Bladder cohort - R Notebook

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Based on tutorial found at https://www.costalab.org/wp-content/uploads/2020/11/R_class_D3.htm

First install BiocManager, edgeR, and TCGAbiolinks

```
if (!require("BiocManager", quietly = TRUE))
  install.packages("BiocManager")

BiocManager::install("edgeR")
```

```
## Bioconductor version 3.19 (BiocManager 1.30.23), R 4.4.0 (2024-04-24 ucrt)

## Warning: package(s) not installed when version(s) same as or greater than current; use
##   'force = TRUE' to re-install: 'edgeR'

## Installation paths not writeable, unable to update packages
##   path: C:/Program Files/R/R-4.4.0/library
##   packages:
##     KernSmooth, nlme, survival

## Old packages: 'evaluate', 'leaps'
```

```
BiocManager::install("TCGAbiolinks")
```

```
## Bioconductor version 3.19 (BiocManager 1.30.23), R 4.4.0 (2024-04-24 ucrt)

## Warning: package(s) not installed when version(s) same as or greater than current; use
##   'force = TRUE' to re-install: 'TCGAbiolinks'

## Installation paths not writeable, unable to update packages
##   path: C:/Program Files/R/R-4.4.0/library
##   packages:
##     KernSmooth, nlme, survival
## Old packages: 'evaluate', 'leaps'
```

```
BiocManager::install("genefilter")
```

```
## Bioconductor version 3.19 (BiocManager 1.30.23), R 4.4.0 (2024-04-24 ucrt)
```

```
## Warning: package(s) not installed when version(s) same as or greater than current; use
##   'force = TRUE' to re-install: 'genefilter'

## Installation paths not writeable, unable to update packages
##   path: C:/Program Files/R/R-4.4.0/library
##   packages:
##     KernSmooth, nlme, survival
## Old packages: 'evaluate', 'leaps'
```

Step 1 - Load packages, download data from TCGA, and prepare it for DEGList

```
library("TCGAbiolinks")
library("limma")
library("edgeR")
library("glmnet")
```

```
## Loading required package: Matrix

## Loaded glmnet 4.1-8
```

```
library("factoextra")
```

```
## Loading required package: ggplot2

## Welcome! Want to learn more? See two factoextra-related books at https://goo.gl/ve3WBa
```

```
library("FactoMineR")
library("caret")
```

```
## Loading required package: lattice
```

```
library("SummarizedExperiment")
```

```
## Loading required package: MatrixGenerics

## Loading required package: matrixStats

##
## Attaching package: 'MatrixGenerics'

## The following objects are masked from 'package:matrixStats':
##
##     colAlls, colAnyNAs, colAnys, colAvgsPerRowSet, colCollapse,
##     colCounts, colCummaxs, colCummins, colCumprods, colCumsums,
##     colDiffs, colIQRDiffs, colIQRs, colLogSumExps, colMadDiffs,
##     colMads, colMaxs, colMeans2, colMedians, colMins, colOrderStats,
##     colProds, colQuantiles, colRanges, colRanks, colSdDiffs, colSds,
```

```
##     colSums2, colTabulates, colVarDiffs, colVars, colWeightedMads,
##     colWeightedMeans, colWeightedMedians, colWeightedSds,
##     colWeightedVars, rowAlls, rowAnyNAs, rowAnys, rowAvgsPerColSet,
##     rowCollapse, rowCounts, rowCummaxs, rowCummins, rowCumprods,
##     rowCumsums, rowDiffs, rowIQRDiffs, rowIQRs, rowLogSumExps,
##     rowMadDiffs, rowMads, rowMaxs, rowMeans2, rowMedians, rowMins,
##     rowOrderStats, rowProds, rowQuantiles, rowRanges, rowRanks,
##     rowSdDiffs, rowSds, rowSums2, rowTabulates, rowVarDiffs, rowVars,
##     rowWeightedMads, rowWeightedMeans, rowWeightedMedians,
##     rowWeightedSds, rowWeightedVars

## Loading required package: GenomicRanges

## Loading required package: stats4

## Loading required package: BiocGenerics

##
## Attaching package: 'BiocGenerics'

## The following object is masked from 'package:limma':
##
##     plotMA

## The following objects are masked from 'package:stats':
##
##     IQR, mad, sd, var, xtabs

## The following objects are masked from 'package:base':
##
##     anyDuplicated, aperm, append, as.data.frame, basename, cbind,
##     colnames, dirname, do.call, duplicated, eval, evalq, Filter, Find,
##     get, grep, grepl, intersect, is.unsorted, lapply, Map, mapply,
##     match, mget, order, paste, pmax, pmax.int, pmin, pmin.int,
##     Position, rank, rbind, Reduce, rownames, sapply, setdiff, table,
##     tapply, union, unique, unsplit, which.max, which.min

## Loading required package: S4Vectors

##
## Attaching package: 'S4Vectors'

## The following objects are masked from 'package:Matrix':
##
##     expand, unname

## The following object is masked from 'package:utils':
##
##     findMatches
```

```
## The following objects are masked from 'package:base':
##
##     expand.grid, I, unname

## Loading required package: IRanges

##
## Attaching package: 'IRanges'

## The following object is masked from 'package:grDevices':
##
##     windows

## Loading required package: GenomeInfoDb

## Loading required package: Biobase

