A Meta-Analysis of Genomic and Transcriptomic Variations in Lung Adenocarcinoma

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ABSTRACT
Lung cancer is the leading cause of largest number of deaths worldwide and lung adenocarcinoma (LUAD) is the most common form of lung cancer. In this study, we carried out an integrated meta-analysis of the mutations including single-nucleotide variations (SNVs), the copy number variations (CNVs), RNA-seq and clinical data of patients with LUAD downloaded from The Cancer Genome Atlas (TCGA). We integrated significant SNV and CNV genes, differentially expressed genes (DEGs) and the DEGs in active subnetworks to construct prognosis signature. Cox proportional hazards model (LOOCV) with Lasso penalty was used to identify best gene signature among different gene categories. The patients in both training and test data were clustered into high-risk and low-risk groups by using risk scores of the patients calculated based on selected gene signature. We generated 12-gene signature (DEPTOR, ZBTB16, BCHE, MGLL, MASP2, TNNI2, RAPGEF3, SGK2, MYO1A, CYP24A1, PODXL2, CCNA1) for overall survival prediction. The survival time of high-risk and low-risk groups were significantly different. These 12-gene signature could predict prognosis and they are potential predictors for the survival of the patients with LUAD.

CCS CONCEPTS
• Applied computing ~ Bioinformatics

KEYWORDS
TCGA, LUAD, Differential Expression, Mutation, Active Subnetwork, Survival, Cox Regression

1 Introduction
Lung cancer is the most common cancer and responsible for largest number of deaths worldwide with 1.8 million deaths, 18.4% of the total (IARC, 2018). Lung cancer is categorized into two main categories: non-small cell lung cancer (NSCLC) which occurs in 85% of patients and small cell lung cancer (SCLC) in 15% of cases. NSCLC is grouped into 3 histological sub-types: lung adenocarcinoma (LUAD) which is most common form of lung cancer, lung squamous cell carcinoma (LUSC) and large cell carcinoma (Travis, 2011). Integration of different types of molecular data has been used to characterize molecular basis of lung cancer and to determine clinical status of patients. Shi et al. analyzed 101 LUAD samples by using data from different levels -DNA mutations, gene expression profile, copy number variations and DNA methylation- in order to identify the relation between genomic status and clinical status. They determined deleterious mutations at ZKSCAN1 and POU4F2 genes which are two novel candidate driver genes (Shi, 2016). Furthermore, recent studies have been performed to generate new methods to analyze integrative cancer data. Berger et al. (Berger, 2016) proposed a new method called expression-based variant-impact phenotyping (eVIP) using DEGs to distinguish driver mutations from passenger mutations. They characterized 194 somatic
mutations related with primary LUAD and claimed that 69% of mutations were mutations. They found the driver mutations in LUAD are EGFR (p.S645C), ERBB2 (p.S418T), ARAF (p.S214C) and ARAF (p.S214F). TCGA network analyzed 230 LUAD samples using mRNA, microRNA and DNA sequencing integrated with copy number, methylation and proteomic data (The Cancer Genome Atlas Research Network, 2014) and reported the samples with high rates of somatic mutation. Eighteen genes with high mutation load were reported such as RIT1 activating mutations and MGA loss-of-function mutations. They also identified aberrations in NF1, MET, ERBB2 and RIT1 occurred in 13% of cases and MAPK and PI(3)K pathway activity (The Cancer Genome Atlas Research Network, 2014). Deng Z. et al., presented genomic alterations in LUAD samples from TCGA and found the significantly aberrant CNV segments which are associated with the immune system and 63 mutated genes associated with lung cancer signaling related to cancer progression. They identified the mutations of the PI3K protein family members include PIK3C2B, PIK3CA, PIK3R1 (Deng, 2017).

In recent years, studies have been performed to generate gene signatures predicting prognosis risk of patients with lung adenocarcinoma. In 2016, Krzystanek et.al. identified 7-gene signature by using microarray data of early stage lung adenocarcinoma from GEO datasets. The genes (ADAM10, DLGAP5, RAD51AP1, FGFR10p, NCGAP, KIF15, ASPM) which have high hazards ratios showed significant results at cox regression analysis and Kaplan-Meier survival plots (Krzystanek, 2016). Shukla et.al. identified 96 genes including five long noncoding RNAs (lncRNAs) among training data which had prognostic association at test data, by using lung adenocarcinoma RNA-seq and clinical data from TCGA (Shukla, 2017). Shi et.al. studied on long noncoding RNAs (lncRNAs) expression signature model to predict stage I lung adenocarcinoma from TCGA and determined 31-lncRNA signature to predict overall survival in patients with LUAD (Shi, 2018). Zhao et. al. used gene expression profiles from TCGA and identified 20 genes which were significantly associated with the overall survival (OS). When they combined with GEO data set, they obtained four genes, FUT4, SLC25A42, IGFBP1, and KIHDC8B as common (Zhao, 2018). Li et. al. performed RNA-sequencing on LUAD tumor samples and normal tissue samples. They construct protein–protein interaction network by using DEGs which were intersection of GEO datasets and identified hub genes. Then, they test these genes on patient cohorts and TCGA data. They identified eight genes (DLGAP5, KIF11, RAD51AP1, CCNB1, AURKA, CDC6, OIP5 and NCGAP) which were closely related to survival in LUAD (Li, 2018). He et. al. studied on previous GEO datasets and TCGA data and they identified 8-gene prognostic signature (CDCP1, HMMR, TPX2, CIRBP, HLF, KBTBD7, SEC24B-AS1, and SH2B1) by using the step-wise multivariate Cox analysis. These genes were good predictors of survival between high-risk and low-risk groups of patients with early-stage NSCLC (He, 2019). The studies above determined different gene signatures for prognosis risk prediction by using different methods and presented different genes. However, mostly gene expression data has been used for this purpose. We tried to integrate SNVs, CNVs, DEGs and active subnetwork DEGs to generate gene signature for risk model by using TCGA LUAD data. TCGA (Chang, 2013) provides simple nucleotide variation, gene expression, miRNA expression, DNA methylation, copy number variation and reverse phase protein array, clinical and biospecimen data from more than 10,000 cancer patients with 39 cancer types.

