and acceleration of computational abilities over the past few years have led to the availability of extensive genomic information [1–5]. Multiple research utilizing these high-dimensional data establish cancer as a group of highly heterogeneous genomic diseases, characterized by large inter-tumor and intra-tumor diversities [6–8]. Moreover, common genetic features were repeatedly identified among patients of different cancer types and significant diversities were found among patients diagnosed with the same cancer type [9,10]. These findings highlight the need for personalized, gene-targeted cancer treatments.

By now, hundreds of genes had been recognized as cancer drivers [11] and many more are currently researched. Some, like *TP53* [12], *BRAF* [13], *EGFR* [14] or *IDH1* [15] have already been targeted for gene therapy. Nonetheless, there are still numerous obstacles to overcome in order to fully unravel the cancer

genomic landscape. Currently, most research is based on data derived by Whole Exome Sequencing (WES) [2]. In addition, most studies focus exclusively or predominantly on non-silent mutations; alterations in the coding regions that cause a change in the amino-acid sequence of the produced protein. Silent mutations, such as modifications in the introns, the untranslated-regions (UTR'5 and UTR'3) or even synonymous mutations in the coding region itself are by and large excluded from the analyses [16].

Yet, cancerous silent mutations could have detrimental effects on gene expression [16–18], which in some cases could even lead to consequences more significant than non-silent mutations. Mutations in regulatory regions, such as promoters or enhancers, can destruct or form new transcription-factor binding sites and cause changes in transcription regulation [19,20]. Mutations in the untranslated regions can affect translation regulation or modify microRNA binding sites and thus impact mRNA stability [21]. Synonymous mutations can alter all aspects of gene expression [22], impacting translation rates [23,24], protein-folding [25], transcription [26–28], mRNA stability [29] and splicing [30,31]. Overall, silent mutations could modify all phases of the gene expression process, causing amplification or reduction in protein quantities. Hence, even though most silent mutations do not cause a change in protein functionality, they could dramatically change protein abundance and could therefore influence cancer fitness.

We believe that including these mutations in cancer research is imperative for acquiring a broader understanding of the genomic landscape profoundly linked with cancer development and progression. Indeed, there are previous studies that have demonstrated that silent mutations or non-silent mutations that modulate gene expression can significantly affect the phenotype of the cancer cell and its survival [30,32–38]. However, to the best of our knowledge, no previous study has performed a broad, quantitative comparison between the predictive abilities of various mutation types on cancer classification and progression. In this study, we explore silent and non-silent mutations, aiming to quantify the predictive ability of various types of silent mutations to perform cancer diagnosis and to estimate patients' survival probabilities over time, while comparing it to the performance of non-silent mutations.

2. Results

2.1 Data processing and feature engineering

Genomic and clinical data of 9,915 patients across 33 cancer types were obtained from The Cancer Genome Atlas (TCGA). See Fig. 1 for data characteristics [39]. The genomic data consisted of detailed information about the patients' DNA mutations while the clinical data held personal information such as patients' vital status. These data were used to perform two tasks- patients' cancer type classification and survival estimation. The full flow chart of the study is depicted in Fig. 2.

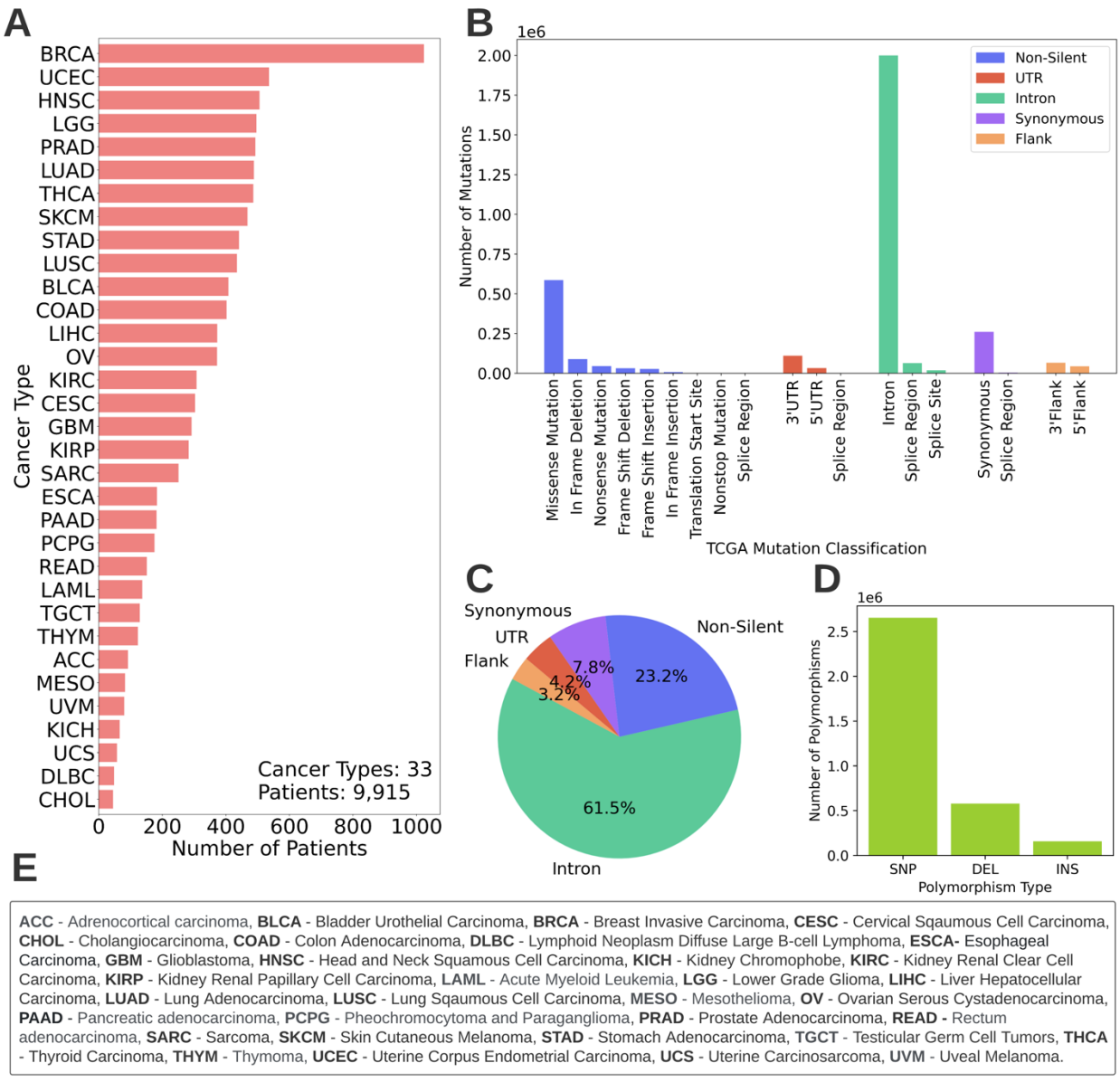


Fig. 1: TCGA data characteristics. Description of the data retrieved from TCGA after initial preprocessing (discarding patients with missing genomic or clinical data and patients with multiple genomic samples). Overall, 9,915 patients across 33 cancer types are included in the study. **a** Patient distribution across cancer types. **b** Sorting TCGA mutations to five categories for the study**.** The x axis depicts the mutation classification according to TCGA*. The y axis depicts the number of mutations in the TCGA mutation categories. The legend depicts the five categories to which the mutations are sorted for this study. *Note: In TCGA, Synonymous mutations are referred to as "Silent". As the terms are in fact not interchangeable (synonymous mutations are a subcategory of silent mutations) we replace the term "Silent" with "Synonymous" where needed. **c** Mutation type distribution. The distribution includes all mutations of the 9,915 patients. **d** Polymorphism type distribution. Mutations could be either Single Nucleotide Polymorphisms (SNP), Deletions (DEL) or Insertions (INS). The distribution includes all mutations of the 9,915 patients, **e** Names and abbreviations of the 33 cancer types.

As Fig. 2 indicates, the genomic data was split into five categories. One category holds all non-silent mutations (amino-acid-altering exonic mutations). The other four categories consist of silent mutations

from different regions within and adjacent to the genes; synonymous mutations (exonic mutations that do not directly affect the amino acids), mutations in introns, UTRs or flanking regions. In the next preprocessing step, for each category, the initial data was used to create three kinds of features (see Fig. 3 and Methods), representing different resolutions-

1. Low resolution features - indicating the number of mutations each patient had in an entire gene.
2. Medium resolution features - indicating the number of mutations each patient had in a 50-nucleotide-long gene segment.
3. High resolution features - binary features indicating whether a specific mutation occurred or not, for each patient.

Analyzing features from multiple resolution levels improves the models' results (as demonstrated in Fig. 4A and in Table S1 in Additional File 1) and could also identify specific mutations, regulatory regions and entire genes that are related to cancer fitness.

The features created for each of the five categories were used as five separate datasets (referred to as single-mutation-type datasets). A sixth dataset that combines features of all mutation types (referred to as all-features dataset) was also created. The six datasets were used to perform cancer type diagnosis and patient survival estimation. Evaluating the performance of models trained on the six datasets enables us to compare the predictive ability of features derived from silent and non-silent mutations (referred to as silent features and non-silent features).