## Welcome to Bioconductor
##
##     Vignettes contain introductory material; view with
##     'browseVignettes()'. To cite Bioconductor, see
##     'citation("Biobase")', and for packages 'citation("pkgname")'.

##
## Attaching package: 'Biobase'

## The following object is masked from 'package:MatrixGenerics':
##
##     rowMedians

## The following objects are masked from 'package:matrixStats':
##
##     anyMissing, rowMedians
```

```
library("gplots")
```

```
##
## Attaching package: 'gplots'

## The following object is masked from 'package:IRanges':
##
##     space

## The following object is masked from 'package:S4Vectors':
##
##     space

## The following object is masked from 'package:stats':
##
##     lowess
```

```r
library("survival")
```

```
##
## Attaching package: 'survival'

## The following object is masked from 'package:caret':
##
##     cluster
```

```r
library("survminer")
```

```
## Loading required package: ggpubr

##
## Attaching package: 'survminer'

## The following object is masked from 'package:survival':
##
##     myeloma
```

```r
library("RColorBrewer")
library("gProfileR")
library("genefilter")
```

```
##
## Attaching package: 'genefilter'

## The following objects are masked from 'package:MatrixGenerics':
##
##     rowSds, rowVars

## The following objects are masked from 'package:matrixStats':
##
##     rowSds, rowVars
```

```r
setwd('C:/Adam/R/')  # make sure it already exists

# Before we perform a GDC query let's look at the TCGA-BLCA data
# As of June 2024 we should see a case count of 412
TCGAbiolinks:::getProjectSummary("TCGA-BLCA")
```

```
## $file_count
## [1] 23394
##
## $data_categories
##    file_count case_count                data_category
## 1        6729        412  Simple Nucleotide Variation
## 2        4285        412             Sequencing Reads
## 3        1760        412                  Biospecimen
```

```
## 4         994        412                     Clinical
## 5        4478        412        Copy Number Variation
## 6        1736        412      Transcriptome Profiling
## 7        1320        412              DNA Methylation
## 8         343        343           Proteome Profiling
## 9          26         12 Somatic Structural Variation
## 10       1723        406         Structural Variation
##
## $case_count
## [1] 412
##
## $file_size
## [1] 4.082979e+14
```

```r
# Download TCGA-BLCA data from GDC
# We want the complete RNA sequencing and raw gene count data
# So we run a query of the Transcriptome Profiling category and RNA-Seq experimental type
# We use the STAR - Counts workflow type because it contains the raw gene counts we need
# We ignore other sample types besides tumor and normal
# The original paper by Wang uses the HTSeq-counts workflow, but this is a legacy version of
#  the new STAR - COUNTS workflow type
query_TCGA = GDCquery(
  project = "TCGA-BLCA",
  data.category = "Transcriptome Profiling",
  data.type="Gene Expression Quantification",
  experimental.strategy = "RNA-Seq",
  workflow.type = "STAR - Counts",
  sample.type = c("Primary Tumor", "Solid Tissue Normal"))
```

```
## ------------------------------------

## o GDCquery: Searching in GDC database

## ------------------------------------

## Genome of reference: hg38

## --------------------------------------------

## oo Accessing GDC. This might take a while...

## --------------------------------------------

## ooo Project: TCGA-BLCA

## --------------------

## oo Filtering results

## --------------------
```

```
## ooo By experimental.strategy

## ooo By data.type

## ooo By workflow.type

## ooo By sample.type

## -----------------

## oo Checking data

## -----------------

## ooo Checking if there are duplicated cases

## ooo Checking if there are results for the query

## --------------------

## o Preparing output

## --------------------
```

```r
# Run the query and format it as a table
# The results are a table with 431 rows (because some patients have multiple cases each)
# There are 29 columns with meta data about each case such as sample_type (tumor vs normal)
lihc_res = getResults(query_TCGA)

# We can create a summary table shows there are 412 tumor and 19 normal (412+19=431)
summary(factor(lihc_res$sample_type))
```

```
##       Primary Tumor Solid Tissue Normal
##                 412                  19
```

```r
# Go ahead and download all the data from GDC to our working directory
GDCdownload(query = query_TCGA)
```

```
## Downloading data for project TCGA-BLCA