2 Data

Simple Nucleotide Variation, Transcriptome Profiling, Copy Number Variation and Clinical data of both 55 LUAD patients who have paired (both normal and tumor samples) RNAseq data and of 510 patients who have all four types of data was downloaded from TCGA harmonized database by using R/Bioconductor TCGAbiolinks package (Colaprico, 2016). We analyzed the genomic alteration data including Simple Nucleotide Variations, Copy Number Variations; and transcriptomic variations from RNAseq data, processed using the reference of hg38; and clinical data of LUAD patients (Table 7).
3 Methods

3.1 Identification of Significant Simple Nucleotide Variations
The Mutation Annotation Format (maf) file contained somatic mutations of all patients in TCGA LUAD project, was downloaded using TCGAbiolinks package. The other R/Bioconductor package, maf tools (Mayakonda, 2018), were used to subset original maf file by tumor sample barcodes of patients of interest. Maftools package also summarizes the mutations and represents as summary plot and oncoplot. Significant mutated genes divided into two groups, oncogene (OG) or tumor suppressor gene (TSG), among tumor samples of 55 and 510 patients were identified separately by using SomInaClust R package (Van den Eynden, 2015). SomInaClust works on the basic assumption that important genes in tumor samples have clustered on sequence and high number of inactivating mutations because of the selective pressure during tumorigenesis. Based on this assumption, oncogenes have clustered mutations, while tumor suppressors have inactivating (protein truncating) mutations. SomInaClust uses a reference step in which background mutation rate and hot spots are determined for genes existing in reference mutation database such as COSMIC(v88) database (Forbes, 2017).

3.2 Identification of Significant Copy Number Variations
The TCGA LUAD CNV dataset for primary solid tumor samples, generated by Affymetrix Genome-Wide Human SNP Array 6.0 platform, was downloaded using TCGAbiolinks package. The significant aberrant genomic regions in tumor samples of 55 and 510 patients were identified separately by R/Bioconductor GAIA package (Morganella, 2011). NCBI IDs and Hugo Symbols of the genes with differential copy number were determined using biomaRt package (Durinck, 2009).

3.3 Differential Expression Analysis (DEA)
The Transcriptome Profiling data in mRNA expression level (as unnormalized HTSeq raw counts) of 55 LUAD patients who have paired samples was downloaded by TCGAbiolinks package. Differentially expressed genes were determined with FDR adjusted p-values (q-values) in tumor samples (TP) according to normal samples (NT) of 55 LUAD patients by limma-voom method using limma (Ritchie, 2015) and edgeR (McCarthy, 2012) R/Bioconductor packages. NCBI IDs and Hugo Symbols of the differentially expressed genes determined by the biomaRt R package.

3.4 Active Subnetwork and Pathway Analysis
We identified the active subnetworks of differentially expressed genes in tumor samples of 55 LUAD patients using R/Bioconductor DEsubs package (Vrahatis, 2016). The output of limma package containing differentially expressed genes with their Ensembl IDs and FDR adjusted p-values (q-values) were used as input of DEsubs package. DEsubs package determines and represents the active subnetworks with their graphs both at subnetwork and pathway levels.

3.5 Statistical Analysis
Clinical data of 55 and 510 patients was downloaded from TCGA database using the TCGAbiolinks package. Univariate Cox Proportional Hazards Regression analysis (Cox, 1972) and logRank test (Mantel, 1966) were performed using survival R package for significant SNV containing genes, significant CNV containing genes, DEGs and active subnetwork genes to identify genes with prognostic ability. For the genes with prognostic ability (p value < 0.05), Cox proportional hazards model (LOOCV) with Lasso penalty was used to identify best gene signature among different combinations of molecular levels (SNV genes, CNV genes, DEGs and active subnetwork genes) by using glmnet R package. Concordance index (c-index) was performed using pec R package to validate the predictive ability of different gene signatures. The larger c-index is used to determine the gene signature which has more accurate predictive ability. Multivariate cox proportional regression analysis was performed using survival R package for genes of selected signature and risk score of
each patient was calculated using coefficient and expression values of the genes. Then, patients were clustered into high-risk group and low-risk group and Kaplan-Meier (KM) survival curves (Kaplan & Meier, 1958) were generated using survminer R package to demonstrate the overall survival of risk groups stratified based on gene signature. ROC curve analysis was also performed for risk scores calculated based on selected gene signature by using survivalROC R package.

Significant differences in tumor stages, mutation counts and expression levels of patients in the high-risk and low-risk groups were identified using ggstatsplot R package. In order to validate the prognosis risk signature, the risk scores of 442 TCGA patients with LUAD were calculated using the expression values of gene signature and their coefficient values from multivariate cox proportional regression analysis. Similarly, 442 patients (after exclusion of 55 and other patients with missing data from 510 patients) were clustered into high-risk and low-risk groups and the overall survival difference between the two groups of patients was assessed by KM survival curve. Significance level used for identification of genes containing copy number variations and differentially expressed genes, was 0.01 for FDR corrected q-value. Significance level was 0.05 for FDR corrected p values (q value) for identification of genes containing significant single nucleotide variations; and was 0.05 for p-values for active subnetwork and pathway analysis, and for all statistical analysis.

Figure 1: The workflow of construction and validation of the prognosis gene signature

4 Results

4.1 Identification of Significant Simple Nucleotide Variations

Mutation data of LUAD patients as maf file generated by mutect pipeline was downloaded by TCGAbiolinks package and maftools package was used to subset original maf file by tumor sample barcodes of 55 LUAD patients and 510 LUAD patients. Then, significant mutations for both 55 and 510 LUAD patients were
determined separately with their roles as tumor suppressor or oncogene by SomInaClust R package. In order to determine important genes including significant mutation clusters, we used SomInaClust R package. EGFR, KRAS, TP53, STK11, RB1 and MGA genes were determined as candidate driver genes in tumor samples (Figure 2). EGFR and KRAS genes were classified as oncogene and STK11, RB1 and MGA genes were classified as tumor suppressor. Although TP53 gene has both OG score and TSG score, TP53 was classified as tumor suppressor in Table 1 depending on reference information of cancer gene census. EGFR, KRAS, TP53, STK11 and RB1 have highly significant estimation. While EGFR and TP53 have higher number of mutations, KRAS, STK11, RB1 and MGA have lower number of mutations. While EGFR, KRAS, TP53, STK11, RB1 are well known cancer related genes, MGA gene is not in cancer gene census. Loss of function mutations on MGA which is MYC-interacting transcription factor, have been newly described suggesting that MGA is a candidate tumor suppressor in lung cancer (Romero, 2014), as identified by SomInaClust analysis.

