Prediction of high-risk liver cancer patients from their mutation profile: Benchmarking of mutation calling techniques

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Abstract

Identification of somatic mutations with high precision is one of the major challenges in prediction of high-risk liver-cancer patients. In the past, number of mutation calling techniques have been developed that include MuTect2, MuSE, VarScan2, and SomaticSniper. In this study, an attempt has been made to benchmark potential of these techniques in predicting prognostic biomarkers for liver cancer. Initially, we extracted somatic mutations in liver-cancer patients using VCF and MAF files from the cancer genome atlas. In terms of size, the MAF files are 42 times smaller than VCF files and containing only high-quality somatic mutations. Further, machine learning based models have been developed for predicting high-risk cancer patients using mutations obtain from different techniques. The performance of different techniques and data files have been compared based on their potential to discriminate high and low risk liver-cancer patients. Finally, univariate survival analysis revealed the prognostic role of highly mutated genes. Based on correlation analysis, we selected 80 genes negatively associated with the overall survival of the liver cancer patients. Single-gene based analysis showed that MuTect2 technique based MAF file has achieved maximum HR_{LAMC3} 9.25 with p-value 1.78E-06. Further, we developed various prediction models using selected genes for each technique, and the results indicate that MuTect2 technique based VCF files outperform all other methods with maximum AUROC of 0.72 and HR 4.50 (p-value 3.83E-15). Eventually, VCF file generated using MuTect2 technique performs better among other mutation calling techniques to explore the prognostic potential of mutations in liver cancer. We hope that our findings will provide a useful and comprehensive comparison of various mutation calling techniques for the prognostic analysis of cancer patients.

Keywords: Mutation calling techniques; Prognosis; Liver cancer; Survival analysis; Machine learning; Regression

49 **Introduction**

50 According to the world health organization, cancer is a life-threatening disease and the first
 51 leading cause of death worldwide in 2019. Global cancer statistics estimate that in 2020, 19.3
 52 million new cases and 10 million deaths have been occurred due to cancer [1]. Cancer is
 53 extremely heterogeneous; therefore, the same treatment strategy is not effective for
 54 individuals with similar types of cancer. Till now, there is no universal treatment available
 55 for all types of malignancies. Currently, several targeted therapies are available for cancer
 56 treatment, which majorly focus on the detection of mutations at the genetic level [2]. In the
 57 last few years, several therapies have been designed based on the mutated genes for the
 58 cancer treatment. For instance, B-Raf Proto-Oncogene, Serine/Threonine Kinase (BRAF)
 59 inhibitors (Sorafenib) is identified to treat melanoma patients with V600E mutation in the
 60 BRAF gene [3, 4]. However, drugs like afatinib and erlotinib are used to target the mutation
 61 in the EGFR in non-small-cell lung cancer [5, 6]. Moreover, BRCA1/BRCA2 gene mutations
 62 in ovarian cancer patients have been treated by poly (ADP-ribose) polymerase (PARP)
 63 inhibitor, i.e., olaparib [7]. Of note, research on the mutations associated with the genes in
 64 cancer patients is essential for identifying the correct mechanism of the disease. Due to the
 65 advancements in next-generation sequencing, such as whole-genome, whole-exome, and
 66 mutation calling techniques, the detection of more than 98% mutations associated with the
 67 disease using sequencing data is possible [8, 9]. The easy availability and low cost of next-
 68 generation sequencing techniques enable researchers to perform experiments on large cohorts
 69 of cancer patients [10].

70 The genetic variants are mainly categorised into single nucleotide variant (SNV),
 71 insertion/deletion (indel), and structural variants (SV, which incorporates copy number
 72 alterations, duplications, and translocations). In recent years, a huge number of somatic
 73 mutation calling algorithms (for example, Mutect2, Varscan2, SomaticSniper, MuSE,
 74 Strelka2, etc.) have been developed to identify mutations at the genetic level using
 75 sequencing data [11-17]. Mutect2 calls somatic mutation such as single nucleotide alterations
 76 and indels using the local assembly of haplotypes. SomaticSniper pipeline detects somatic
 77 SNVs using Bayesian algorithm to compare the genotype likelihoods in the tumor and normal
 78 samples. However, Varscan2 mutation calling algorithm uses exomes, whole-genome
 79 sequencing data to capture germline variants, somatic mutations and copy number variants in
 80 tumor-normal data. Moreover, MuSE is a Markov Substitution model for Evolution, to
 81 identify novel mutations in the large-scale tumor sequencing data.