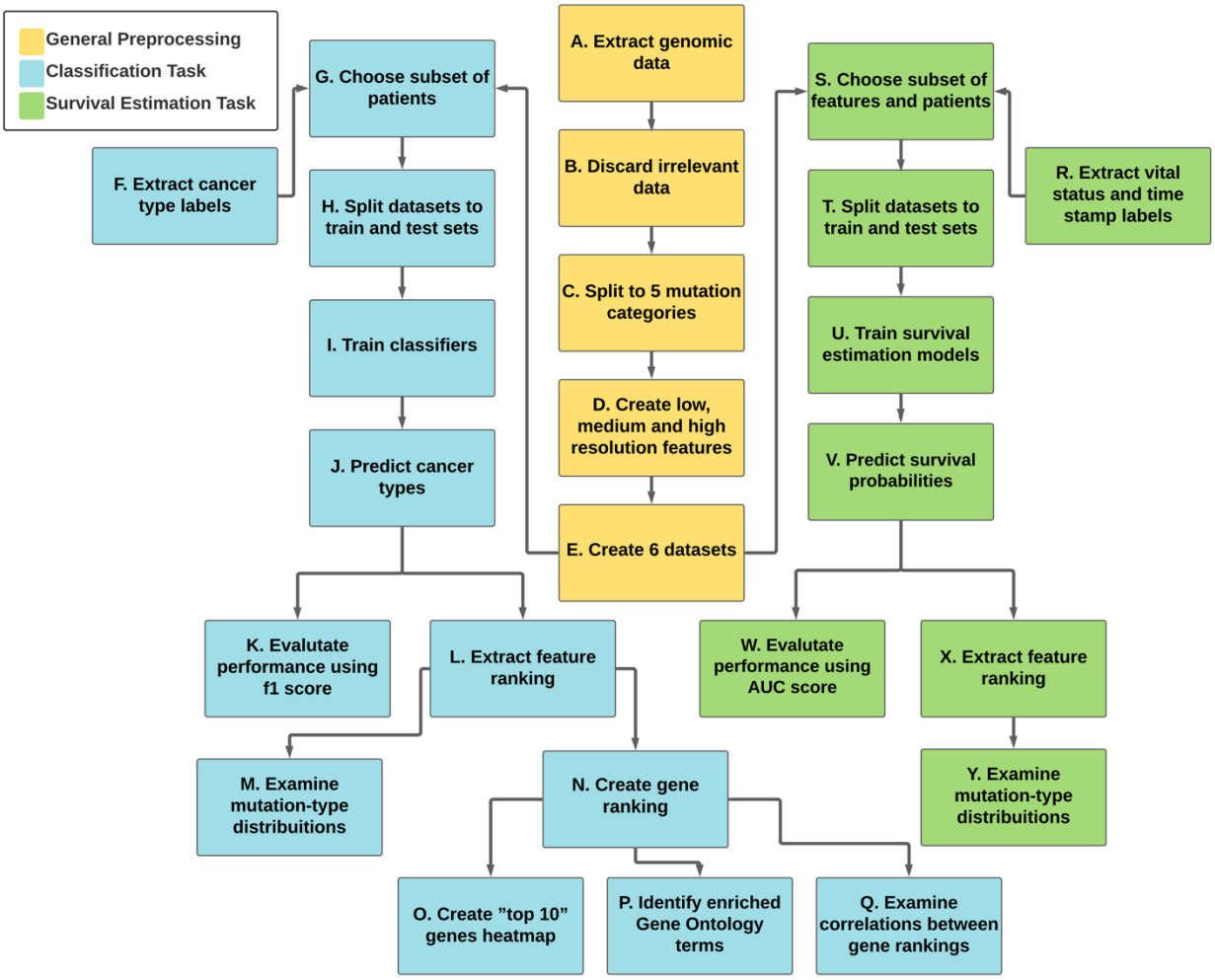
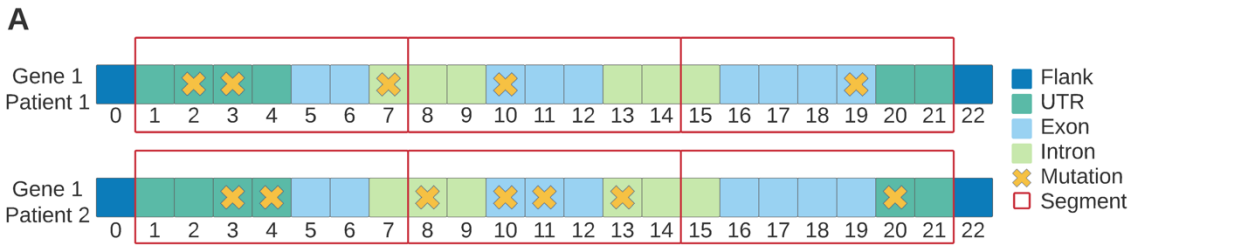


Fig. 2: The flow chart of the study. Yellow boxes denote preprocessing steps performed for both tasks. Blue boxes denote steps performed for the cancer type classification task and green boxes denote steps performed for the survival probability estimation task.



B

Intron Dataset

	Gene 1	Segment 1	Segment 2	Position 7	Position 8	Position 13
Patient 1	1	1	0	1	0	0
Patient 2	2	0	2	0	1	1

UTR Dataset

	Gene 1	Segment 1	Segment 3	Position 2	Position 3	Position 4	Position 20
Patient 1	2	2	0	1	1	0	0
Patient 2	3	2	1	0	1	1	1

Low Resolution Features
Medium Resolution Features
High Resolution Features

Fig. 3: A simplified illustration of the feature extraction process. **a** A representation of the initial genomic information. The X's denote mutations that two patients have in the same gene. The red rectangular frames represent the 50-nucleotide-long segments used for the medium resolution features. **b** An example of the features that would have been extracted for the intron dataset and the UTR dataset according to the initial information shown in a.

2.2 For all cancer types, the silent features improved cancer classification in comparison to the null model

In the cancer type classification task, only cancer types with more than 200 patients were included (a total of 19 types). A one-vs-all (OVA), supervised learning model was created for every pair of cancer type and dataset (see sub-section 4.3.1). Specifically, each model deployed the features in the dataset in order to predict whether patients suffered from the specific cancer type (classified as "Positive") or suffered from any of the other types (classified as "Negative", since the model predicts only the existence of the specific cancer). This section presents the results of this analysis.

As mentioned in sub-section 2.1, combining features from three levels of resolutions led to the best performance of cancer type classification (see Fig. 4A). Fig. 4B depicts the F1 scores (see sub-section 4.3.1.3 for the definition of the F1 score) obtained by the OVA models by using features from all levels of resolutions. The worst performing model, which used flanking-region features in order to diagnose Glioblastoma (GBM), was 1.9 folds better than the comparable null model (see sub-section 4.3.1.2 for details about the null models). The best performing model that used silent features was the intron model for diagnosing Ovarian Serous Cystadenocarcinoma (OV), and its F1 score was 20 folds higher than the comparable null model. Even though the non-silent models generally achieved better results than silent models, for several cancer types the performances were substantially similar. For example, for detection of Breast Invasive Carcinoma (BRCA), Liver Hepatocellular Carcinoma (LIHC) and OV the performance difference between the non-silent model and the intron model was less than 10%. For Sarcoma (SARC) diagnosis, the non-silent model outperformed the UTR model by a mere 2%, and the flank model was exceeded by only 12%. In addition, *the all-features models, which used both silent and non-silent features, obtained higher F1 scores than the non-silent models for 13 out of the 19 cancer types* (denoted in red in Fig. 4B) and for the other cancer types, the performances were very similar.

To control for the number of features, the same analysis was conducted using balanced datasets as well (see sub-section 4.3.6) and the results (see Fig. S1 in Additional File 1) accentuate the high diagnostic ability of silent mutations; *In the balanced version, the Intron model outperformed the non-silent model for four cancer types and the UTR and flank models were superior to the non-silent model for two cancer types*. Quite similarly to the unbalanced datasets, combining silent and non-silent mutations rather than solely using the latter improved classification results for 12 out of 19 cancer types (keeping in mind that the all-features dataset had the same number of features as the non-silent dataset in this analysis). All these findings support the hypothesis that silent mutations do affect cancer mechanisms and hold additional predictive information that could not be obtained from non-silent mutations alone.

Another interesting phenomenon demonstrated in Fig. 4B is the considerable differences in the models' ability to diagnose different cancer types. While the majority of the BRCA, LGG (Lower Grade Glioma) or COAD (Colon Adenocarcinoma) patients were correctly diagnosed (by at least one model), KIRP (Kidney Renal Papillary Cell Carcinoma) and STAD (Stomach Adenocarcinoma) patients were often poorly diagnosed. To explore the origin of this difference, we examined the similarity between genetic

profiles of the different cancer types and assessed whether cancers with higher genetic similarity have higher misclassification rates: For every pair of cancer types, the correlation between their Jaccard similarity score and their misclassification rate was inspected (see sub-section 4.3.5). The results (see Fig. S2 in Additional File 1) indicate a Spearman correlation coefficient of 0.72 (p value < 10^{-28}), suggesting the similarity between genetic profiles of patients of different cancers is indeed a major cause for misclassifications. However, this is not the only cause as it only explains ~52% of the variance in their misclassification rate. Another factor that could lead to misclassifications is high mutation heterogeneity among patients of the same cancer type.