![Pyramid plot of important mutated genes classified as oncogene (OG) or tumor suppressor gene (TSG) in tumor samples of 55 patients with LUAD](image)

**Figure 2:** Pyramid plot of important mutated genes classified as oncogene (OG) or tumor suppressor gene (TSG) in tumor samples of 55 patients with LUAD

**Table 1. Significant mutated genes in 55 tumor samples**

| Gene | # Mutations | Q value   | OG Score | TSG Score | Classification | CGC* |
|------|-------------|-----------|----------|-----------|----------------|------|
| EGFR | 11          | 1.57e-12  | 80       | 0         | OG Dom         |     |
| KRAS | 8           | 1.57e-12  | 100      | 0         | OG Dom         |     |
| TP53 | 20          | 4.8e-07   | 62.5     | 55        | TSG Rec        |     |
| STK11| 7           | 0.000106  | 0        | 85.7      | TSG Rec        |     |
| RB1  | 7           | 0.0049    | 0        | 71.4      | TSG Rec        |     |
| MGA  | 6           | 0.0217    | 0        | 80        | TSG NA         |     |

* Cancer gene census (Dom: Dominant, Rec: Recessive)
Figure 3: Pyramid plot of top 40 important mutated genes classified as oncogene (OG) or tumor suppressor gene (TSG) in tumor samples of 510 patients with LUAD