Liver cancer is one of the deadliest disease which is the seventh most common cancer among the 36 cancers reported by Global Cancer Statistics 2020 [1]. Ample treatment methods were developed in the past, but still the survival rate of liver cancer patients is very low, leading to high-mortality rate [18]. Being the most comprehensive resource for the cancer related research, TCGA provides two types of file formats for mutation data such as Variant Call Format (VCF) and Mutation Annotation Format (MAF). VCF files are the raw mutation files that store and report the genomic sequence variations that directly came out of the various automated variant calling pipelines. On the other hand, MAF files are the processed version of the VCF files, which are curated by removing the false positives or by recovering the known calls that the automated pipelines may have missed. VCF files report mutations irrespective of their importance, but MAF files describe only the most affected ones by removing the low-quality mutations. In GDC portal, both type of files are available generated using the four major mutation calling techniques named as MuTect2, MuSE, VarScan2, and SomaticSniper. Despite number of techniques are available, it is difficult to understand which method and file is better to explore the role of mutations in cancer.

In the current study, we have systematically evaluated the four mutation calling tools which are widely used in TCGA, to identify highly mutated genes associated with high-risk liver cancer patients. For this, we have collected VCF and MAF files of 418 liver cancer patients for all the mutation calling techniques. The gene-based annotations were identified using highly accurate and widely used methods ANNOVAR [19] and Maftools [20]. Correlation and survival analysis is performed to identify mutated genes that can impact the survival of liver cancer patients. Finally, several prediction algorithms have been developed for the top genes. The inferences of our study can give a valuable reference and guidance to the researchers to choose a reliable somatic mutation algorithm to determine the mutation-associated genes having a significant impact on the survival of the cancer patients.

Material and Methods

Dataset Collection

We obtained liver cancer (TCGA-LICH and TCGA-CHOL) mutation data from Genome Data Commons (GDC) data portal. Precisely, we collected the controlled access VCF of liver cancer patients under the approval of dbGap (Project No. 17674) according to the GDC protocols [21]. In addition to that, we have also downloaded the MAF files of TCGA liver cancer patients. In TCGA, four different techniques are used for mutation calling, i.e., MuSE,

Mutect2, Varscan2, and SomaticSniper. In this study, we have utilized VCF and MAF files of 418 liver cancer samples generated from four different mutation calling methods. Moreover, the clinical data like age, gender, tumor stage, overall survival (OS) time, and vital status were collected using TCGA assembler 2 [22].

Mutation Annotations

We used the ANNOVAR software package (<https://annovar.openbioinformatics.org/en/latest/>) for functional annotations of genetic variant mutations. First, we convert VCF files into ANNOVAR genetic variants file; using “convert2annovar.pl” script; the processed file contains five major columns such as chromosome number, start position, end position, reference nucleotide, and altered nucleotides. It provides three major type of annotations (i.e., gene-based, region-based, and filter-based annotations). In this work, we used gene-based annotations, in which we obtained mutations/gene/samples. In this way, we get per-gene mutations for each sample for the four different mutation calling techniques. After that, we count number of mutations per gene for each liver cancer patient with the help of in-house python script (gene_to_matrix.py). Similarly, for MAF files we counted the number of mutations/gene/samples. Finally, we generated matrices for each mutation calling technique from VCF and MAF files, in which number of mutations per gene per sample were reported.

Correlation Analysis

To understand the impact of number of genetic mutations on overall survival (OS) of liver cancer patients, we have implemented correlation test. After that, we removed the genes with the non-significant p-value i.e., >0.05 , and ranked the remaining genes on the bases of correlation coefficients. We choose top-10 negatively correlated genes from each technique for VCF and MAF files for further analysis.

Survival Analysis

In this study, we have performed survival analysis by the ‘survival’ package in R (V.3.5.1) using cox proportional hazard (Cox PH) model. We perform univariate survival, in order to understand the impact of per gene mutations on the survival of liver cancer patients. The log-rank test was used to estimate the significant survival distributions between high-risk and low-risk groups in terms of the p-value. Kaplan-Meier (KM) survival curves were used for the graphical representation of high-risk and low-risk groups [23].