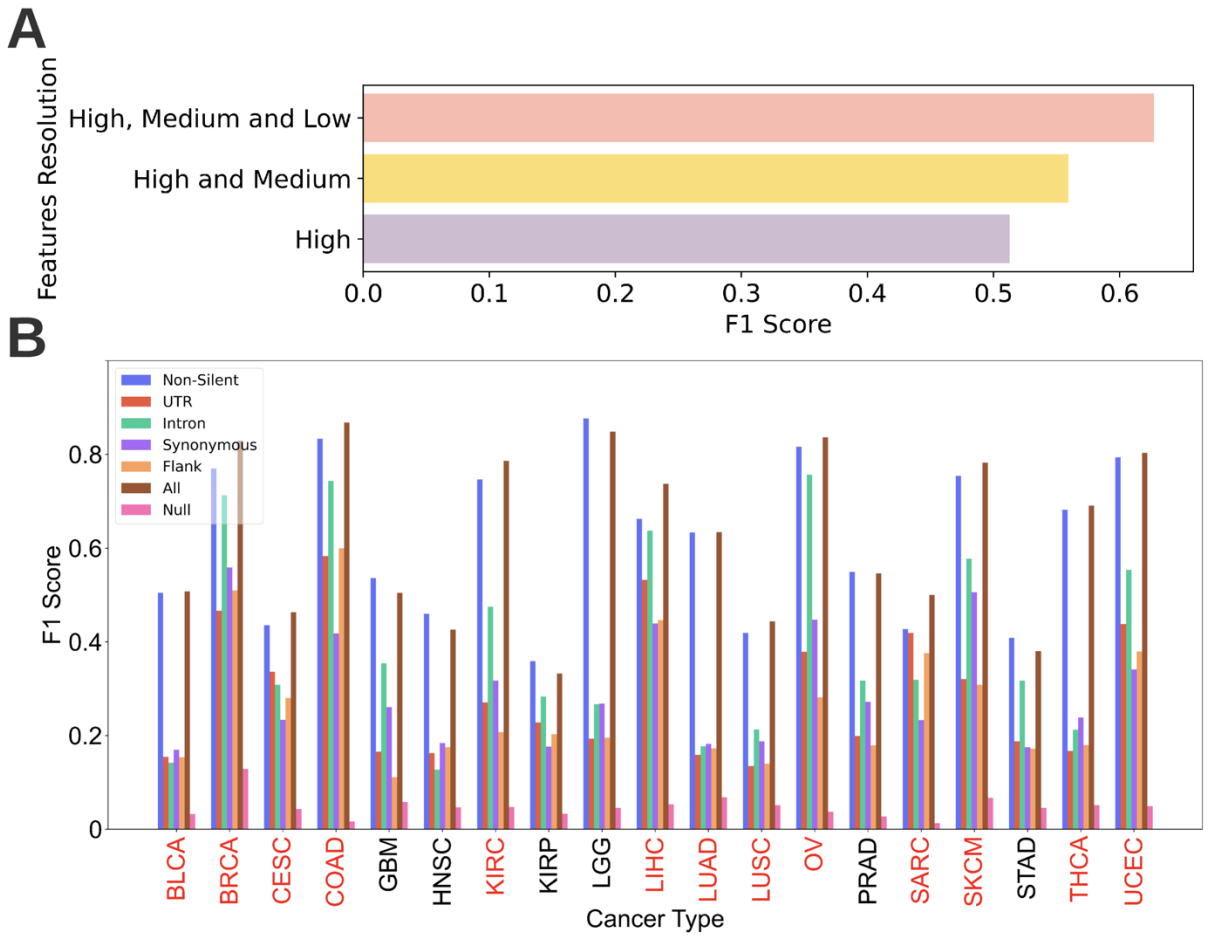


Fig. 4: The F1 scores achieved by the OVA models. **a** The F1 scores achieved in the cancer type classification task when using only high resolution features, high and medium resolution features and all resolutions combined. The scores shown are the average F1 scores achieved by the all-features models across all cancer types. **b** The F1 scores achieved by the OVA models per cancer type, using features from all levels of resolution. The x axis depicts the cancer types, the y-axis depicts the F1 scores achieved by the models. Each bar color denotes a different dataset. Cancer types for which the all-features model outperformed the non-silent model are denoted on red. See Fig. 1E for the unabbreviated names of the cancer types.

2.3 Silent features comprise 32% of the 10 most predictive features for cancer classification, on average across cancer types

Each OVA model provides an importance ranking for all its features. Examining the ranking of silent features among all features is another way to evaluate their predictive power. Reviewing the feature importance ranking produced by the all-features models, *silent features comprised nearly half of the top ranked 100 features and nearly a third of the top ranked 10 features (chosen from hundreds of thousands of features), when averaged across cancer types* (see Fig. 5). However, the ranking of silent features varied substantially between cancer types (see Tables S2,S3 in Additional File 1 for results per cancer type); while there were only non-silent features in the top 10 features of Lung Adenocarcinoma (LUAD), silent features constituted eight out of the top 10 features of Cervical Squamous Cell Carcinoma (CESC). Altogether, 18 out of the 19 cancer types had at least one silent feature in their top 10 features list, demonstrating their high significance. The analysis was repeated with balanced datasets and the results were similar (see Fig. S3 in Additional file 1).

When evaluating the influence of the polymorphism type (whether a mutation is an insertion, a deletion or an SNP) on the importance ranking, it was seen that the presence of deletions in the highly ranked features was notably higher than their presence in the initial datasets (See Fig. S4 in Additional File 1). In fact, their prevalence in the top 10 features was 2.9-6.8 folds higher than their prevalence in the initial datasets (varying between the different models). The presence of SNPs and insertions in the highly ranked features was lower than their presence in the initial datasets, with the exception of the UTR dataset, for which the insertions were 1.3 folds more common in the top 10 features lists than in the initial datasets, on average across cancer types.

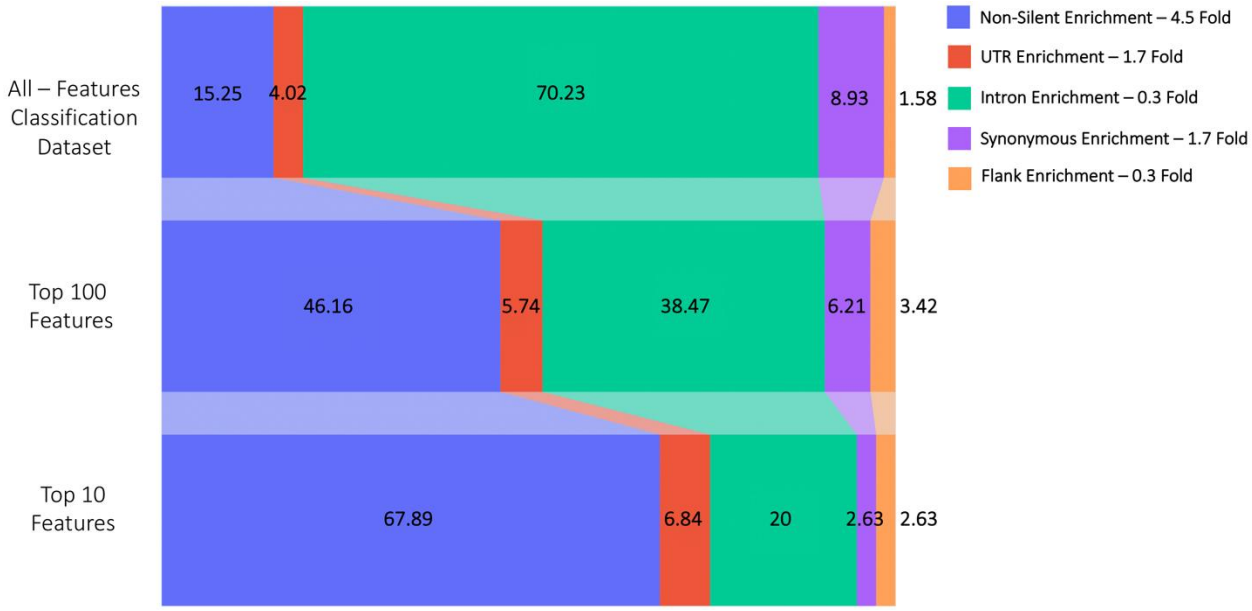


Fig. 5: Feature-type distribution of the all-features dataset and of the top ranked features chosen in the classification task. Feature-type distribution of the all-features dataset* (top row), top ranked 100 features (middle row) and top ranked 10 features (bottom row). The feature rankings were obtained from the all-features models classifying the 19 cancer types and were averaged across them. The legend indicates the enrichment in the amount of each feature-type in the top 10 features compared to its original amount in the all-features dataset (ratio between bottom and top row). *Note: The distribution depicted in the top row is the distribution of the all-features dataset after it underwent preprocessing relevant for the classification task.