Table 2. Significant mutated genes in tumor samples of 510 patients with LUAD

| Gene   | # Mutations | qDG      | OG Score | TSG Score | Classification | CGC* |
|--------|-------------|----------|----------|-----------|----------------|------|
| KRAS   | 143         | 1.97e-250| 97.8     | 0         | OG             | Dom  |
| TP53   | 253         | 2.52e-135| 79.7     | 38        | TSG            | Rec  |
| EGFR   | 73          | 8.97e-84 | 73.8     | 10        | OG             | Dom  |
| STK11  | 83          | 4.6e-61  | 27.8     | 72        | TSG            | Rec  |
| BRAF   | 44          | 8.07e-51 | 67.5     | 7.4       | OG             | Dom  |
| RBM10  | 39          | 9.06e-31 | 0        | 78.9      | TSG            | NA   |
| NF1    | 63          | 5.37e-25 | 0        | 54.2      | TSG            | Rec  |
| MGA    | 52          | 6.46e-23 | 0        | 58.3      | TSG            | NA   |
| SETD2  | 44          | 1.34e-20 | 16.7     | 58.1      | TSG            | Rec  |
| RB1    | 32          | 4.99e-20 | 0        | 68.8      | TSG            | Rec  |
| PIK3CA | 27          | 1.36e-19 | 61.5     | 0         | OG             | Dom  |
| ATM    | 48          | 5.18e-18 | 25       | 45.7      | TSG            | Rec  |
| CTNNB1 | 21          | 3.32e-15 | 61.1     | 12.5      | OG             | Dom  |
| ARID1A | 30          | 1.76e-14 | 12.5     | 60        | TSG            | Rec  |
| ARID2  | 29          | 2.83e-12 | 0        | 57.1      | TSG            | Rec  |
| SMARCA4| 48          | 2.23e-11 | 16.7     | 42.9      | TSG            | Rec  |
| Gene      | ID | Adj.P   | Score | Z score | FDR | Status   | Gene Type |
|-----------|----|---------|-------|---------|-----|----------|-----------|
| CSMD3     | 324| 6.25e-10| 17.5  | NA      | NA  | NA       | NA        |
| ATF7IP    | 17 | 1.84e-08| 71.4  | TSG     | NA  | NA       | NA        |
| KEAP1     | 90 | 1.91e-08| 24.1  | TSG     | NA  | NA       | NA        |
| NFE2L2    | 14 | 2.83e-07| 58.3  | O.G    | Dom | NA       | NA        |
| KDM5C     | 16 | 1.76e-06| 60    | TSG     | Rec | NA       | NA        |
| ERBB2     | 13 | 6.94e-06| 14.3  | O.G    | Dom | NA       | NA        |
| LRP1B     | 267| 6.04e-05| 15.6  | NA      | Rec | NA       | NA        |
| HMCN1     | 97 | 8.93e-05| 24.1  | TSG     | NA  | NA       | NA        |
| MAP2K1    | 9  | 0.000263| 66.7  | O.G    | Dom | NA       | NA        |
| APC       | 24 | 0.000272| 37.5  | TSG     | Rec | NA       | NA        |
| PNISR     | 6  | 0.000626| 83.3  | TSG     | NA  | NA       | NA        |
| RPL5      | 7  | 0.000626| 83.3  | TSG     | Dom | NA       | NA        |
| GNAS      | 19 | 0.000962| 28.6  | O.G    | Dom | NA       | NA        |
| COL11A1   | 129| 0.00139 | 18.1  | NA      | NA  | NA       | NA        |
| EPHA5     | 66 | 0.00221 | 23.4  | TSG     | NA  | NA       | NA        |
| TTK       | 18 | 0.00221 | 41.2  | TSG     | NA  | NA       | NA        |
| FFXW7     | 12 | 0.0028  | 50    | TSG     | Rec | NA       | NA        |
| DMD       | 99 | 0.00349 | 18.8  | NA      | NA  | NA       | NA        |
| SMAD4     | 20 | 0.00379 | 35    | TSG     | Rec | NA       | NA        |
| FER       | 16 | 0.0043  | 46.2  | TSG     | NA  | NA       | NA        |
| MARK1     | 21 | 0.0043  | 46.2  | TSG     | NA  | NA       | NA        |
| TEP1      | 29 | 0.0043  | 46.2  | TSG     | NA  | NA       | NA        |
| ATRX      | 35 | 0.00463 | 26.5  | TSG     | Rec | NA       | NA        |
| CDKN2A    | 21 | 0.00585 | 35    | TSG     | Rec | NA       | NA        |
| MYO9A     | 19 | 0.00615 | 42.9  | TSG     | NA  | NA       | NA        |
| ZNF800    | 17 | 0.00615 | 42.9  | TSG     | NA  | NA       | NA        |
| CMTR2     | 26 | 0.00674 | 55.6  | TSG     | NA  | NA       | NA        |
| RASA1     | 9  | 0.00674 | 55.6  | TSG     | NA  | NA       | NA        |
| CDKN1B    | 5  | 0.00674 | 80    | TSG     | Rec | NA       | NA        |
| DHX15     | 7  | 0.00674 | 80    | TSG     | NA  | NA       | NA        |
| IQGAP2    | 28 | 0.00816 | 40    | TSG     | NA  | NA       | NA        |
| LTN1      | 19 | 0.00816 | 40    | TSG     | NA  | NA       | NA        |
| SMARCA1   | 19 | 0.00816 | 40    | TSG     | NA  | NA       | NA        |
| SPTA1     | 164| 0.00971 | 17.6  | NA      | NA  | NA       | NA        |
| FHOD3     | 31 | 0.0122  | 30.4  | TSG     | NA  | NA       | NA        |
| CPVL      | 8  | 0.0161  | 66.7  | TSG     | NA  | NA       | NA        |
| MAP3K12   | 8  | 0.0161  | 66.7  | TSG     | NA  | NA       | NA        |
| TOP2B     | 9  | 0.0161  | 66.7  | TSG     | NA  | NA       | NA        |
| ROCK1     | 21 | 0.0163  | 35.3  | TSG     | NA  | NA       | NA        |
| PBRM1     | 12 | 0.0172  | 45.5  | TSG     | Rec | NA       | NA        |
| AKAP6     | 40 | 0.0195  | 28    | TSG     | NA  | NA       | NA        |
| SENP1     | 3  | 0.0241  | 100   | TSG     | NA  | NA       | NA        |
| SP1       | 4  | 0.0241  | 100   | TSG     | NA  | NA       | NA        |
| WISP3     | 4  | 0.0241  | 100   | TSG     | NA  | NA       | NA        |
| RAD50     | 13 | 0.0243  | 41.7  | TSG     | NA  | NA       | NA        |
| COL28A1   | 19 | 0.0243  | 41.7  | TSG     | NA  | NA       | NA        |
| SCAF8     | 18 | 0.0243  | 41.7  | TSG     | NA  | NA       | NA        |
| Gene    | ID  | Freq. | # Del | # Amp | Type | Chromosome |
|---------|-----|-------|-------|-------|------|------------|
| STK31   | 19  | 0.0243| 0     | 41.7  | TSG  | NA         |
| IDH1    | 6   | 0.0248| 40    | 25    | TSG  | Dom        |
| USH2A   | 240 | 0.0263| 0     | 13.2  | NA   | NA         |
| YLPM1   | 23  | 0.0269| 0     | 31.6  | TSG  | NA         |
| IQUB    | 12  | 0.0272| 0     | 57.1  | TSG  | NA         |
| MARK2   | 10  | 0.0272| 0     | 57.1  | TSG  | NA         |
| NAA15   | 8   | 0.0272| 0     | 57.1  | TSG  | NA         |
| CDH10   | 99  | 0.028 | 0     | 16.4  | NA   | NA         |
| AKT1    | 3   | 0.0296| 66.7  | 0     | OG   | Dom        |
| RAF1    | 7   | 0.031 | 66.7  | 0     | OG   | Dom        |
| VPS13C  | 39  | 0.0332| 0     | 25    | TSG  | NA         |
| ZBBX    | 28  | 0.0333| 0     | 30    | TSG  | NA         |
| DST     | 67  | 0.0333| 0     | 19.1  | NA   | NA         |
| KMT2C   | 52  | 0.0388| 0     | 18.8  | NA   | Rec        |
| DGKB    | 38  | 0.0431| 0     | 28.6  | TSG  | NA         |
| MAP2K4  | 8   | 0.045 | 33.3  | 50    | TSG  | Rec        |
| FBN2    | 93  | 0.045 | 0     | 20.5  | TSG  | NA         |
| B2M     | 8   | 0.045 | 0     | 50    | TSG  | Rec        |
| BAP1    | 8   | 0.045 | 0     | 50    | TSG  | Rec        |

*Cancer gene census (Dom: Dominant, Rec: Recessive)

4.2 Identification of Significant Copy Number Variations

CNVs (Copy Number Variations) are important aberrations which results alterations in gene expression in tumorigenesis and tumor growth. In order to determine significant CNVs among tumor samples of 55 and 510 LUAD patients, gaia R package was used. Significant recurrent CNVs in tumor samples of 55 LUAD patients, over the orange q value thresholds (0.01), are mostly observed on Chromosome 1, 8, 9, and 17. Chromosome 1 has the highest number of amplifications followed by Chromosome 8. Chromosome 9 has the highest number of deletions followed by Chromosome 17 as seen in Figure 4. Top ten significant amplified and deleted genes which are all from chromosome 1 are showed in Table 3. Chromosome 1 has the highest number of gene aberration with 2006 amplified or deleted genes followed by Chromosome 8 with 1029 aberrant genes and Chromosome 19 with 785 aberrant genes.
Figure 4: Significant CNVs on all chromosomes in tumor samples of 55 patients with LUAD