150

151 **Machine learning Techniques**

152 **Classification Models**

153 In this study, we have implemented various machine learning techniques for the classification
154 of high-risk and low-risk samples based on the number of mutations in the chosen genes.
155 Classification algorithms includes Decision tree (DT), Support Vector Classifier (SVC),
156 Random Forest (RF), XGBoost (XGB), Gaussian Naive Bayes (GNB), Logistic Regression
157 (LR), k-nearest neighbors (KNNs) and ExtraTree (ET) using Scikit learn [24].

158

159 **Regression Models**

160 Further, we implemented several regressors to develop regression models for overall survival
161 time prediction in liver cancer patients. These techniques were developed using python-
162 library scikit-learn and includes Random Forest (RF), Ridge, Lasso, Decision Tree (DT),
163 Elastic Net (ENR), Logistic Regression (LR), and Support Vector Regression (SVR)[24].

164

165 **Performance Evaluation**

166

167 **Cross-Validation Technique**

168 To avoid over-optimization in the machine learning models, we have used standard five-fold
169 cross-validation technique [25, 26]. In case of classification, the complete dataset was divided
170 into 80:20 ratio, the five-fold cross-validation was performed on the 80% training dataset. In
171 this method, the training dataset split-up into five equal sets. However, four sets used for
172 training and remaining set used for the testing purpose. The similar task was repeated for at
173 least five times, so that every set can be used in training and testing. Finally, the performance
174 or outcome computed by taking the mean of all five sets. The similar process was repeated
175 for the cross validation of regression models. In this the complete dataset was used for the
176 five-fold cross validation.

177

178 **Performance Measure Parameters**

179 To evaluate the performance of classification models, we have used standard parameters. We
180 have calculated threshold-dependent such as sensitivity (Sens), specificity (Spec), accuracy
181 (Acc), F1-score, and MCC, and independent parameters like Area Under the Receiver
182 Operating Characteristic (AUROC). These parameters were calculated using the following
183 equations (1-5).

184

$$Sensitivity = \frac{P_T}{P_T + N_F} \times 100 \quad [1]$$

$$Specificity = \frac{N_T}{N_T + P_F} \times 100 \quad [2]$$

$$Accuracy = \frac{P_T + N_T}{P_T + P_F + N_T + N_F} \times 100 \quad [3]$$

$$F1 - score = \frac{2P_T}{2P_T + N_F + N_P} \quad [4]$$

$$Matthews Correlation Coefficient = \frac{(P_T * N_T) - (P_F * N_F)}{\sqrt{(P_T + P_F)(P_T + N_F)(N_T + P_F)(N_T + N_F)}} \quad [5]$$

185

186 **P_T =True Positive, P_F =False Positive, N_T =True Negative, N_F =False Negative**

187

188 Similarly, to evaluate the regression models, we have used parameters such as mean absolute
189 error (MAE), root mean-square error (RMSE), correlation coefficient (R), and p-value, to
190 evaluate the performance of regression models as previously used in different studies [27-29].

191

192 **Results**

193 In this study, we have used 418 TCGA liver cancer patients somatic mutation data (VCF files
194 and MAF files) and OS data. The mutation data were taken from four different mutation
195 calling techniques i.e., MuSE, Mutect2, Varscan2 and SomaticSniper. ANNOVAR software
196 and in-house scripts were used to extract the number of mutations/gene/samples from the
197 VCF and MAF files. The total number of genes and mutations extracted from different
198 techniques is shown in Table 1. Where, in VCF files Mutect2 and SomaticSniper reported the
199 highest number of genes and mutation counts i.e., more than 25000 genes and 5 million
200 mutations. On the other hand, in MAF files the reported number of genes and mutations is
201 comparatively less for each technique.

202

203 **Table 1: Total number of genes and mutations for each gene extracted from VCF and**
204 **MAF files using different mutation calling technique**

File Type	Technique	Number of Genes	Number of Mutations
VCF	MuTect2	25366	5237093
	MuSE	19425	379368
	Varscan2	19422	576231
	SomaticSniper	25785	5003969
MAF	MuTect2	16474	59741

Figure 2: Visualization of mutation summary (variants classification, type and SNVs) for MuTect2, MuSE, Varscan2 and SomaticSniper MAF files

In Varscan2 and MuTect2, the variant classification distribution represents nine types of mutations such as Missense_Mutation, Nonsense_Mutation, Splice_Site, Translational_Start_Site, Frame_Shift_Ins, Frame_Shift_Del, In_Frame_Ins, In_Frame_Del, and Nonstop_Mutations, while MuSE and SomaticSniper MAF files consist Missense_Mutation, Nonsense_Mutation, Splice_Site, Translational_Start_Site, Nonstop_Mutations. The SNV class visualizes the single-nucleotide variants in the TCGA cohort, we observed that all the methods present diverse distribution of SNV as shown in (Figure 2). Oncoplots generated by the Maftools visualization module illustrating the somatic landscape of the cancer patients for Varscan2, MuTect2, MuSE and SomaticSniper MAF files. In Figure 3, we display the topmost mutated genes with their mutation percentage ($\geq 5\%$) in total number of samples. From the results we observed that, TP53 is highly mutated gene and have almost 20% or $>20\%$ mutations among different techniques.