2.4 A gene's predictive power for cancer type classification varies drastically when mutated by different types of mutations

Table 1 lists the 10 most predictive features of three of the 19 cancer types, as chosen by the all-features models (see Table S4 in Additional File 2 for full feature importance rankings of all cancer types). As seen in Table 1, some genes appeared in the top 10 ranked genes for multiple cancer types. *MUC4* was in the top 10 list for 16 out of the 19 cancer types and *TP53* was on 11 lists, suggesting these genes could play an essential role in cancer mechanisms. Interestingly, *MUC4* was predictive of many cancer types when it had either non-silent mutations or synonymous mutations (see Table 1). This last finding raises the following fundamental question: is the mutation type a determining factor in a gene's ability to predict a cancer type? Or perhaps different kinds of alterations in various regions of the same gene would cause a similar loss or gain of function, leading to the same outcomes on cancer development?

To try and answer this question, the top 10 features list from every single-mutation-type OVA model was examined (the all-features models were excluded from this analysis). For each cancer, a top 10 genes list was derived from the top 10 features list (see sub-section 4.3.2). Fig. 6 depicts a heatmap, presenting the number of top 10 genes lists a gene has appeared in (19 meaning the gene appeared in the top 10 genes lists of all cancer types, and zero meaning it had appeared in none). As seen in Fig. 6, the number of appearances a gene has in the top 10 lists changes dramatically when it is mutated by mutations of different types. For example, the aforementioned *MUC4* gene appears in all 19 lists when it is mutated by non-silent mutations or synonymous mutations, but when it is mutated in the UTR, introns or flanks it loses its predictive significance and does not appear in any of the lists. In fact, it is evident that most genes are highly predictive of multiple cancer types only when mutated by a specific mutation type. For example, *MUC16* is highly predictive of 15 cancer types, but only if its mutations are synonymous. Altogether, it is evident from Fig. 6 that *the mutation type does influence the predicative power a gene has on cancer diagnosis.* Nonetheless, it can also be seen that for some genes, such as *AK2* or *KTM2C*, more than a single mutation type leads to high predictivity of multiple cancers. So, even though it has been established that not all mutations cause the same effect, perhaps some lead to more similar consequences than others.

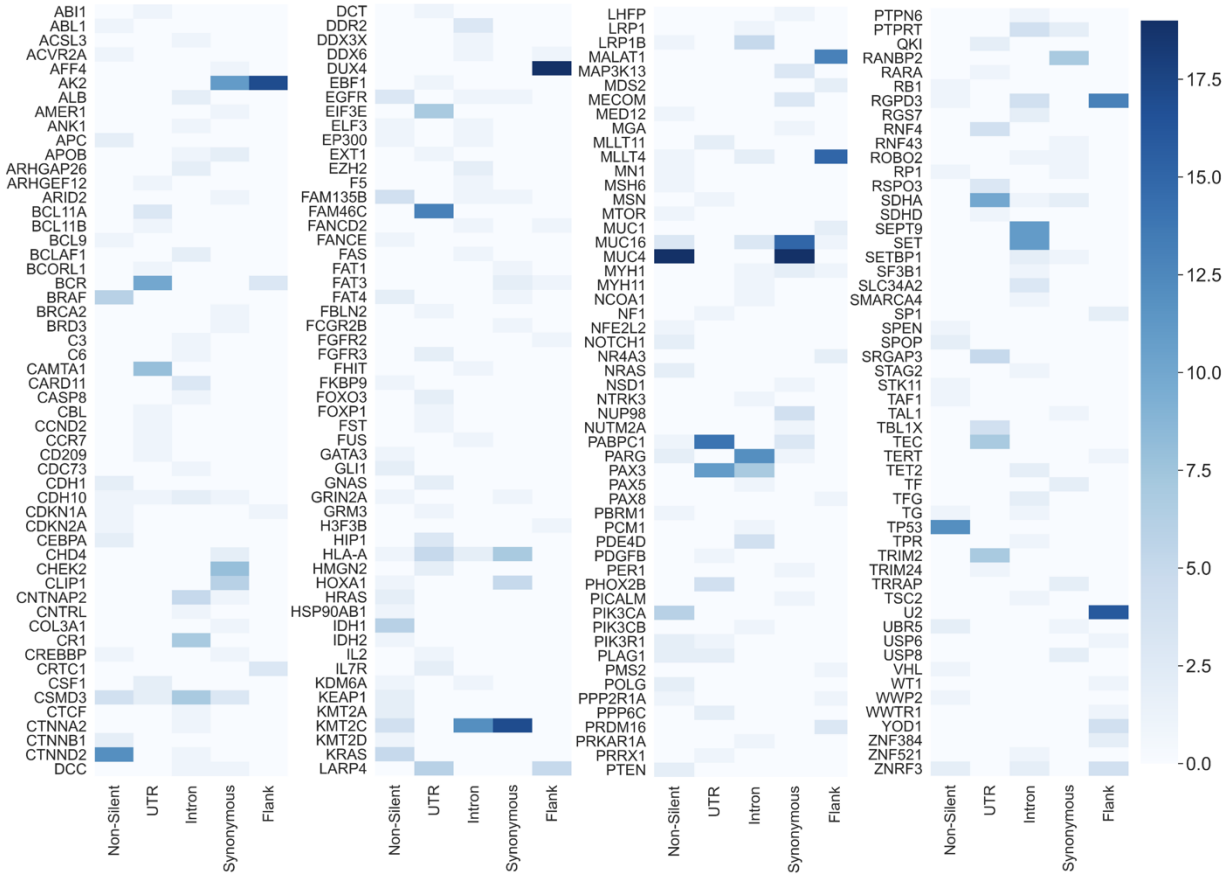


Fig. 6: The number of top 10 ranked genes lists a gene had appeared in when it was mutated by a specific mutation type. Every row represents a gene, every column represents a mutation type. A lighter shade indicates the gene was in the top 10 lists of a few cancer types and a darker shade indicates the gene was in the top 10 lists of many cancer types. The minimum value possible is zero (the gene is not included in the top 10 genes list of any cancer type) and the maximum is 19 (the gene is included in the top 10 genes lists for all examined cancers).

2.5 Synonymous, non-silent and intronic mutations affect a gene's predictive power on cancer type classification in a positively correlated manner

To assess whether some mutation types lead to similar consequences, every cancer type was separately examined. It was assumed that if two different mutation types have similar effects on a gene, then the predictive power of that gene for a specific cancer type would be similar when mutated by either one of them. Therefore, the gene's importance in both models should be similar as well. Inferring to all genes, the gene importance ranking of both models should be correlated.

For every cancer type, a Spearman correlation was performed between every pair of gene ranking lists obtained from the five single-mutation-type models (see subsection 4.3.3). The correlation coefficients were then averaged across all cancer types (see Fig. S5 in Additional File 1 for the correlations of each cancer type). The results, shown in Fig. 7, indicate a significant 0.4 correlation between the gene ranking lists of the non-silent and synonymous models, a 0.32 correlation between the lists of the non-silent and intron models and a correlation of 0.3 between the lists of the synonymous and intron models. These three correlations obtained a p-value smaller than $8.5 * 10^{-9}$. Correlations between all other pairs of models

were neither high nor significant. A possible reason for these results is a common mechanism shared by the different mutation types. For example, both synonymous and non-silent mutations may affect co-translational folding, and both synonymous and intronic mutations may influence splicing. Thus, it is conceivable that these mutations could have similar consequences over the gene's expression or functionality.

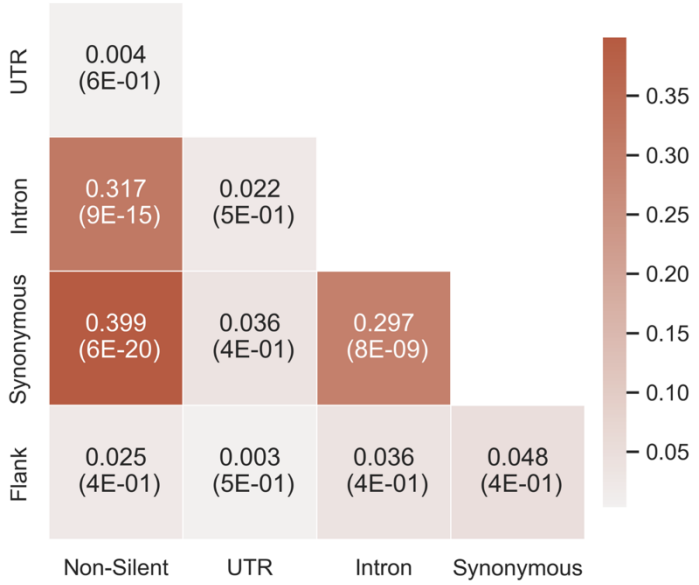


Fig. 7: The average Spearman correlation of every pair of gene ranking lists of two models. For every cancer type, the correlation between the gene ranking lists of every pair of models was calculated. The average value across cancer types is shown. The respective average p-values are denoted in parentheses. The colors represent the correlation coefficient. A darker color indicates a higher correlation.