## Of the 431 files for download 431 already exist.

## All samples have been already downloaded
```

```r
# Now load the RNA-Seq data from the files into R workspace
tcga_data = GDCprepare(query_TCGA)
```

```
## |                                                  |   0%                    |
```

```
## Starting to add information to samples

##  => Add clinical information to samples

##  => Adding TCGA molecular information from marker papers

##  => Information will have prefix 'paper_'

## blca subtype information from:doi:10.1016/j.cell.2017.09.007

## Available assays in SummarizedExperiment :
##   => unstranded
##   => stranded_first
##   => stranded_second
##   => tpm_unstrand
##   => fpkm_unstrand
##   => fpkm_uq_unstrand
```

```
# This data object has 60660 rows and 431 columns
# This indicates there are 60660 different genes found throughout all the cases
# The object contains both clincal and expression data
dim(tcga_data)
```

```
## [1] 60660   431
```

```
# We can access the data in the object like this which verifies 412 tumor and 19 normal
table(tcga_data@colData$definition)
```

```
##
## Primary solid Tumor Solid Tissue Normal
##                 412                  19
```

```
# Or see the gender data of 117 female and 314 male
table(tcga_data@colData$gender)
```

```
##
## female   male
##    117    314
```

```
# let's look at the various names of the first 6 genes...
head(rowData(tcga_data))
```

```
## DataFrame with 6 rows and 10 columns
##                       source     type     score     phase            gene_id
##                     <factor> <factor> <numeric> <integer>        <character>
## ENSG00000000003.15   HAVANA     gene        NA        NA ENSG00000000003.15
## ENSG00000000005.6    HAVANA     gene        NA        NA  ENSG00000000005.6
## ENSG00000000419.13   HAVANA     gene        NA        NA ENSG00000000419.13
## ENSG00000000457.14   HAVANA     gene        NA        NA ENSG00000000457.14
## ENSG00000000460.17   HAVANA     gene        NA        NA ENSG00000000460.17
```

```
## ENSG00000000938.13   HAVANA     gene        NA        NA ENSG00000000938.13
##                         gene_type   gene_name       level     hgnc_id
##                       <character> <character> <character> <character>
## ENSG00000000003.15 protein_coding      TSPAN6           2  HGNC:11858
## ENSG00000000005.6  protein_coding        TNMD           2  HGNC:17757
## ENSG00000000419.13 protein_coding        DPM1           2   HGNC:3005
## ENSG00000000457.14 protein_coding       SCYL3           2  HGNC:19285
## ENSG00000000460.17 protein_coding    C1orf112           2  HGNC:25565
## ENSG00000000938.13 protein_coding         FGR           2   HGNC:3697
##                             havana_gene
##                             <character>
## ENSG00000000003.15 OTTHUMG00000022002.2
## ENSG00000000005.6  OTTHUMG00000022001.2
## ENSG00000000419.13 OTTHUMG00000032742.2
## ENSG00000000457.14 OTTHUMG00000035941.6
## ENSG00000000460.17 OTTHUMG00000035821.9
## ENSG00000000938.13 OTTHUMG00000003516.3
```