Figure 3: Oncoplot visualization of mutation frequency of top-most mutated genes. The rows represented the genes with % mutations, and columns display the samples. (a) Illustrates the oncoplot of MuTect2 technique and indicates that 89.18% of samples having mutated genes (b) Illustrates the oncoplot of MuSE technique and shows that 80.29% of samples having mutated genes (c) Presents the oncoplot of Varscan2 approach and shows that 88.43% of samples having mutated genes (d) Illustrates the oncoplot of SomaticSniper technique and indicates that 75.73% of samples having alerted/mutated genes

Correlation Analysis

By implementing the correlation test we ranked the genes and choose top-10 genes having significant negative-correlation coefficients. The procedure is repeated for all the four techniques from MAF and VCF files of liver cancer patients, which lead to 80 genes in total. The complete correlation analysis is provided in Supplementary Table S1.

Prognostic Biomarkers for High-Risk Prediction

Single gene

Univariate survival analysis was performed using cox-proportional hazard model. We have calculated the HR and p-value for ten genes from each technique for VCF files. SomaticSniper technique has achieved the maximum HR value in single gene based analysis with $HR_{CLDN20} = 7.06$ and p-value $6.62E-07$, followed by Varscan2 with $HR_{FAM160A2} = 6.81$ and p-value $4.01E-05$, followed by MuTect2 based VCF file with $HR_{SNHG10} = 5.49$ and p-value $3.94E-06$, and Muse technique has achieved the HR_{CLMP} of 3.01 with p-value $1.67E-05$ as shown in Table 2.

Table 2: Hazards ratio for top-10 genes from VCF files derived using MuTect2, MuSE, Varscan2, and SomaticSniper technique

MuTect2					MuSE				
Gene	HR	P-value	95% CI	C-index	Gene	HR	P-value	95% CI	C-index
SNHG10	5.49	3.94E-06	2.66 - 11.31	0.53	CLMP	3.01	1.67E-05	1.82 - 4.97	0.54
WIZ	2.69	9.71E-07	1.81 - 4.00	0.56	BIRC6	2.80	4.46E-04	1.58 - 4.99	0.54
MGAT4EP	2.49	4.46E-04	1.50 - 4.15	0.54	LINC02210-CRHR1	2.03	6.42E-03	1.22 - 3.39	0.53
LINC00304	2.39	7.40E-05	1.55 - 3.67	0.55	DHX8	2.00	2.90E-02	1.07 - 3.74	0.52
CACNG7	1.93	5.72E-04	1.33 - 2.81	0.56	LINC00972	1.91	9.31E-03	1.17 - 3.10	0.54
OR52B6	1.83	1.12E-03	1.27 - 2.63	0.56	PAX7	1.90	8.29E-04	1.30 - 2.76	0.56
TYK2	1.80	2.21E-03	1.24 - 2.63	0.56	TAS1R2	1.61	2.63E-02	1.06 - 2.44	0.53
PIGO	1.79	1.66E-02	1.11 - 2.88	0.52	SNTG1	1.53	3.37E-02	1.03 - 2.27	0.54
S100A12	1.71	1.10E-02	1.13 - 2.59	0.54	CNTN5	1.34	2.25E-01	0.83 - 2.16	0.51
DNAJC9-AS1	1.08	6.51E-01	0.77 - 1.51	0.52	ZNF521	1.26	2.63E-01	0.84 - 1.91	0.52
Varscan2					SomaticSniper				
Gene	HR	P-value	95% CI	C-index	Gene	HR	P-value	95% CI	C-index
FAM160A2	6.81	4.01E-05	2.73 - 17.02	0.52	CLDN20	7.06	6.62E-07	3.27 - 15.2	0.53
LOC100420587	5.45	1.31E-07	2.90 - 10.22	0.54	NR2C2AP	5.17	3.16E-05	2.38 - 11.2	0.52
SPDYA	3.08	7.70E-04	1.60 - 5.94	0.53	ATG9B	3.34	2.59E-04	1.75 - 6.37	0.53
BRSK2	2.55	1.01E-03	1.46 - 4.46	0.54	HAUS5	2.79	2.22E-05	1.74 - 4.48	0.55
ADGRF4	2.21	1.23E-02	1.19 - 4.10	0.53	LOC100287329	2.58	8.23E-04	1.48 - 4.49	0.53
LINC00972	2.11	2.18E-03	1.31 - 3.41	0.55	P4HTM	2.18	2.43E-02	1.11 - 4.31	0.52
TM4SF18	2.07	1.40E-02	1.16 - 3.70	0.53	OR6C76	2.12	1.18E-03	1.35 - 3.35	0.54
OR5AS1	1.86	1.43E-02	1.13 - 3.06	0.54	CLK2	1.94	3.58E-02	1.05 - 3.61	0.52
PDE11A	1.72	2.74E-03	1.21 - 2.46	0.55	FAM187B	1.64	1.51E-02	1.10 - 2.43	0.55
LOC101929073	1.29	2.98E-01	0.80 - 2.11	0.52	NOMO3	1.34	1.45E-01	0.90 - 1.98	0.52