2.6 Combining both silent and non-silent features enables the detection of Gene Ontology terms that are not detected by non-silent features alone

Enrichment analysis was performed in order to examine whether genes that were considered important by the models are related to specific biological functions and processes. The affiliation of these genes to biological pathways could illuminate their contribution to the development and progression of the disease. The GOrilla [40,41] and REVIGO [42] tools were used to find non-redundant Gene Ontology terms (GO terms) that are enriched for any of the 19 cancer types. To find the terms, a gene ranking list was used as input for the GOrilla tool (see sub-section 4.3.4). As demonstrated in Fig. 6 and Fig. 7, different mutation types dramatically change the predictive power of genes and thus inputting gene rankings of the different models could illuminate different biological pathways.

Fig. 8 lists the GO terms that were enriched for the 19 cancer types when using the gene rankings from the all-features models. Examining these results, it can be seen that most GO terms that are repeatedly enriched across cancer types are related to DNA-protein bindings, to protein-protein bindings and to phosphorylation. As expected, these terms are associated with various regulation mechanisms of the gene expression process, such as transcription (interactions between transcription factors and RNA Polymerase, histone phosphorylation) or translation (attachment of ribosomes to the DNA sequence).

As most research today encompasses mainly non-silent mutations, it is interesting to test whether the GO terms that were detected with the all-features gene rankings are also detected with gene rankings obtained from non-silent models. Fig. 9 depicts the number of cancer types for which a GO term was found significantly enriched when using the gene rankings from both models. It can be seen that most GO terms detected by the all-features models across various cancer types are considerably less detected by the non-silent models. That is to say, *adding silent features to non-silent features caused the gene ranking to encompass a broader biological significance and thus led to a more comprehensive detection of GO terms.*

When examining the results, one must consider the uneven number of features in both models; The all-features models have almost seven times as many features as the non-silent models. Because the gene ranking is derived from the feature ranking it is bound to have some effect over the enrichment results. However, it is not the only determinant; if the silent features were unimportant for the model, adding them (even many of them) would not cause such a difference in the enrichment results. As the rank of a gene is derived from the rank of its most important feature (see sub-section 4.3.2), unimportant silent features would have made a small impact on the gene ranking, leading to similar gene rankings of the all-features and non-silent models and thus to similar enrichment results. The fact that many more GO terms were found enriched by the all-features models demonstrates once again the importance of the silent features and the importance of examining the whole picture.

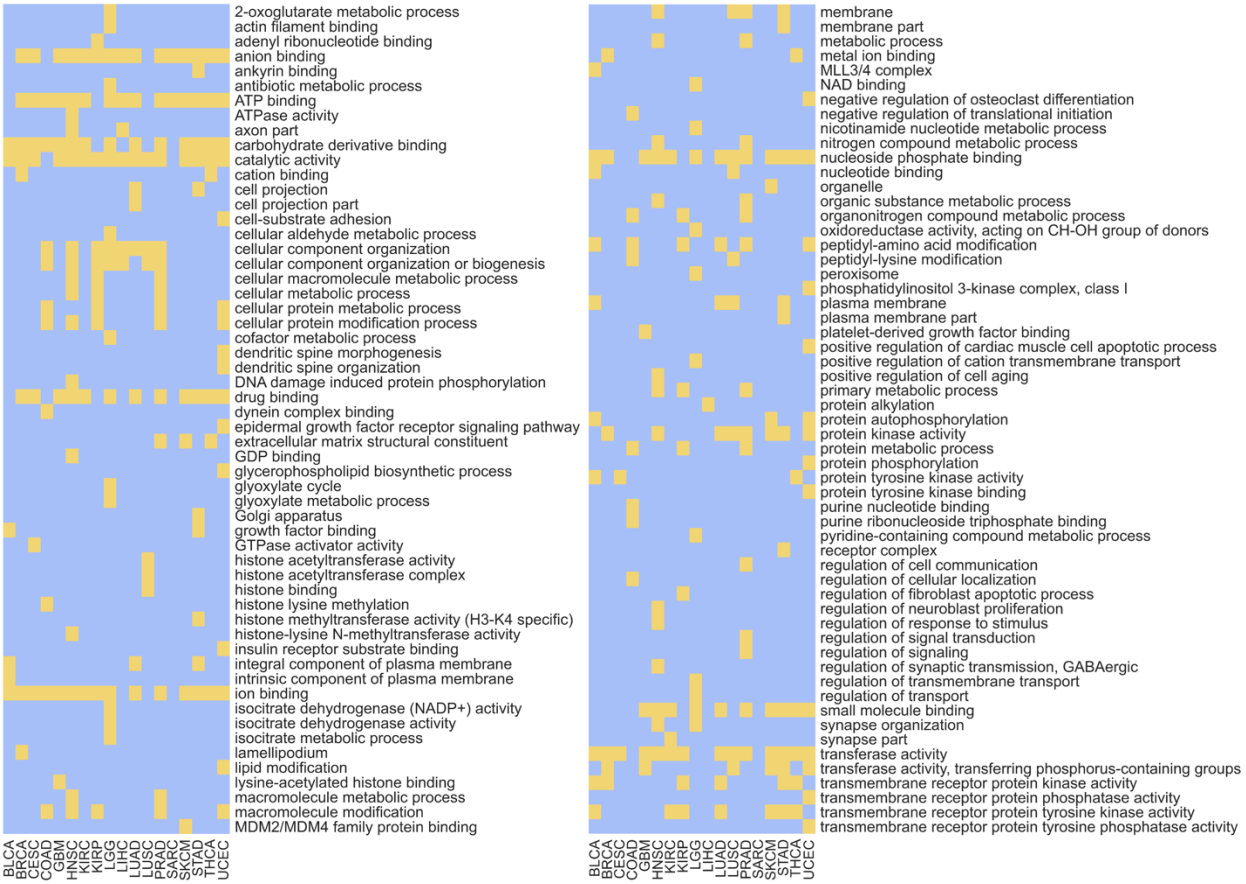


Fig. 8: GO terms enrichment for the 19 cancer types. Received by using the gene rankings of the all-features models. Every row represents a GO term, every column represents a cancer type. Yellow positions indicate non-redundant enriched GO terms with a p-value smaller than 0.001 and a q-value (FDR correction) smaller than 0.05. Blue positions indicate GO terms that are not enriched under these requirements.

Fig. 9: The number of cancer types for which a GO terms was enriched using gene rankings from the non-silent models and the all-features models. Every row represents a GO term. Every column represents a model from which the gene ranking list was used as input to the GOrilla tool.

2.7 All silent-features models outperformed the null model in predicting survival probabilities for more than 10 years after an initial cancer diagnosis

The purpose of this analysis was to assess whether the survival probabilities of patients could be estimated solely based on their silent mutations, and to compare the estimations of the silent-features models to the estimations of the non-silent and all-features models. Similarly to the cancer type classification task, no additional information, such as patient's age, sex, race or treatment history was used. In this analysis, patients across all 33 cancer types were included and a Random Survival Forest

(RSF) [43] algorithm was utilized. Due to the high computational requirements of the algorithm, only a subset of the features was chosen from each of the six initial datasets (see sub-section 4.4.1.1 for details about feature selection). The models were trained to predict patients' survival probability at any time after an initial cancer diagnosis. Then, the models were used to estimate the survival probabilities of patients at 10 different time points. The estimations were evaluated using the Area Under the Curve (AUC) [44] score and the results are presented in the following section.

All the silent-features models outperformed the null model for more than 10 years after the initial diagnosis (see Fig. 10). Additionally, the all-features model achieved the highest AUC score for more than nine years (3,500 days) after the diagnosis. This demonstrates that the addition of silent features to non-silent features is superior to the use of non-silent features alone for survivability prediction.