Table 3. Top ten significant deleted and amplified genes in tumor samples of 55 patients with LUAD

| Gene Symbol | Aberration | q-value     | Aberrant Region       | Gene Region                  |
|-------------|------------|-------------|-----------------------|------------------------------|
| RN7SKP285   | Del        | 0.00474651  | 1:103501576-107318961 | 1:103523562-103523879        |
| RNU2-17P    | Amp        | 0.00474651  | 1:150131878-150768299 | 1:150218417-150236156        |
| AC242988.1  | Amp        | 0.00474651  | 1:150131878-150768299 | 1:150255095-150257286        |
| CA14        | Amp        | 0.00474651  | 1:150131878-150768299 | 1:150257251-150265078        |
| APH1A       | Amp        | 0.00474651  | 1:150131878-150768299 | 1:150265399-150269580        |
| CIART       | Amp        | 0.00474651  | 1:150131878-150768299 | 1:150282543-150287093        |
Significant recurrent CNVs in tumor samples of 510 LUAD patients, over the orange q value thresholds (0.01), are mostly observed on Chromosome 4, 9, 10, 11, 12, 13, 14, 16, 18 and 20. But Chromosome 11 has the highest number of aberrations followed by Chromosome 9, 16 and 18. Chromosome 4, 9, 10, 12 and 16 has mostly amplifications (Figure 5). The pattern of CNVs in tumor samples of 510 patients has a marked difference from the CNV pattern in tumor samples of 55 patients (Figure 4). Top ten significant amplified and deleted genes which are all from chromosome 1 are showed in Table 4. Chromosome 1 has the highest number of gene aberration with 3124 amplified or deleted genes followed by Chromosome 6 with 2911 aberrant genes and Chromosome 3 with 2149 aberrant genes.

**Figure 5: Significant CNVs on all chromosomes in tumor samples of 510 patients with LUAD**

**Table 4. Top ten significant amplified and deleted genes in 510 LUAD patients**

| Gene Symbol   | Aberration | q-value       | Aberrant Region          | Gene Region            |
|---------------|------------|---------------|--------------------------|------------------------|
| AL359821.1    | Del        | 0.0029609     | 1:71621685-71778398      | 1:71738173-71738354    |
| GD12P2        | Del        | 0.0029609     | 1:71928758-119984738     | 1:72274552-72275159    |
| AL513166.2    | Del        | 0.0029609     | 1:71928758-119984738     | 1:72283170-72753772    |
| RPL31P12      | Del        | 0.0029609     | 1:71928758-119984738     | 1:72301472-72301829    |
| AL583808.1    | Del        | 0.0029609     | 1:71928758-119984738     | 1:72636547-72899240    |
| RNU6-1246P    | Del        | 0.0029609     | 1:71928758-119984738     | 1:72717663-72717769    |
| AL583808.2    | Del        | 0.0029609     | 1:71928758-119984738     | 1:72765031-72791282    |
| AL583808.3    | Del        | 0.0029609     | 1:71928758-119984738     | 1:72793104-72854475    |
| AL732618.1    | Del        | 0.0029609     | 1:71928758-119984738     | 1:72979014-72979314    |
| KRT8P21       | Del        | 0.0029609     | 1:71928758-119984738     | 1:73104792-73106282    |
| SF3B4         | Amp        | 0.0029609     | 1:149907993-247650984    | 1:149923317-149927803  |
| MTMR11        | Amp        | 0.0029609     | 1:149907993-247650984    | 1:149928651-149936879  |
| OTUD7B        | Amp        | 0.0029609     | 1:149907993-247650984    | 1:149937812-150010726  |
| AC244033.2    | Amp        | 0.0029609     | 1:149907993-247650984    | 1:150045660-150067701  |
| AC244033.1    | Amp        | 0.0029609     | 1:149907993-247650984    | 1:150053864-150055034  |
| VPS45         | Amp        | 0.0029609     | 1:149907993-247650984    | 1:150067279-150145329  |
4.3 Differential Expression Analysis (DEA)

The Transcriptome Profiling data of LUAD patients in mRNA expression level (as unnormalized HTSeq raw counts), was downloaded by TCGABiolinks R package. Differentially expressed genes were determined with FDR adjusted p-values (q-values) in tumor samples (TP) of 55 patients with LUAD according to normal samples (NT) of the same patients by limma-voom method using limma and edgeR packages. The volcano plot in Figure 6, shows the differentially expressed genes (DEGs) as dots of which black ones represent the genes which have differential expression less than two fold and not significant while red ones represent upregulated and green ones downregulated more than two fold (log$_2$=1) significantly (q value < 0.01). As the result of this analysis, 3575 genes are dysregulated more than two fold with 0.01 q-value significance.

![Volcano Plot](image)

Figure 6: Volcano plot of differentially expressed genes in tumor samples of 55 patients with LUAD.

As the result of DEA, differently expressed genes (DEGs) are determined with their log Fold Change (logFC), adjusted p-value (q-value), entrez gene IDs and HGNC symbols after enrichment analysis. The top 10 down-regulated and up-regulated genes are showed in Table 5 and Table 6. The list of DEGs were used for pathway analysis and active subnetwork analysis.
Table 5. Top ten significant down-regulated genes in tumor samples of 55 LUAD patients

| ensembl_gene_id | entrezgene | hgnc_symbol | logFC          | adj.P.Val      |
|-----------------|------------|-------------|----------------|---------------|
| ENSG00000182010 | 219790     | RTKN2       | -4.52455117194123 | 1.07397390772473e-42 |
| ENSG00000158764 | 142683     | ITLN2       | -7.4364942528429  | 3.19924465283634e-41 |
| ENSG00000102683 | 6445       | SGCG        | -4.10485571819757  | 4.07515928515459e-41 |
| ENSG00000198873 | 2869       | GRK5        | -2.65790712999242124 | 4.07515928515459e-41 |
| ENSG00000107742 | 9806       | SPOCK2      | -3.56967403596283  | 3.85300139768808e-40 |
| ENSG00000170323 | 2167       | FABP4       | -5.72790493543673  | 1.03033381509032e-39 |
| ENSG00000158764 | 142683     | ITLN2       | -7.4364942528429  | 3.19924465283634e-41 |
| ENSG00000102683 | 6445       | SGCG        | -4.10485571819757  | 4.07515928515459e-41 |
| ENSG00000198873 | 2869       | GRK5        | -2.65790712999242124 | 4.07515928515459e-41 |
| ENSG00000107742 | 9806       | SPOCK2      | -3.56967403596283  | 3.85300139768808e-40 |
| ENSG00000170323 | 2167       | FABP4       | -5.72790493543673  | 1.03033381509032e-39 |