```
# To preview the raw gene counts let's look at the expression levels of the first
#  6 genes in the first 3 cases...
rownames = values(tcga_data)$gene_name[1:6]
firs6genes = head(assay(tcga_data)[,1:3])
rownames(firs6genes) = rownames
colnames(firs6genes) = c("Case 1", "Case 2", "Case 3")
firs6genes
```

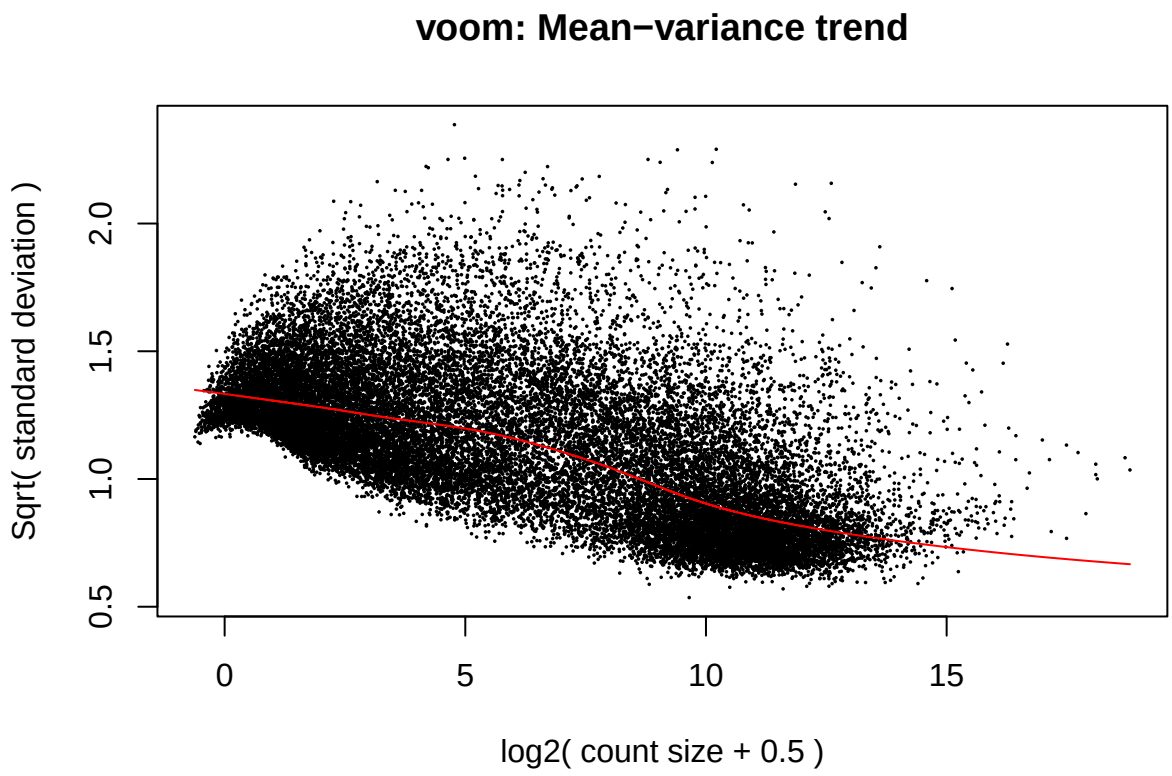
```
##          Case 1 Case 2 Case 3
## TSPAN6     3679  28986    951
## TNMD          0     21      1
## DPM1       4190   2917   2976
## SCYL3       850   1910    705
## C1orf112   1196   1495    655
## FGR         353    905   2282
```

Step 2 - Generate DEGList, filter low counts, and normalize data

```
# Before we can perform DEG analysis we need to normalize the data
# Let's create a limma pipeline to do this...
# The pipeline function will take in three input parameters:
#   tcga_data - the data object we created in Step 1
#   condition_variable - the variable by which we will group patients (tumor vs normal)
#   reference_group - indicates which of the condition variable
#    values is the reference group (no tumors)
# The pipeline will return a list of three objects:
#   voom - the TMM normalized data returned by running voom
#   eBayes - the fitted model returned by running eBayes
#   topTable - a simple table which contains the top 1000 differentially expressed genes
#    sorted by p.value
limma_pipeline = function(
  tcga_data,
  condition_variable,
```

```
reference_group=NULL){

# Create a design matrix
# The factor is the category classifier for the data (tumor vs normal)
#  limma requires it to be a factor object
design_factor = colData(tcga_data)[, condition_variable, drop=T] # definition
group = factor(design_factor) # Solid Normal Tissue

# otherwise just pick the first class as the reference class
if (!is.null(reference_group)) {
  group = relevel(group, ref=reference_group)
}

# make the design matrix
design = model.matrix(~ group)

# generate the DGEList object using the input...
#  counts is the raw gene counts (numericla matrix - rows as genes, columns as cases)
#  samples is the clinical data (data frame)
#  genes is the annotation information (data frame - gene id and names)
# the DGEList object returned is a transformed version of tcga_data
dge = DGEList(counts=assay(tcga_data),
              samples=colData(tcga_data),
              genes=as.data.frame(rowData(tcga_data)))

# filtering - by default genes with less than 10 counts per million reads are removed
# after filtering we have 28087 genes remaining
# no need to filter further by logfc or adjusted p-value because all
#  entries already meet the cutoff criteria
keep = filterByExpr(dge,design) # genes which meet are left after filtering
dge = dge[keep,,keep.lib.sizes=FALSE] # filter the DGEList object, only keep the genes we want
rm(keep) # remove this object from memory because we are done with it