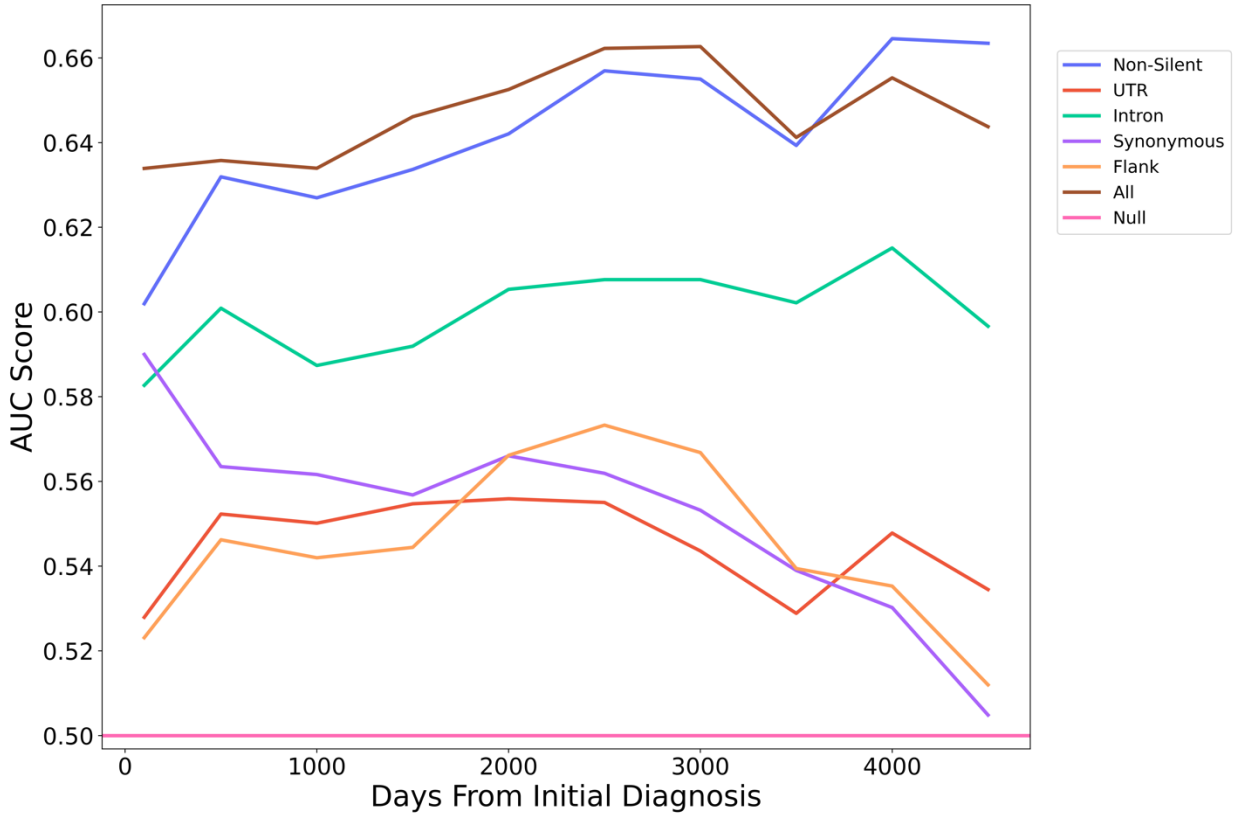


Fig. 10: AUC scores achieved by the six RSF models for various times after the initial cancer diagnosis. The x axis depicts the days passed since the diagnosis and the y-axis depicts the AUC score achieved by the models. Each colored curve denotes a different dataset. The horizontal line depicts the AUC score of a null model.

2.8 Silent features comprise 30% of the 10 most predictive features for survival estimation

Reviewing the feature importance ranking produced by the all-features model for survival estimation, *silent features comprised more than half of the top ranked 100 features and a third of the top ranked 10 features (see Fig. 11).* Table 2 holds the 10 most predictive features for survival estimation (the full feature importance list can be found in Table S5 in Additional File 3).

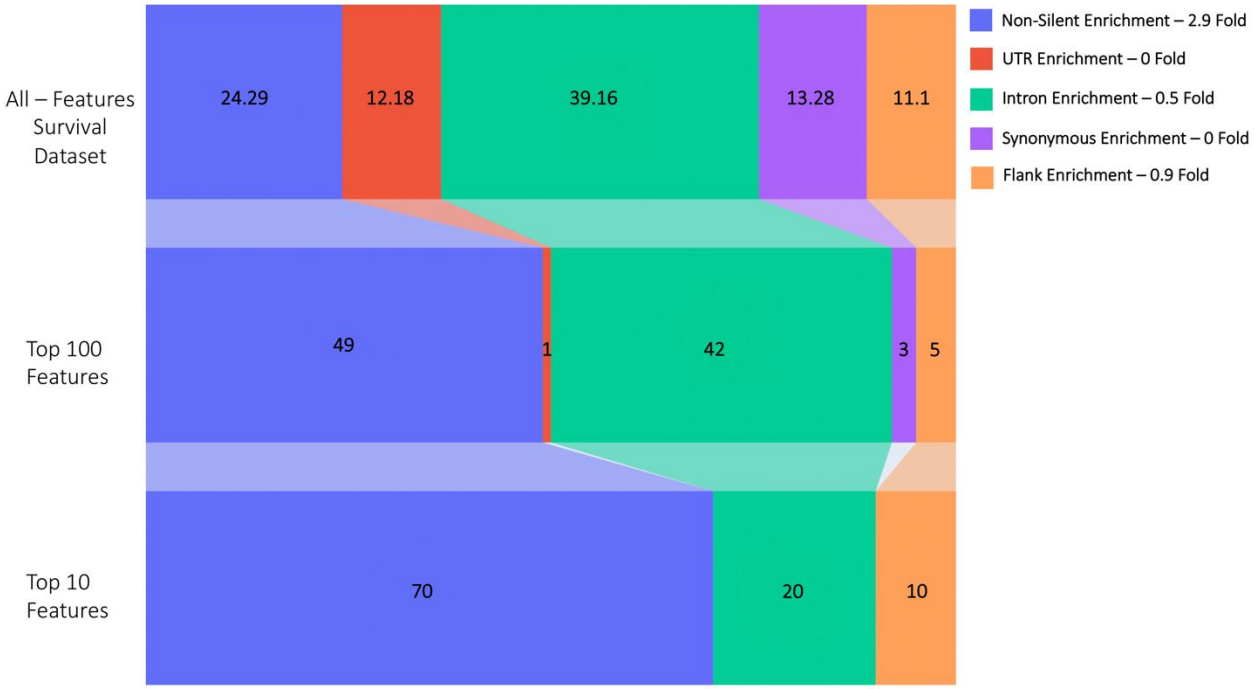


Fig. 11: Feature-type distribution of the all-features dataset and of the top ranked features chosen in the survival probability estimation task. Feature-type distribution of the all-features dataset* (top row), top ranked 100 features (middle row) and top ranked 10 features (bottom row). The feature rankings were obtained from the all-features model. The legend indicates the enrichment in the amount of each feature-type in the top 10 features compared to its original amount in the all-features dataset (ratio between bottom and top row). *Note: The distribution depicted in the top row is the distribution of the all-features dataset after it underwent preprocessing relevant for the survival estimation task.

3. Discussion

It has been suggested that silent mutations could affect tumorigenesis and cancer cell fitness through changes in gene expression regulation [30,32–38]. However, to the best of our knowledge, this study provides the first quantitative assessment of the predictive power of silent mutations over cancer classification and prognosis in comparison to non-silent mutations.

The results demonstrate the predictive ability of silent mutations to perform both the classification and survival estimation tasks; we specifically show that for some cancer types, it is comparable to the performances of non-silent mutations. Moreover, combining both non-silent and silent mutations achieved the best classification results for 68% of the cancer types and the best survival estimation results up to nine years after the cancer diagnosis. When using the same amount of features, a combination of silent and non-silent features was still superior to using only non-silent features for 63% of cancer types. In addition, since the protein functionality is quite robust to point mutations [45] and considering that many silent mutations (which affect gene expression regulation) were found highly predictive by the models, it is probable that some of the highly predictive non-silent mutations are such due to their impact on gene expression regulation rather than their impact on protein functionality.

As shown in Fig. 4B, the predictive power of silent mutations varies significantly between cancer types. This could suggest that some cancers are more affected by changes in genes' functionality caused mostly by non-silent mutations, while others are more affected by changes in gene expression levels, caused by both silent and non-silent mutations. The importance of different mutation types also varies when examining specific genes and pathways; the predictive power of a gene changes dramatically when it is mutated by different types of mutations. This suggests that a mutation that causes high predictivity changes the gene's functionality or regulation in a way that is optimal for the fitness of the cancer.

When examining the results of this study, one should keep in mind some inherent biases of the data. For example, non-silent mutations are naturally about 20 times more frequent than synonymous mutations. Thus, even if the effect of a single mutation is similar for both types, non-silent mutations are expected to make a larger impact. Another bias originates from the source of the data; the genomic data in this study is derived using WES, which is highly biased towards exonic mutations. WES sequences the genome's coding regions, ignoring most non-coding regions internal and external to genes [46]. In fact, an astonishing 98% of the genome is overlooked when performing WES, resulting in a narrow prism, heavily biased in favor of exonic mutations. An additional source of bias is the varying quantity of mutations in different genes: The importance of a gene for the models is greatly influenced by the number of mutations it has in TCGA. Specifically, there is an average 0.72 Spearman correlation between the number of mutations that genes have in TCGA and the gene rankings obtained for the 19 cancer types (see Fig. S6 in Additional File 1). Nonetheless, even though this correlation is high and significant, it also indicates that 52% of the variation in gene ranking could not be explained by the amount of mutations per gene in TCGA. In fact, some genes, such as *HRAS*, *YOD1*, *VHL* and *CEBPA*, were among the most important genes for several cancer types even though their number of mutations in TCGA is very small compared to other genes (ranging from the 4^{th} to 16^{th} percentile). We expect that without these biases the significance of silent mutations in cancer diagnosis and survival prediction will be even higher than the results reported here.

Finally, this study provides a broad, statistical analysis of the predictive abilities of silent and non-silent mutations of various kinds. The results suggest that models based on silent mutations could be very useful in practice. For example, for analyzing liquid biopsy samples [47,48] in order to perform cancer diagnosis or track cancer prognosis. Nevertheless, extensive work is required in order to expand and deepen our understanding of silent mutations and their ramifications on cancer development. For example, specific silent mutations that were chosen predictive by the models should be investigated in order to ascertain which regulatory regions and mechanisms they impact. Driver silent mutations should be distinguished from passenger silent mutations. Classification should be performed on both healthy individuals and cancer patients to understand the full diagnostic ability of silent mutations. Classification should also be performed using genomic information obtained from blood samples to see whether the diagnostic ability is similar under these circumstances. Finally, it will make sense to validate some of the mutations experimentally. All these research suggestions form the tip of the iceberg in an understudied field, full of clinical potential that is yet to be revealed.