HR: Hazard ratio; 95% CI: 95% Confidence Interval; C-index: Concordance index

Similar analysis was done for MAF files from each technique and HR values were calculated. As exhibited in Table 3, Mutect2 technique based MAF file has achieved the maximum

HR_{LAMC3} = 9.25 with p-value 1.78E-06, followed by Varscan2 with HR_{SYDE1} 8.46 and 3.71E-05, followed by MuSE technique with HR_{ITGB8} 8.30 and p-value 5.69E-07, then followed by SomaticSniper with HR_{CAD} 5.56 and p-value 8.10E-04.

Table 3: Hazards ratio for top-10 genes from MAF files derived using MuTect2, MuSE, Varscan2, and SomaticSniper technique

MuTect2					MuSE				
Gene	HR	P-value	95% CI	C-index	Gene	HR	P-value	95% CI	C-index
LAMC3	9.25	1.78E-06	3.71 - 23.05	0.52	ITGB8	8.37	5.69E-07	3.64 - 19.24	0.52
EVC2	4.30	8.66E-05	2.08 - 8.91	0.53	TBX3	8.10	6.06E-05	2.91 - 22.53	0.52
NYNRIN	3.94	1.22E-03	1.72 - 9.05	0.52	SIPA1L3	4.90	5.54E-05	2.26 - 10.61	0.52
KIAA2026	3.85	1.49E-03	1.68 - 8.86	0.52	CAD	4.45	3.58E-03	1.63 - 12.14	0.52
SUPT20H	3.41	7.53E-03	1.39 - 8.40	0.51	EVC2	4.16	2.97E-04	1.92 - 9.01	0.52
BRINP2	2.83	2.43E-02	1.14 - 6.98	0.52	ARHGEF11	3.17	2.37E-02	1.17 - 8.64	0.51
LRP1B	1.93	7.81E-03	1.19 - 3.14	0.54	BRINP2	2.80	2.56E-02	1.13 - 6.92	0.52
TP53	1.48	3.60E-02	1.03 - 2.14	0.55	PCDH15	1.72	1.20E-01	0.87 - 3.39	0.51
TG	1.46	4.53E-01	0.54 - 3.97	0.51	TG	1.46	4.55E-01	0.54 - 3.97	0.51
PCDH15	1.43	3.30E-01	0.70 - 2.93	0.51	CSMD3	1.24	4.54E-01	0.71 - 2.15	0.51
Varscan2					SomaticSniper				
Gene	HR	P-value	95% CI	C-index	Gene	HR	P-value	95% CI	C-index
SYDE1	8.46	3.71E-05	3.07 - 23.35	0.52	CAD	5.56	8.10E-04	2.04 - 15.17	0.52
ALPP	4.33	1.44E-03	1.76 - 10.66	0.52	TOP2A	4.63	2.73E-03	1.70 - 12.62	0.52
KIAA2026	3.85	1.49E-03	1.68 - 8.86	0.52	KIAA2026	4.01	2.62E-03	1.62 - 9.93	0.52
CAD	3.32	1.91E-02	1.22 - 9.04	0.51	EVC2	4.00	1.04E-03	1.75 - 9.17	0.52
BRINP2	2.83	2.43E-02	1.14 - 6.98	0.52	KTN1	2.56	1.09E-01	0.81 - 8.10	0.51
TP53	1.60	9.85E-03	1.12 - 2.30	0.56	EPHA3	2.25	1.67E-01	0.71 - 7.13	0.51
PCDH15	1.48	2.81E-01	0.72 - 3.05	0.51	KIF26B	2.03	1.66E-01	0.74 - 5.55	0.51
TG	1.46	4.53E-01	0.54 - 3.97	0.51	PCDH15	1.76	1.78E-01	0.77 - 4.02	0.51
PLCB1	1.25	7.00E-01	0.40 - 3.96	0.50	TP53	1.63	1.20E-02	1.11 - 2.38	0.55
XIRP2	1.11	7.55E-01	0.58 - 2.12	0.51	TG	1.18	8.17E-01	0.29 - 4.79	0.50