# TODO do we need rpkm() filtering?

# Normalization (TMM followed by voom)
# normalizing - minimize batch effects and variation with the TMM normalization
# TMM - trimmed mean of M-values
# use the voom method to convert the data to have a similar variance as arrays
#  (TODO what is this?)
dge = calcNormFactors(dge)
v = voom(dge, design, plot=TRUE)

# Fit model to data given design
#  fits a series of linear models, one to each probe
#  then pass it to eBayes to rank the differential expression
fit = lmFit(v, design)
fit = eBayes(fit)

# Show top genes
topGenes = topTable(fit, coef=ncol(design), number=1000, sort.by="p")
print(topGenes[10])
```

```
  return(
    list(
      voomObj=v, # normalized data
      fit=fit, # fitted model and statistics
      topGenes=topGenes # the 1000 most differentially expressed genes
    )
  )
}

# TODO only run the pipeline if we didn't already run it before and save the data to a local file
#  tcga_data = readRDS(file = "tcga_data.RDS")
#  saveRDS(object = tcga_data,
#    file = "tcga_data.RDS",
#    compress = FALSE)

# Run the pipeline on the tcga_data from step 1 and normal tissue as the reference
#  "definition" is the column name for the tissue type (tumor vs normal)
#  "Solid Tissue Normal" is our baseline/control/reference class value
# The limma_res object returned is a list of 3 objects - voomObj, fit, topGenes
limma_res = limma_pipeline(
  tcga_data=tcga_data,
  condition_variable="definition",
  reference_group="Solid Tissue Normal"
)
```



```
##                            havana_gene
## ENSG00000164530.15  OTTHUMG00000014611.4
## ENSG00000168079.17  OTTHUMG00000132172.4
## ENSG00000163815.6   OTTHUMG00000133087.3
## ENSG00000153446.16  OTTHUMG00000159314.4
## ENSG00000196616.14  OTTHUMG00000161413.4
## ENSG00000168309.18  OTTHUMG00000159159.5
## ENSG00000108924.14  OTTHUMG00000177840.3
## ENSG00000018625.15  OTTHUMG00000024080.4
## ENSG00000224958.6   OTTHUMG00000019962.6
## ENSG00000197766.8   OTTHUMG00000181840.6
## ENSG00000126218.12  OTTHUMG00000017374.8
## ENSG00000168477.19 OTTHUMG00000031088.12
## ENSG00000068976.14  OTTHUMG00000066835.3
## ENSG00000123560.14  OTTHUMG00000022111.6
## ENSG00000034971.17  OTTHUMG00000034789.4
## ENSG00000241158.7   OTTHUMG00000158723.8
## ENSG00000168497.5   OTTHUMG00000154309.3
## ENSG00000004776.13  OTTHUMG00000048122.5
## ENSG00000077943.8   OTTHUMG00000017733.2
## ENSG00000154330.13  OTTHUMG00000019966.5
## ENSG00000106809.11  OTTHUMG00000020224.4
## ENSG00000119147.10  OTTHUMG00000130921.4
## ENSG00000181856.15  OTTHUMG00000102181.6
## ENSG00000232855.7   OTTHUMG00000078747.6
## ENSG00000144218.19 OTTHUMG00000153011.13
## ENSG00000108018.15  OTTHUMG00000019018.5
## ENSG00000167281.19 OTTHUMG00000150183.10
## ENSG00000141052.18  OTTHUMG00000058767.6
## ENSG00000119508.18  OTTHUMG00000021030.3
## ENSG00000101605.13  OTTHUMG00000178209.7