Table 6. Top ten significant up-regulated genes in tumor samples of 55 LUAD patients

| ensembl_gene_id | entrezgene | hgnc_symbol | logFC    | adj.P.Val      |
|-----------------|------------|-------------|----------|---------------|
| ENSG00000183010 | 5831       | PYCR1       | 3.5139225242735  | 3.06017765569688e-41 |
| ENSG00000059573 | 5832       | ALDH18A1    | 1.68852856318992  | 6.30895314373162e-38 |
| ENSG00000164466 | 94081      | SFXN1       | 1.5322079314688  | 5.01920971916517e-37 |
| ENSG00000135052 | 51280      | GOLM1       | 2.51608337184892  | 1.73125209540521e-35 |
| ENSG00000180198 | 1104       | RCC1        | 1.62119814668367  | 1.82637777402336e-34 |
| ENSG00000155660 | 9601       | PDIA4       | 1.6846754492746  | 2.3785532052335e-34 |
| ENSG00000096063 | 6732       | SRPK1       | 1.62823462104507  | 2.66784756146056e-34 |
| ENSG00000128050 | 10606      | PAICS       | 1.65390171937903  | 4.22169230063646e-31 |
| ENSG00000111344 | 8437       | RASAL1      | 3.57173273242386  | 1.0825178193746e-30 |
| ENSG00000173457 | 26472      | PPP1R14B    | 1.86684316566064  | 7.07845976872399e-30 |

4.4 Active Subnetwork and Pathway Analysis

The output of Differentially Expression Analysis (DEA) containing differentially expressed genes with their Ensembl IDs and adjusted p-values (q-values) were used as input of DEsubs R package. The active subnetworks of differentially expressed genes in tumor samples of both 55 LUAD patients were determined by DEsubs package and results were represented as graphs at subnetwork and organism levels. DEsubs package identified 35 subnetworks including 192 genes, 14 of them including more than three genes, 8 of them including three genes and the others including two genes. In Figure 7, the top ten significant genes which play role in determined subnetworks are represented with their Q-values. These genes are FABP4, WNT3A, EDNRB, TEK, AGER, EPAS1, ACADL, PDIA4, ANGPT4, KL. In this analysis, 35 subnetworks were determined and the first three subnetworks are presented in Figure 8, 9 and 10. When we look at the subnetworks’ graphs, in subnetwork 1 (Figure 8), the prominent genes are WNT genes which are members of WNT pathway, a major evolutionary conserved signaling pathway playing role in cell differentiation, cell migration and organogenesis during development and highly related to lung cancer; in subnetwork 3 (Figure 10), the prominent gene is AKT3 which is one of the AKT family members which play role in tumorigenesis and are modulators of several tumors. The pathways of subnetwork genes are mostly cancer related pathways such as melanoma, glioma, colorectal cancer, chronic myeloid leukemia, basal cell carcinoma, apoptosis, erbb signaling, jak stat signaling and map kinase signaling pathways (Figure 11).
Figure 7. Top 10 significant subnetwork genes in tumor samples of 55 LUAD patients

Figure 8. Graph of subnetwork 1 from 55 LUAD patients
Figure 9. Graph of subnetwork 2 from 55 LUAD patients

Figure 10. Graph of subnetwork 3 from 55 LUAD patients
4.5 Statistical Analysis

In order to identify a molecular prognosis risk model, the clinical data of all patients in TCGA LUAD project (Table 7) was downloaded by TCGAbiolinks R package and separated as training data of 55 LUAD patients who have paired samples for RNAseq data and used for gene signature construction; and test data of remaining 422 LUAD patients after removing patients who have missing values in clinic data. Different gene signatures were generated from the genes which have prognostic ability. The univariate cox regression analysis was performed for significant SNV genes, significant CNV genes, significant DEGs and active subnetwork genes in tumor samples of 55 patients with LUAD. There were 38 CNV genes, 463 DEGs and 37 subnetwork genes (DEsubs) with prognostic ability after univariate analysis and logRank test ($p < 0.05$). SNV genes did not have significant prognostic ability. Then different data categories (DEGs; DEsubs; CNVs; CNVs + DEGs, CNVs + DEsubs; CNVs + DEGs + SNVs; CNVs + DEsubs + SNVs) were generated by using significant prognostic genes. These data categories underwent the Cox proportional hazards regression with Lasso penalty and LOOCV. Gene models from different categories were generated by using glmnet R package which gives active genes with their coefficients. The genes in the models were DEPTOR, ZBTB16, BCHE, MGLL, MASP2, TNNI2, RAPGEF3, SGK2, MYO1A, CYP24A1, PODXL2, CCNA1 from DEGs category; THRA, RAPGEF3, LAMB2 from DEsubs category; SNX13, AC080080.1, RNMTL1P2, AC080080.2 from CNVs category; THRA, RAPGEF3, LAMB2 from CNVs + DEsubs. The genes in CNVs + DEGs and CNVs + DEGs + SNVs categories were same with the genes in DEGs category; the genes in CNVs + DEsubs + SNVs were in the CNVs + DEsubs category. Then, c-index analysis was performed to identify the survival predictive ability of the gene models identified from different categories in Figure 12. The higher c-index score was 0.858 from DEGs gene model. This gene model was chosen as best candidate prognosis gene signature for LUAD.
Table 7. Summary of clinical features of 55 and 510 patients with LUAD

| Category                  | Number          |                  |
|---------------------------|-----------------|-----------------|
|                           | 55 patients     | 510 patients    |
| Age at diagnosis (median; range) | 66 (42-86)      | 66 (33-88)      |
| Gender                    |                 |                 |
| Female                    | 33              | 273             |
| Male                      | 22              | 237             |
| Tumor stage               |                 |                 |
| I                         | 28              | 275             |
| II                        | 12              | 119             |
| III                       | 12              | 84              |
| IV                        | 2               | 25              |
| NA                        | 1               | 7               |
| Vital status              |                 |                 |
| Alive                     | 31              | 326             |
| Dead                      | 24              | 184             |

Figure 12. The c-index of different gene categories in training data and selected signature in testing data.