HR: Hazard ratio; 95% CI: 95% Confidence Interval; C-index: Concordance index

Multiple Gene

In order to explore the effect of mutations in all the selected genes altogether, we have predicted the survival time to estimate the high-risk group in liver cancer patients. Using the predicted OS time, HR and p-value was computed with cox proportional hazard models for each technique corresponds to each file type. We achieved highest HR 4.50 with highly significant p-value 3.83E-15 for the VCF files generated using the MuTect2 technique

(Figure 4A). However, in case of MAF files, MuSE technique performed best among other techniques with HR 2.47 and p-value 9.64E-07 (Figure 4B). Additionally, KM survival plots clearly represents the segregation of high- and low-risk groups; the comparison of different mutation calling techniques based on two file formats is shown in Figure 4.

Figure 4: Kaplan Meier survival curves for the risk estimation of liver cancer patients based on the combined effect of mutation (A) survival plots for the VCF files (B) survival plots for the MAF files

Prediction of Overall Survival of Patients

To predict the overall survival for liver cancer patients, we have used number of mutations in the top-10 genes as the input feature and developed regression models for VCF and MAF files for each technique, using seven different regressors such as, Linear (LR), Lasso (LAS), Ridge (RID), Elastic Net (ENT), Decision Tree (DTR), Random Forest (RFR), and Support Vector (SVR). Table 4 exhibits the performance of best performing regressor in each file type. Performance of all the regressors for each file type and technique is reported in Supplementary Table S2. In case of MuTect2 technique, the OS predicted using VCF files have MAE 12.52 and significant correlation of 0.57 between the true and predicted OS; whereas in MAF file the MAE is 16.47 with R 0.37. Whereas, MuSE technique has achieved the minimum MAE of 13.88 and 16.89 along with R of 0.51 and 0.34, for VCF and MAF file respectively. In files generated using Varscan2 technique, for VCF file the minimum MAE is 14.57 with R 0.48, whereas for MAF file it is 16.53 with R 0.36. VCF and MAF file generated using SomaticSniper technique reported minimum MAE of 15.76 (R=0.40) and

16.72 (R=0.33), respectively. As shown in Table 4, for VCF as well as MAF files, MuTect2 technique outperformed the other techniques in terms of MAE, RMSE and R-value.

Table 4: Performance of best regressors on top-10 genes from VCF and MAF files extracted using all techniques

Technique	File Type	MAE	RMSE	R	p-value
MuTect2	VCF	12.52	19.58	0.57	7.00E-37
	MAF	16.47	22.16	0.37	1.31E-14
MuSE	VCF	13.88	20.38	0.51	1.38E-29
	MAF	16.89	22.48	0.34	1.68E-12
Varscan2	VCF	14.57	20.78	0.48	4.77E-26
	MAF	16.53	22.26	0.36	9.11E-14
SomaticSniper	VCF	15.76	21.82	0.40	3.31E-17
	MAF	16.72	22.26	0.33	8.46E-12

MAE: Mean Absolute Error; RMSE: Root Mean Square Error; HR: Hazard Ratio; R: Correlation Coefficient

Discrimination of Low- and High-Risk patients

Initially, the dataset was divided into two groups, i.e., the high-risk and low-risk group based on the median OS. Samples with OS time less than the median OS time were designated to the high-risk group, whereas the remaining were assigned to the low-risk group. To assess the ability of the number of mutations/gene/samples to classify the patients into the high and low-risk groups, classification models were developed on top 10 genes for each technique and file type, using eight different classifiers such as RF, LR, XGB, DT, KNN, GNB, ET and SVC. The performance of all the classifiers for every model generated on each technique for both the files are reported in Supplementary Table S3.