## ENSG00000171368.12  OTTHUMG00000131011.5
## ENSG00000163145.13  OTTHUMG00000097095.5
## ENSG00000179915.24 OTTHUMG00000129263.23
## ENSG00000125851.10  OTTHUMG00000031941.6
## ENSG00000112936.19  OTTHUMG00000150340.4
## ENSG00000136546.16  OTTHUMG00000154078.6
## ENSG00000182253.15  OTTHUMG00000171887.6
## ENSG00000205221.12  OTTHUMG00000152149.3
## ENSG00000179388.9   OTTHUMG00000097825.3
## ENSG00000123358.20  OTTHUMG00000150393.8
## ENSG00000189129.14  OTTHUMG00000018596.3
## ENSG00000118526.7   OTTHUMG00000015608.2
## ENSG00000123243.15  OTTHUMG00000017635.7
## ENSG00000225398.3   OTTHUMG00000047819.3
## ENSG00000268926.3   OTTHUMG00000187223.2
## ENSG00000149294.17  OTTHUMG00000167196.8
## ENSG00000172403.11  OTTHUMG00000161165.7
## ENSG00000127528.6   OTTHUMG00000182330.1
## ENSG00000172348.15  OTTHUMG00000014782.2
## ENSG00000004799.8   OTTHUMG00000153977.3
## ENSG00000206579.9   OTTHUMG00000164288.3
## ENSG00000172260.15  OTTHUMG00000009698.6
## ENSG00000181072.11  OTTHUMG00000155658.3
```

```
## ENSG00000231943.9   OTTHUMG00000047830.7
## ENSG00000065325.13 OTTHUMG00000130269.12
## ENSG00000186642.16  OTTHUMG00000102045.6
## ENSG00000111452.13  OTTHUMG00000168339.5
## ENSG00000268388.6   OTTHUMG00000183870.4
## ENSG00000181234.9   OTTHUMG00000163736.4
## ENSG00000173175.15  OTTHUMG00000159517.6
## ENSG00000163431.13  OTTHUMG00000035802.3
## ENSG00000156218.13  OTTHUMG00000147363.4
## ENSG00000176533.13  OTTHUMG00000180435.3
## ENSG00000103241.7   OTTHUMG00000137651.5
## ENSG00000140538.16 OTTHUMG00000148677.12
## ENSG00000141338.14  OTTHUMG00000180192.5
## ENSG00000121671.12  OTTHUMG00000153225.5
## ENSG00000280429.1   OTTHUMG00000177388.1
## ENSG00000149090.12  OTTHUMG00000166328.4
## ENSG00000070193.5   OTTHUMG00000131153.5
## ENSG00000144655.15  OTTHUMG00000131293.3
## ENSG00000118407.15  OTTHUMG00000015056.4
## ENSG00000149451.18  OTTHUMG00000031758.4
## ENSG00000254510.2   OTTHUMG00000166924.2
## ENSG00000147588.7   OTTHUMG00000164600.2
## ENSG00000166091.21  OTTHUMG00000028751.9
## ENSG00000108405.4   OTTHUMG00000177673.2
## ENSG00000154175.18  OTTHUMG00000159094.7
## ENSG00000135472.9   OTTHUMG00000169808.5
## ENSG00000153234.15 OTTHUMG00000131950.10
## ENSG00000267505.1   OTTHUMG00000179859.1
## ENSG00000174576.10  OTTHUMG00000167045.2
## ENSG00000100307.13  OTTHUMG00000150418.4
## ENSG00000198932.13  OTTHUMG00000022061.6
## ENSG00000154721.15  OTTHUMG00000078441.4
## ENSG00000077157.22  OTTHUMG00000041393.7
## ENSG00000153823.19  OTTHUMG00000133191.5
## ENSG00000022267.19 OTTHUMG00000022504.13
## ENSG00000154734.16  OTTHUMG00000078688.5
## ENSG00000059915.17  OTTHUMG00000018954.5
## ENSG00000151892.15  OTTHUMG00000019097.4
## ENSG00000143171.13  OTTHUMG00000034626.5
## ENSG00000132840.10  OTTHUMG00000108158.5
## ENSG00000133392.18  OTTHUMG00000129935.9
## ENSG00000138356.14  OTTHUMG00000154536.6
## ENSG00000164736.6   