Multivariate Cox regression analysis was performed for the genes in the chosen gene signature and risk scores of each patient in training data (55 LUAD patients) were calculated by using coefficient values and normalized expression values (log₂+1) in tumor samples. Then the patients were clustered into high-risk and low-risk groups by using maxstat (maximally selected rank statistics) method based on optimal cutpoints for numerical variables by using survminer R package. When we performed Kaplan-Meier (KM) survival analysis to demonstrate the overall survival of risk groups stratified based on gene signature, patients with high-risk score demonstrated poor overall survival (p < 0.0001) than those with low-risk score in training dataset (Figure 13).
Figure 13. Risk clustering and Kaplan-Meier survival analysis of the signature for training data

The ROC curve analysis was performed to compare sensitivity and specificity of the predictive ability of risk score based on chosen gene signature. AUC values were 0.883 for 1 year, 0.813 for 2 year, 0.943 for 5 year and 0.976 for 10 year survival prediction (Figure 14). These high AUC values showed that the risk scores calculated based on chosen gene signature can highly predict the overall survival.

Figure 14. ROC curve analysis for 1, 2, 5 and 10 year survival prediction by the signature in both training and test data

When we performed the correlation analysis between tumor stages, mutation counts and gene expressions of signature genes, there was a significant difference of tumor stages between risk groups although there was no difference of total SNV mutation count between groups (Figure 15). However, as expected gene expression levels were significantly different between high-risk and low-risk groups in training data (55 LUAD patients) (Figure 16). The expression levels of the DEPTOR, ZBTB16, BCHE, MGLL, MASP2, TNNI2, RAPGEF3, SGK2, MYO1A and PODXL2 genes were lower in high-risk group while the expression levels of the CYP24A1 and CCNA1 genes were higher in high-risk group.
Figure 15. Correlation analysis between risk groups and total mutation count and tumor stage in tumor samples of training data
In order to validate our signature, we calculated c-index for the prediction of overall survival of the 442 TCGA patients with LUAD (test data) and the c-index was 0.591 which is lower than the c-index of training data (0.858). Then, multivariate cox regression analysis was performed for the signature genes in test data and the risk score for each patient was calculated by using normalized gene expression levels in tumor samples and coefficient values of the genes in signature from multivariate cox regression analysis. Patients in test dataset were divided into high-risk and low-risk groups by using maxstat (maximally selected rank statistics) method from using survminer R package (Figure 17). Patients in high-risk group had poor overall survival significantly ($p < 0.00055$). The ROC curve analysis was performed to compare sensitivity and specificity of the predictive ability of risk score in the test dataset. AUC values were 0.479 for 1 year, 0.571 for 2 year, 0.622 for 5 year and 0.676 for 10 year survival prediction (Figure 14). The AUC values of risk scores calculated based on chosen gene signature were very low according to the AUC values of training data. Although the survival predictive ability (c-index) of our gene signature and AUC values of risk score in test data was low, our 12-gene signature can separate patients into two groups which have significant overall survival difference.

We performed the correlation analysis between tumor stages, mutation counts and gene expressions of signature genes for test data, there was a slight significant difference of tumor stages between risk groups although there was no difference of total SNV mutation count between groups (Figure 18). The gene expression levels of 6 signature genes (BCHE, DEPTOR, MASP2, CCNA1, MGLL, TNNI2) were significantly different between high-risk and low-risk groups however, the gene expression levels of other 6 signature genes (CYP24A1, MYO1A, RAPGEF3, SGK2, ZBTB16, PODXL2) do not have significant difference in test data (Figure 19). The expression levels of the CCNA1, TNNI2 genes were lower in high-risk group while the expression levels of the BCHE, DEPTOR, MASP2, MGLL genes were higher in high-risk group.
Figure 18: Correlation analysis between risk groups and total mutation count and tumor stage in tumor samples of test data

Figure 19: Violin plot showing the expression levels of the signature genes between low-risk and high-risk groups in tumor samples of test data
5 Discussion

In this study, we performed an integrative analysis by using level-3 data of SNVs, CNVs and transcriptome profiling of TCGA LUAD patients. We aimed to identify genomic and transcriptomic abnormalities that might be used to generate a molecular signature. We determined significant mutated genes; amplified and deleted genes; and differentially expressed genes (DEGs) significantly and their active subnetworks by using R packages. Then we performed univariate and Cox Proportional Hazards Regression (CPHR) analysis with LOOCV and Lasso penalty to identify predictor genes on patients’ survival time.

We identified that KRAS and EGFR oncogenes with TP53, STK11, RB1 and MGA tumor suppressors were mutated significantly. These genes were determined as candidate driver mutated genes although they have low number of mutations in samples of cohort. KRAS and EGFR works in important signaling pathways playing roles in the regulation of cell proliferation and transcription of tumor suppressors. TP53, STK11 and RB1 are tumor suppressors playing important roles in cell metabolism, apoptosis, DNA damage response and regulation of cell division. Although KRAS, EGFR, TP53, STK11 and RB1 are well known cancer related genes, loss-of-function MGA mutations with MYC amplification have been newly described (The Cancer Genome Atlas Research Network, 2014). MGA, encodes MAX gene-associated protein which is a MYC-interacting transcription factor and antagonizes the transcriptional regulation of MYC involved in cancer processes (Romero, 2014).

When we integrated significant SNVs, CNVs and DEGs from active subnetworks by performing Cox Proportional Hazards Regression (CPHR) analysis with LOOCV and Lasso penalty after univariate CPHR, we determined 12-gene signature (DEPTOR, ZBTB16, BCHE, MGLL, MASP2, TNI12, RAPGEF3, SGK2, MYO1A, CYP24A1, PODXL2, CCNA1) which have high potential to be used as molecular signature for personalized cancer treatment. When we clustered the patients into high-risk and low-risk group based on risk scores calculated by using expressions and coefficients of 12-genes, there were highly significant overall survival difference.