Number of mutations reported through each technique were used to develop models to predict the high- and low-risk group. In case of VCF file derived using Mutect2, SVC-based model achieved AUROC of 0.72 and 0.69 in training and validation data, respectively as shown in Table 5. Similarly, ET-based model developed on genes from MAF files extracted using MuTect2 technique performed with AUROC of 0.57 and 0.67 on training and validation dataset, respectively. For MuSE technique, GNB-based model developed on genes from VCF files achieved AUROC of 0.66 and 0.68 on training and validation data whereas, ET-based model developed on genes from MAF files achieved 0.60 and 0.51 AUROC on training and validation dataset, respectively. For the genes obtained from the Varscan2 technique, SVC-based model with genes from VCF file performed best with AUROC 0.68 and 0.64 on the training and validation dataset, with the minimum difference in sensitivity and specificity, whereas for MAF files, LR-based model achieved AUROC of 0.63 and 0.63

on training and validation dataset. For SomaticSniper technique, LR-based model developed on genes from VCF files achieved AUROC of 0.63 and 0.65 on training and validation data whereas, LR-based model developed on genes from MAF files achieved 0.60 and 0.64 AUROC on training and validation dataset, respectively. For VCF as well as MAF files, MuTect2 technique performed best among other techniques in terms of difference between sensitivity and specificity as well as AUROC.

Table 5: Performance of best classifiers on top-10 genes from VCF and MAF files extracted using all techniques

Technique	File Type	Dataset	MLT	Sensitivity	Specificity	Accuracy	AUROC	F1	Kappa	MCC
MuTect2	VCF	Training	SVC	70.06	71.86	71.26	0.72	0.71	0.41	0.42
		Validation		69.05	66.67	67.86	0.69	0.68	0.36	0.36
	MAF	Training	ET	58.03	52.76	55.39	0.57	0.57	0.11	0.11
		Validation		60.98	63.42	62.20	0.67	0.62	0.24	0.24
MuSE	VCF	Training	GNB	63.47	64.07	63.77	0.66	0.64	0.28	0.28
		Validation		71.43	52.38	61.91	0.68	0.65	0.24	0.24
	MAF	Training	ET	58.03	53.42	55.73	0.60	0.57	0.11	0.12
		Validation		30.00	75.61	53.09	0.51	0.39	0.06	0.06
Varscan2	VCF	Training	SVC	62.28	70.66	66.47	0.68	0.65	0.33	0.33
		Validation		71.43	61.91	66.67	0.64	0.68	0.33	0.34
	MAF	Training	LR	57.41	63.80	60.62	0.63	0.59	0.21	0.21
		Validation		48.78	78.05	63.42	0.63	0.57	0.27	0.28
SomaticSniper	VCF	Training	LR	60.48	61.08	60.78	0.63	0.61	0.22	0.22
		Validation		52.38	76.19	64.29	0.65	0.60	0.29	0.29
	MAF	Training	LR	54.94	61.49	58.20	0.60	0.57	0.16	0.17
		Validation		45.00	80.49	62.96	0.64	0.55	0.26	0.27

MLT: Machine Learning Technique; LR: Logistic Regression; ET: ExtraTree; DT: Decision Tree; XGB: eXtreme Gradient Boosting; RF: Random Forest

Discussion

Liver cancer is a global problem and occurs after severe liver diseases [31]. Chronic liver diseases are associated with cancer development and prompt progressive mutations at the genomic level [32, 33]. Previous studies report that liver cancer is associated with poor prognosis and a high mortality rate amongst the most frequent cancer types [34, 35]. Nowadays, several mutation calling techniques are available to identify the mutation landscape in tumor/normal patients. Hitherto, there is not an appropriate comparison of mutation detection methods for the predictive and prognostic analysis. In this study, we examine the performance of four widely used mutation calling techniques such as MuTect2,

MuSE, VarScan2, and SomaticSniper using TCGA liver cancer cohort. We have applied various techniques in order to compare all the methods for predicting and analysing prognostic biomarkers in liver cancer patients. First, we have used VCF and MAF files generated by the different mutation calling methods. We have used the most popular methods (ANNOVAR and Maftools) to identify the gene-associated mutations in liver cancer samples. Further, we observed that the VCF files of Mutect2 and SomaticSniper report highest number of mutated genes and cover over 5 million mutations. Whereas, MAF files reports comparatively less mutated genes for each technique as shown in Table 1.