OTTHUMG00000164377.3
## ENSG00000241684.6   OTTHUMG00000158725.3
## ENSG00000108381.11  OTTHUMG00000090655.5
## ENSG00000188729.6   OTTHUMG00000156190.2
## ENSG00000179796.12  OTTHUMG00000130572.8
## ENSG00000106034.18  OTTHUMG00000156982.6
## ENSG00000101938.15  OTTHUMG00000022199.4
## ENSG00000170271.11  OTTHUMG00000164141.3
## ENSG00000258274.1   OTTHUMG00000170323.1
## ENSG00000130176.8   OTTHUMG00000182033.3
## ENSG00000148339.12  OTTHUMG00000020736.2
## ENSG00000162706.13  OTTHUMG00000037177.3
```

```
## ENSG00000136842.14  OTTHUMG00000020325.3
## ENSG00000145936.9   OTTHUMG00000130439.4
## ENSG00000118729.12  OTTHUMG00000011970.1
## ENSG00000122121.11  OTTHUMG00000022373.2
## ENSG00000109906.14  OTTHUMG00000168243.4
## ENSG00000100146.18  OTTHUMG00000149913.7
## ENSG00000163710.9   OTTHUMG00000159312.4
## ENSG00000122367.20  OTTHUMG00000018655.7
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## ENSG00000178999.13  OTTHUMG00000108189.6
## ENSG00000187699.10  OTTHUMG00000154361.3
## ENSG00000176697.20  OTTHUMG00000178797.3
## ENSG00000174306.21  OTTHUMG00000032481.7
## ENSG00000109794.13 OTTHUMG00000160565.11
## ENSG00000068383.19  OTTHUMG00000019293.6
## ENSG00000152049.6   OTTHUMG00000133161.4
## ENSG00000113742.14  OTTHUMG00000130541.7
## ENSG00000179921.15  OTTHUMG00000155203.4
## ENSG00000150347.16  OTTHUMG00000018298.3
## ENSG00000121297.8   OTTHUMG00000150184.5
## ENSG00000076382.17 OTTHUMG00000166586.27
## ENSG00000134138.20  OTTHUMG00000129781.7
## ENSG00000166912.17  OTTHUMG00000175662.2
## ENSG00000093009.11  OTTHUMG00000150386.3
## ENSG00000048342.17 OTTHUMG00000160255.16
## ENSG00000225873.4   OTTHUMG00000156179.8
## ENSG00000257222.1   OTTHUMG00000170477.1
## ENSG00000102010.15  OTTHUMG00000021180.2
## ENSG00000007237.19 OTTHUMG00000177945.15
## ENSG00000196843.17  OTTHUMG00000155229.8
## ENSG00000108691.9   OTTHUMG00000132887.3
## ENSG00000088325.16  OTTHUMG00000032190.3
## ENSG00000133687.16  OTTHUMG00000169324.2
## ENSG00000257596.1   OTTHUMG00000169701.1
## ENSG00000185022.12  OTTHUMG00000151163.5
## ENSG00000197321.15 OTTHUMG00000017882.10
## ENSG00000150594.7   OTTHUMG00000019050.3
## ENSG00000089685.15  OTTHUMG00000177505.9
## ENSG00000127564.17  OTTHUMG00000128975.4
## ENSG00000144395.18  OTTHUMG00000154475.6
## ENSG00000065320.9   OTTHUMG00000130257.6
## ENSG00000189184.12  OTTHUMG00000161348.2
## ENSG00000171243.8   OTTHUMG00000090807.2
## ENSG00000102802.10  OTTHUMG00000016679.2
## ENSG00000008710.20  OTTHUMG00000155795.6
## ENSG00000197299.12  OTTHUMG00000149834.7
## ENSG00000126524.10  OTTHUMG00000023165.4
## ENSG00000241399.7   OTTHUMG00000154080.2
## ENSG00000286447.1   OTTHUMG00000195727.1
## ENSG00000278023.7   OTTHUMG00000188399.3
## ENSG00000228063.2   OTTHUMG00000041228.5
## ENSG00000177301.16 OTTHUMG00000011567.10
## ENSG00000121152.10  OTTHUMG00000130451.6
## ENSG00000198673.10  OTTHUMG00000170207.6
## ENSG00000103657.14  OTTHUMG00000172433.6
## ENSG00000169116.11  OTTHUMG00000160827.1
## ENSG00000275569.1   OTTHUMG00000186851.2
## ENSG00000160957.14  OTTHUMG00000165178.7
## ENSG00000144645.14 OTTHUMG00000130672.11
## ENSG00000108342.13  OTTHUMG00000133247.3
```