6 Conclusion

In this study we analyzed significant SNVs, CNVs and DEGs in active subnetworks, which have impact on overall survival of TCGA LUAD patients. We determined 12-genes of which expressions are strong candidates to be used as molecular signature for prediction of survival of patients with lung adenocarcinoma.

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REFERENCES

Berger, A. H., Brooks, A. N., Wu, X., Shrestha, Y., Chouinard, C., Piccioni, F., … Boehm, J. S. (2016). High-throughput Phenotyping of Lung Cancer Somatic Mutations. Cancer Cell, 30(2), 214–228.

Chang, K., Creighton, C. J., Davis, C., Donehower, L., Drummond, J., Wheeler, D., … Stuart, J. M. (2013). The Cancer Genome Atlas Pan-Cancer analysis project. Nature Genetics, 45(10), 1113–1120.

Colaprico, A., Silva, T. C., Olsen, C., Garofano, L., Cava, C., Garolini, D., … Noushmehr, H. (2016). TCGAbiolinks : an R/Bioconductor package for integrative analysis of TCGA data. Nucleic Acids Research, 44(8), e71–e71.

Cox, D. R. (1972). Regression models and life-tables. J. R. Stat. Soc. B Methodol. 187–220.
Deng, Z. min, Liu, L., Qiu, W. hai, Zhang, Y. qun, Zhong, H. yan, Liao, P., & Wu, Y. hong. (2017). Analysis of genomic variation in lung adenocarcinoma patients revealed the critical role of PI3K complex. PeerJ, 5, e3216.

Durinck, S., Spellman, P. T., Birney, E., & Huber, W. (2009). Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. Nature Protocols, 4, 1184.

Forbes, S. A., Beare, D., Boutselakis, H., Bamford, S., Bindal, N., Tate, J., … Campbell, P. J. (2017). COSMIC: somatic cancer genetics at high-resolution. Nucleic Acids Research, 45(D1), D777–D783.

He, R., & Zuo, S. (2019). A Robust 8-Gene Prognostic Signature for Early-Stage Non-small Cell Lung Cancer. Frontiers in Oncology, 9(July), 1–14.

Kaplan, E. L., & Meier, P. (1958). Nonparametric Estimation from Incomplete Observations. Journal of the American Statistical Association, 53(282), 457–481.

Krzystanek, M., Moldvay, J., Szićs, D., Szallasi, Z., & Eklund, A. C. (2016). A robust prognostic gene expression signature for early stage lung adenocarcinoma. Biomarker Research, 4(1), 4.

Li, S., Xuan, Y., Gao, B., Sun, X., Miao, S., Lu, T., … Jiao, W. (2018). Identification of an eight-gene prognostic signature for lung adenocarcinoma. Cancer Management and Research, Volume 10, 3383–3392.

McCarthy DJ, Chen Y, Smyth GK (2012). “Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation.” Nucleic Acids Research, 40(10), 4288-4297.

Mantel, N. (1966). Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemotherapy Reports, 50(3), 163–170.

Mayakonda A, Lin D, Assenov Y, Plass C, Koeffler PH (2018). “Maftools: efficient and comprehensive analysis of somatic variants in cancer.” Genome Research.

Morganella, S., Pagnotta, S. M., & Ceccarelli, M. (2011). Finding recurrent copy number alterations preserving within-sample homogeneity. Bioinformatics, 27(21), 2949–2956.

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Research, 43(7), e47–e47.

Romero, O. A., Torres-Diz, M., Pros, E., Savola, S., Gomez, A., Moran, S., … Sanchez-Cespedes, M. (2014). MAX Inactivation in Small Cell Lung Cancer Disrupts MYC–SWI/SNF Programs and Is Synthetic Lethal with BRG1. Cancer Discovery, 4(3), 292–303.

Shi, J., Hua, X., Zhu, B., Ravichandran, S., Wang, M., Nguyen, C., … Landi, M. T. (2016). Somatic Genomics and Clinical Features of Lung Adenocarcinoma: A Retrospective Study. PLoS Medicine, 13(12), 1–24.

Shi, X., Tan, H., Le, X., Xian, H., Li, X., Huang, K., … Zhang, J. (2018). An expression signature model to predict lung adenocarcinoma-specific survival. Cancer Management and Research, Volume 10, 3717–3732.

Shukla, S., Evans, J. R., Malik, R., Feng, F. Y., Dhanasekaran, S. M., Cao, X., … Chinnaiyan, A. M. (2017). Development of a RNA-Seq Based Prognostic Signature in Lung Adenocarcinoma. Journal of the National Cancer Institute, 109(1), djw200.

The Cancer Genome Atlas Research Network. (2014). Comprehensive molecular profiling of lung adenocarcinoma. Nature, 511(7511), 543–550.
The International Agency for Research on Cancer (IARC). Latest global cancer data: Cancer burden rises to 18.1 million new cases and 9.6 million cancer deaths in 2018. 12 September 2018. PRESS RELEASE No 263. World Health Organization. Geneva, Switzerland.

Travis, W. D. (2011). Pathology of Lung Cancer. Clinics in Chest Medicine, 32(4), 669–692.

Van den Eynden, J., Fierro, A. C., Verbeke, L. P. C., & Marchal, K. (2015). SomInaClust: detection of cancer genes based on somatic mutation patterns of inactivation and clustering. BMC Bioinformatics, 16(1), 125.

Vrahatis, A. G., Balomenos, P., Tsakalidis, A. K., & Bezerianos, A. (2016). DEsubs: an R package for flexible identification of differentially expressed subpathways using RNA-seq experiments. Bioinformatics, 32(24), 3844–3846.

Zhao, K., Li, Z., & Tian, H. (2018). Twenty-gene-based prognostic model predicts lung adenocarcinoma survival. OncoTargets and Therapy, Volume 11, 3415–3424.