Then, we performed correlation analysis in order to understand the impact of mutations on the survival of liver cancer patients. On performing the univariate survival analysis on VCF files, we observed that LncRNA SNGH10, CLMP, FAM160A2 and CLDN20 achieved the highest HR value in MuTect2, MuSE, VarScan2 and SomaticSniper technique respectively. As shown by Lan et al. LncRNA SNGH10 is an oncogenic lncRNA in liver cancer patients and reduces the survival of the patients [36]. It's down-regulation is also associated with the poor survival non-small cell lung cancer with HR 2.09 with p-value 0.02 [37]. Our study also corresponds with the previous studies and exhibits that the mutations in SNGH10 gene is associated with poor outcome in liver cancer patients with HR 5.49 and p-value 3.94E-06. Whereas, the differential expression of CLMP gene is associated with the progression of cancers of the breast cancer [38]. Yang et al. also reported the significance of CLDN20 gene in the survival of breast cancer patients with HR 1.38 and p-value 0.047 [39]. However, our analysis reveal the role of CLMP and CLDN20 gene in the survival of liver cancer patients. Further, in case of MAF files, the univariate survival analysis reveals that SYDE1, LAMC3, ITGB8, CAD, EVC2, NYNRIN, BRSK2, TP53 genes significantly reduces the overall survival. As shown by the recent study that SYDE1 act as an oncogene and overexpressed in glioma patients makes it an important diagnostic and prognostic biomarker [40]. Moreover, the down-regulation of LAMC3 is correlated with the poor prognosis and metastasis in the ovarian cancer patients [41]. A study also reveals that mutations associated with LAMC3 genes may cause PNH (a rare disorder of clonal stem cell in foetus), which may leads high mortality rate infection and premature birth [42, 43]. We also observed that mutations associated with LAMC3 significantly reduces the survival of patients with HR = 9.25 and p-value 1.78E-06. In addition, ITGB8 is shown to be highly upregulated in high-grade ovarian cancer patients, which leads to shorter OS with significant HR 1.42 [44]. Paul et.al, also reveals that EVC2 gene is highly mutated in breast cancer patients and dysregulates pathways like (mTOR, CDK/RB, cAMP/PKA, WNT, etc) [45]. Our study show that mutations

associated with EVC2 genes reduces the overall survival of patients with HR = 4.3 and p-value 8.66E-05. Researchers have shown that the overexpression of BRSK2 gene correlated with the patients survival and prognosis in pancreatic cancer [46]. Of Note, several studies reports that TP53 is the highly mutated gene among most of the human cancers and affects the survival of cancer patients [47-51]. In current study, we also found that TP53 is the highly mutated gene among the liver cancer patients and covers almost 20% mutations. Correlation and survival analysis shown that mutation associated with TP53 significantly reduces the overall survival with HR = 1.63 and p-value 1.20E-02 among liver cancer patients. While considering the combined effect of selected genes in each file, MuTect2 technique outperformed all the other techniques in VCF file with HR 4.50 (p-value 3.83E-15), whereas MuSE technique outperformed other mutation calling methods with HR 2.47 (p-value 9.64E-07) in case of MAF files (Figure 4).

Furthermore, to compare the different mutation calling techniques we develop various survival prediction and classification models using the top-10 genes respective to each file type (Table 4 and 5). The predicted survival time employed for the stratification of high-risk and low-risk groups. Models based on ten selected genes from VCF file of MuTect2 technique performed best among the other techniques in stratification of patients in high- and low- risk group, as well as in OS time prediction. Our findings suggest that the VCF file generated using MuTect2 mutation calling technique provides the comprehensive information which can be used for the risk-estimation of liver cancer cohort. Furthermore, this needs to be confirmed on the other cancer cohorts to explore the prognostic potential of mutations.

Declarations

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Conflict of Interests

The authors declare no competing financial and non-financial interests.

Ethics Approval

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Consent to participate

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435 **Author contribution**

436 SP, AD, and GPSR collected and processed the datasets. SP, AD, and GPSR implemented the
437 algorithms. SP, AD, and GPSR developed the prediction models. SP, AD, and GPSR
438 analyzed the results. SP, AD, and GPSR penned the manuscript. GPSR conceived and
439 coordinated the project and provided overall supervision to the project. All authors have read
440 and approved the final manuscript.

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