4. Methods

4.1 Data extraction

The genomic and clinical data of patients across 33 cancer types were obtained from The Cancer Genome Atlas (TCGA)[39]. Patients with multiple genomic samples and patients with no genomic samples or

clinical records were excluded, leaving a total of 9,915 patients. The genomic data consists of the patients' mutation information.

4.2 Features engineering

Five categories of mutations were established –

1. non-silent mutations (coding sequence mutations that cause a change in the protein's amino-acid sequence)
2. synonymous mutations (coding sequence mutations that do not cause a direct change in the protein's amino-acid sequence)
3. Intronic mutations
4. UTR mutations
5. Flank mutations

For each category, the genomic data obtained from TCGA was used to create three kinds of features, representing three levels of resolution (see Fig. 3): low resolution features, medium resolution features and high-resolution features.

Low-resolution features count the number of mutations that appear in an entire gene.

Medium-resolution features count the number of mutations that appear in a specific segment of a gene. Each gene is assembled from the 5'UTR, introns, exons and the 3'UTR. The flanking regions are adjacent to the gene from both ends. A gene is split to 50-nucleotide long segments and the medium resolution features count the number of mutations in each segment. Two additional features count the number of mutations in the 5' flanking regions (upstream to the gene) and in the 3' flanking region (downstream to the gene).

High-resolution features indicate whether a specific mutation occurred in a specific location in the gene (For example, an A to G SNP would be considered a different mutation than an A to C SNP, even if it had occurred in the same position). If the specific mutation occurred only for a single patient in the TCGA database, its respective feature was discarded.

The features of each category were used as a separate dataset and they were also combined in order to create the sixth dataset- the all-features dataset.

4.3 Cancer Type Classification

4.3.1 One vs. all classifiers

4.3.1.1 Data preparation-

Only cancer types with more than 200 patients were included in the analysis, resulting in 8,364 patients spanning 19 cancer types.

4.3.1.2 Training and predicting-

114 OVA classifiers were generated and trained; one for each possible combination of cancer type (19) and dataset (6). The objective of each classifier was to distinguish a single cancer type from the rest. Specifically, predicting a "Positive" or "Negative" label for a particular cancer type. The OVA classifiers were constructed using the LightGBM[49] python package. For each classifier, the patients were randomly split into stratified training and testing sets (0.7/0.3 respectively) for 10 times. A null classifier was also generated using scikit-learn's Dummy Classifier[50] for each cancer type; the null classifier randomly assigned labels to the test-set patients, only considering the label distribution of the training-set patients.

4.3.1.3 Evaluating-

The classifiers' performance was evaluated with Accuracy, Recall, Precision and F1 scores (see Fig. 4B for F1 scores or Table S6 in Additional File 1 for all measures). Performances were averaged across the 10 splits. Precision is the fraction of correctly identified positive patients out of all patients that were identified as positive by the model. Recall is the fraction of correctly identified positive patients out of all the patients that are truly positive for the disease. The F1 score is a harmonic mean of precision and recall, taking both measures into account:

$$\mathrm{f1} = 2 * \frac{P * R}{P + R}$$

Where P is Precision and R is Recall. The f1 score ranges from zero to one, one indicating perfect Precision and Recall scores and zero indicating that either the Precision or Recall are also zero.

4.3.2 Gene ranking

Each classifier provides a feature ranking. First, features with zero importance were discarded. Then, a gene ranking was obtained by assigning the features (that can be mutations, segments or entire genes) to the gene they are related to while keeping the original order. Finally, only the highest rank of each gene was kept. The most important gene is ranked "0" and as the numbers increase the importance decreases.

4.3.3 Spearman correlation between gene rankings

Spearman correlations were conducted between gene rankings of pairs of classifiers detecting the same cancer type (see Fig. 7).

For every cancer type:

- The all-features classifier was excluded
- For each of the single-mutation-type classifiers, a gene ranking list was created as described above.
- Every combination of two classifiers was examined; genes that were not in the intersection of both gene ranking lists were discarded. Spearman correlation was calculated between the revised gene ranking lists.

The results were averaged across the 19 cancer types.

4.3.4 Gene ontology enrichment

Enriched GO terms (molecular functions, biological processes and cellular components) were detected for the 19 cancer types using the gene rankings obtained from the non-silent models and the all-features models.

For every combination of cancer type and model (either non-silent or all-features):

- The gene ranking list was created as described above.
- The gene ranking list was used as input to the GOrilla tool [40,41]. The tool used maximum Hyper Geometric (mHG) statistics in order to report GO terms that are enriched in the top of the list compared to the rest of the list. The threshold for splitting the genes list to "top" and "rest" is dynamic and was chosen for each GO term individually by the tool.
- The yielded terms are enriched with a p-value smaller than 0.001 and have passed an FDR correction of 0.05.

- The yielded terms were used as input to the REVIGO [42] tool, which removed terms with a semantic similarity score higher than 0.7. The similarity measure used was "SimRel".

The enriched GO terms detected for the 19 cancer types when using the all-features gene ranking are detailed in Fig. 8. A comparison between the GO terms that are detected when using the all-features gene ranking or the non-silent gene-ranking is seen in Fig. 9.

4.3.5 Spearman correlation between Jaccard similarity scores and misclassification rates

A Spearman correlation was conducted in order to evaluate the influence of genetic profile similarity on misclassification rates among pairs of cancer types. For this analysis binary versions of the features were used, meaning that rather than indicating how many mutations occur in genes and segments the features indicate whether any mutations had occurred or not (high resolution features were originally binary and thus do not change).

4.3.5.1 Calculating the Jaccard similarity scores for every pair of cancer types

- 100 patients were randomly selected from each type, forming two equally sized groups of patients (groups A and B).
- A Jaccard score was calculated for every patient in the group A with every patient in group B. The average score was considered the Jaccard score between the groups. The calculation was performed as shown below:

$$J_{A,B} = \frac{\sum_{a=1}^{100} \sum_{b=1}^{100} \frac{|F_a \cap F_b|}{|F_a| + |F_b| - |F_a \cap F_b|}}{100 * 100}$$

  Where F_a is the binary feature set of patient a from group A and F_b is the binary feature set of patient b from group B. $|F_a|$ is the number of features equal to "1" for patient a from group A (indicating all positions, segments and entire genes that were mutated). $J_{A,B}$ is the average Jaccard similarity score between group A and group B.
- The random sampling process was repeated 5 times. The final Jaccard score for a pair of cancer types was the average of the five repetitions.

4.3.5.2 Calculating the mistake rate for every pair of cancer types

- 250 patients were randomly selected from each type (groups A and B).
- The patients were stratified split to train and test sets (the training-set contained 70% of patients from each cancer types).
- An OVA model was fit on the training-set patients.
- The model was used to classify the test-set patients to one of the two cancer types.
- The misclassification rate between the groups was calculated as shown below:

$$M_{A,B} = \frac{|AB| + |BA|}{|AA| + |BB| + |AB| + |BA|}$$

  Where $|AB|$ is the number of group-A-patients that were classified as group-B-patients. $M_{A,B}$ is the misclassification rate between groups A and B.
- The random sampling process was repeated 10 times. The misclassification rate between the pair of cancer types was the average of the 10 repetitions.

4.3.6 Balanced datasets

To evaluate whether the results are significantly influenced by the imbalance between the mutation categories, balanced datasets were created for the two analyses depicted in Fig. 4B and Fig. 5. In order to maintain balance, only high-resolution features were used in these datasets.

4.3.6.1 Balanced version of Fig. 4B

Six same-size datasets were needed and were created in the following manner:
For every cancer type:

- The patients were split to two equally sized groups. The first for feature selection and creation of the balanced datasets and the second for training models on the balanced datasets and evaluating the results.
- Balanced dataset creation - six OVA models (one per dataset) were trained using the first group of patients. For every model, the highest ranked 8,296 features were chosen as the new dataset. This step resulted in six balanced datasets per cancer type, each containing 8,296 features. (The number of features was derived from the number of features in the smallest category, the flanking region mutations).
- Training and evaluating – six OVA models (one per dataset) were trained using the second group of patients and the balanced datasets. The models were trained for 10 rounds, whereby on each round a stratified random 0.7/0.3 split was performed. The performance was evaluated using the same measures as the imbalanced version of this analysis and can be found in the Fig. S1 in Additional file 1.

4.3.6.2 Balanced version of Fig. 5

An all-features dataset with an internal balance between mutation types was needed. For every cancer type, the 8,296 features that were chosen from each of the five mutation categories were combined in order to create the internally balanced all-features dataset. Then, an OVA model was trained using the balanced dataset and the second group of patients. The model was trained for 10 rounds, whereby on each round a stratified random 0.7/0.3 split was performed. The mutation-types distribution among the top 10 and top 100 features chosen by the classifiers were averaged across cancer types.

4.4 Survival Estimation

4.4.1 Random Survival Forest models

4.4.1.1 Data preparation-

Patients spanning all 33 cancer types were included in this analysis. Patients with no available information after the date of diagnosis and patients who passed away less than 20 days after their diagnosis were not included. Overall, 9,551 patients were included in the analysis. The vital status (alive or deceased) and appropriate time stamp were extracted from the clinical data and used as labels. A subset of features was chosen for each mutation category- all low-resolution features and 5,000 high-resolution features. The high-resolution features were selected based on mutation prevalence in TCGA; the features corresponding to the 5,000 most prevalent mutations were selected.

4.4.1.2 Training and predicting-

A model was generated and trained for each one of the six datasets (non-silent, UTR, intron, synonymous, flank and all-features). The objective of a model was to predict the probability of a patient to survive on a

given time after its initial cancer diagnosis. The models were constructed using the Pysurvival[51] Python package. 60 trees were grown with a maximal depth of 32 splits. For each model, the patients were randomly split into training and testing sets (0.7/0.3 respectively). The process was repeated five times and the survival probability estimation is the average of the 5 repetitions.

4.4.1.3 Evaluating-

The models' performances were evaluated using the Area Under the Curve (AUC) score for various times (100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000 and 4500 days) after the initial cancer diagnosis.

Data Availability

The data used in this study was generated by The Cancer Genome Atlas (https://www.cancer.gov/tcga) and can be downloaded from the genomic data commons (https://portal.gdc.cancer.gov/).

Author Contributions

TT conceived this study. TG, GG, OE and TT analyzed the data. TT supervised the study. TG and TT wrote the paper. All authors read and approved the final manuscript.

Competing Interests

The authors declare they have no competing interests.

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Tables

Cancer Type	Rank	Feature	Feature Type	Importance	Gene
CESC	0	MUC4 Non_Silent	Non-Silent	0.16	MUC4
	1	TP53 Non_Silent	Non-Silent	0.06	TP53
	2	PABPC1 UTR	UTR	0.04	PABPC1
	3	BCR UTR	UTR	0.03	BCR
	4	NF1 5602.0 UTR	UTR	0.01	NF1
	5	RGPD3 0.0 Flank	Flank	0.01	RGPD3
	6	CSF1 UTR	UTR	0.01	CSF1
	7	MUC4 Synonymous	Synonymous	0.01	MUC4
	8	SRGAP3 UTR	UTR	0.01	SRGAP3
	9	CARD11 793.0 Intron	Intron	0.01	CARD11
LIHC	0	MUC4 Non_Silent	Non-Silent	0.25	MUC4
	1	SET 210.0 Intron	Intron	0.08	SET
	2	PIK3CA Non_Silent	Non-Silent	0.03	PIK3CA
	3	ALB Intron	Intron	0.02	ALB

	4	240343-240343-chr5-Intron-DEL-T-T--	Intron	0.02	SDHA
	5	APC Non_Silent	Non-Silent	0.01	APC
	6	FAM46C UTR	UTR	0.01	FAM46C
	7	SRGAP3 UTR	UTR	0.01	SRGAP3
	8	MUC4 Synonymous	Synonymous	0.01	MUC4
	9	SEPT9 3283.0 Intron	Intron	0.01	SEPT1
THCA	0	140753336-140753336-chr7-Missense_Mutation-SNP-A-A-T	Non-Silent	0.18	BRAF
	1	BRAF 378.0 Non_Silent	Non-Silent	0.13	BRAF
	2	TP53 Non_Silent	Non-Silent	0.07	TP53
	3	MUC4 Non_Silent	Non-Silent	0.06	MUC4
	4	NRAS 189.0 Non_Silent	Non-Silent	0.02	NRAS
	5	MUC4 Silent	Synonymous	0.02	MUC4
	6	533874-533874-chr11-Missense_Mutation-SNP-T-T-C	Non-Silent	0.01	HRAS
	7	BRAF Non_Silent	Non-Silent	0.01	BRAF
	8	TP53 26.0 Non_Silent	Non-Silent	0.01	TP53
	9	LRP1B Intron	Intron	0.01	LRP1B

Table 1: Examples of the top 10 ranked features for classifying various cancer types. The top 10 feature rankings for CESC, LIHC and THCA (Thyroid Carcinoma) are shown. For each feature, the table holds its name, mutation type, its importance for classifying the specific cancer type and the gene to which it is related to. The rankings were obtained from the all-features models.

Rank	Feature	Feature Type	Importance	Gene
0	TP53 Non_Silent	Non_Silent	0.0142	TP53
1	MUC4 Non_Silent	Non_Silent	0.0050	MUC4
2	57466291-57466292-chr12-Frame_Shift_Ins-INS-----G	Non_Silent	0.0049	GLI1
3	143147221-143147222-chr5-Intron-INS-----C	Intron	0.0035	ARHGAP26
4	140753336-140753336-chr7-Missense_Mutation-SNP-A-A-T	Non_Silent	0.0032	BRAF
5	NKX2-1 Flank	Flank	0.0031	NKX2-1
6	25743660-25743660-chr2-Missense_Mutation-SNP-T-T-G	Non_Silent	0.0027	ASXL2

7	EGFR Non_Silent	Non_Silent	0.0026	EGFR
8	92956452-92956452-chr15-Splice_Region-SNP-A-A-T	Intron	0.0026	CHD2
9	35457937-35457938-chr6-Frame_Shift_Ins-INS-----C	Non_Silent	0.0026	FANCE

Table 2: The top 10 ranked features for estimating patients' survival probability. For each feature, the table holds its name, mutation type, its importance ranking, the gene to which it is related to and the gene's product description. The ranking was obtained from the all-features model.

Additional Files

Additional File 1 (a docx file): Primary Supplemental File containing all Supplemental Figures, Table S1, Table S2, Table S3 and Table S6.

Fig. S1: The F1 scores achieved by the OVA models using the balanced datasets. Features from all levels of resolution were used. Each dataset contained 8,296 features. The x axis depicts the cancer types, the y-axis depicts the F1 scores achieved by the models. Each bar color denotes a different dataset. Cancer types for which the all-features classifier outperformed the non-silent classifier are denoted on red.

Fig. S2: Spearman correlation between Jaccard similarity scores and misclassification rates of pairs of cancer types. Every dot represents a pair of cancer types. The x axis denotes the pair's Jaccard similarity score and the y axis denotes their misclassification rate. The Spearman coefficient (Rho) and respective p value are noted above each graph.

Fig. S3: Feature-type distribution of the balanced all-features dataset and of the top ranked features for the classification task. Feature-type distribution of the all-features dataset (top row), top ranked 100 features (middle row) and top ranked 10 features (bottom row). The feature rankings were obtained from the all-features models and were averaged across cancer types. The legend indicates the enrichment in the amount of each feature-type in the top 10 features when compared to its original amount in the balanced all-features dataset (ratio between bottom and top row).

Fig. S4: Polymorphism type distributions in the initial datasets, top 100 features and top 10 features obtained from the OVA models. Each sub-figure (a-b) denotes a model. Within a sub-figure, every three clustered columns represent the distribution of the initial dataset (left column), top 100 features (middle column) and top 10 features (right column) of a single cancer type. The analysis was conducted using the feature importance rankings that were obtained from the balanced datasets. The Synonymous models contain only SNPs and thus are excluded from this analysis.

Fig. S5: Spearman correlations between gene rankings of pairs of models per cancer type. The all-features model was excluded from the analysis. Every subplot (a-s) represents a single cancer type. Within a subplot, every graph depicts the correlation between two models. A dot in the graph represents a gene. The x axis denotes the gene's rank given by the first model and the y axis denotes its rank given by the second model. The Spearman coefficient (Rho) and respective p value are noted above each graph.

Fig. S6: Spearman correlation between the number of mutations documented per gene in the TCGA database and the gene's ranking obtained from the all-features models of the 19 cancer types. Each graph represents a single cancer type. A dot represents a single gene. The x axis denotes the number of mutations documented in TCGA for the gene and the y axis denotes the gene's ranking obtained from the all-features model. The Spearman coefficient (Rho) and respective p value are noted above each graph.

Table S1: F1 score improvement gained from adding lower resolution features. The F1 score improvement per cancer type that was achieved by adding medium resolution features and then low resolution features. The results were obtained using the all-features models.

Table S2: Feature type distribution among the top 100 ranked features for each cancer type. Feature rankings were obtained from the all-features models.

Table S3: Feature type distribution among the top 10 ranked features for each cancer type. Feature rankings were obtained from the all-features models.

Table S6: Performance evaluations of the six models (Non-Silent, Intron, UTR, Flank, Synonymous, All-Features) for classifying the 19 cancer types participating in the cancer type classification task, compared to a null model.

Additional File 2 (an xlsx file):

Table S4: Feature importance rankings of the cancer type classification task. Every feature with importance higher than zero is listed. Each sub-table (a-s) holds the feature importance rank of the six models (non-silent, UTR, intron, synonymous, flank and all-features) for a single cancer type.

Additional File 3 (an xlsx file):

Table S5: Feature importance rankings of the survival estimation task. Every feature with importance higher than zero is listed. The table holds the feature importance rank of the six models (non-silent, UTR, intron, synonymous, flank and all-features).