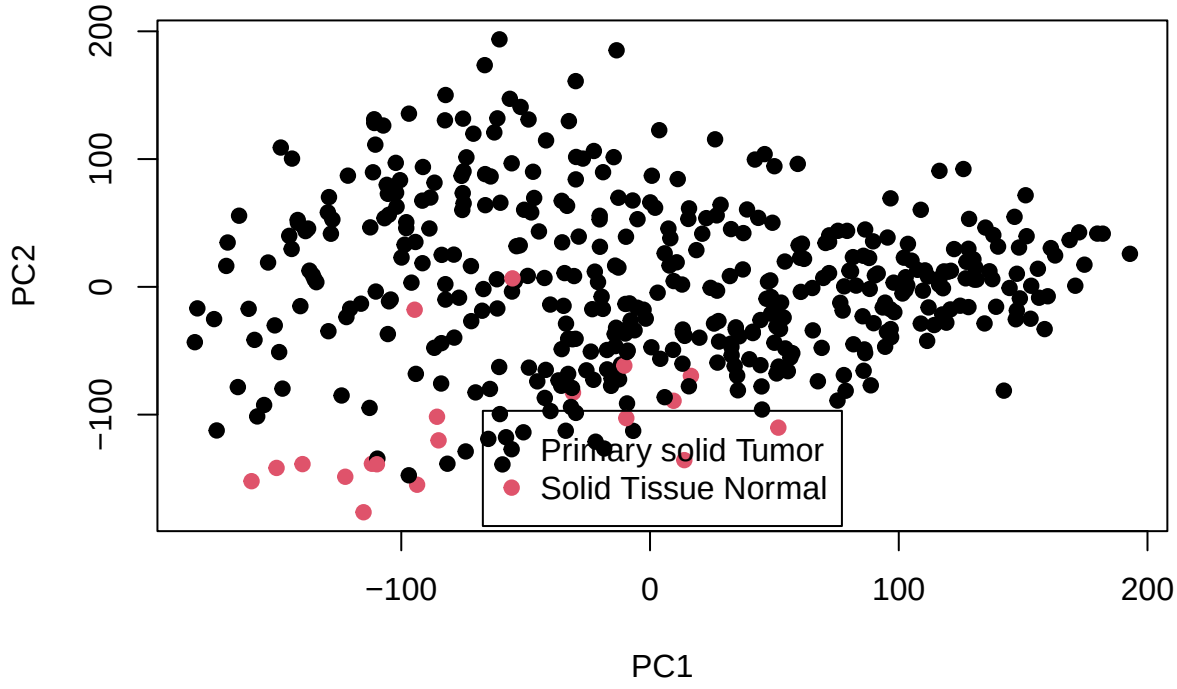
```
## ENSG00000169607.13  OTTHUMG00000131313.3
## ENSG00000184661.14  OTTHUMG00000099429.7
## ENSG00000140682.19  OTTHUMG00000132467.7
## ENSG00000049540.18 OTTHUMG00000150229.14
## ENSG00000204406.14 OTTHUMG00000150440.15
## ENSG00000168952.15  OTTHUMG00000140186.3
## ENSG00000174371.17  OTTHUMG00000039965.9
## ENSG00000129473.11  OTTHUMG00000028738.5
## ENSG00000271474.1   OTTHUMG00000185100.1
## ENSG00000138092.11  OTTHUMG00000125525.4
## ENSG00000196924.19 OTTHUMG00000022712.33
## ENSG00000132718.9   OTTHUMG00000014105.2
## ENSG00000224743.7   OTTHUMG00000016681.7
## ENSG00000101003.10  OTTHUMG00000032124.2
## ENSG00000239218.2   OTTHUMG00000158605.2
## ENSG00000177374.13 OTTHUMG00000177635.11
## ENSG00000172399.6   OTTHUMG00000132968.2
## ENSG00000179909.15  OTTHUMG00000140375.2
## ENSG00000124831.19  OTTHUMG00000133339.5
## ENSG00000125648.15  OTTHUMG00000180852.5
## ENSG00000182389.20 OTTHUMG00000155091.19
## ENSG00000197043.14  OTTHUMG00000164179.7
## ENSG00000266010.2   OTTHUMG00000179440.2
## ENSG00000141433.13  OTTHUMG00000131479.6
## ENSG00000123219.13  OTTHUMG00000131227.5
## ENSG00000151729.11  OTTHUMG00000134299.5
## ENSG00000204131.9   OTTHUMG00000021807.4
## ENSG00000221818.9   OTTHUMG00000163838.3
## ENSG00000064205.10  OTTHUMG00000033071.2
## ENSG00000184226.15  OTTHUMG00000017040.6
## ENSG00000187741.15  OTTHUMG00000173049.7
## ENSG00000197275.14  OTTHUMG00000133658.7
## ENSG00000166402.9   OTTHUMG00000165690.1
## ENSG00000087586.18  OTTHUMG00000032796.7
## ENSG00000135447.17  OTTHUMG00000169934.4
## ENSG00000174456.15  OTTHUMG00000193487.1
## ENSG00000156486.8   OTTHUMG00000044337.4
## ENSG00000229367.1   OTTHUMG00000039469.1
## ENSG00000151789.12 OTTHUMG00000130484.12
## ENSG00000173068.18 OTTHUMG00000019593.13
## ENSG00000162852.14  OTTHUMG00000040090.4
## ENSG00000166483.11  OTTHUMG00000165863.4
## ENSG00000108001.15  OTTHUMG00000019265.4
## ENSG00000013810.20 OTTHUMG00000089535.25
## ENSG00000189159.16  OTTHUMG00000154521.4
## ENSG00000187079.20  OTTHUMG00000165878.8
## ENSG00000140807.7   OTTHUMG00000133169.7
## ENSG00000164463.12  OTTHUMG00000163322.3
## ENSG00000128266.9   OTTHUMG00000150611.6
## ENSG00000009413.16  OTTHUMG00000016318.5
## ENSG00000184005.11  OTTHUMG00000009615.2
## ENSG00000094804.12  OTTHUMG00000133324.7
## ENSG00000179954.16  OTTHUMG00000180855.5
## ENSG00000146374.14  OTTHUMG00000015521.2
```

```
## ENSG00000196550.10  OTTHUMG00000042552.3
## ENSG00000164161.10  OTTHUMG00000161428.3
## ENSG00000080166.16  OTTHUMG00000017206.8
## ENSG00000110076.19 OTTHUMG00000045214.19
## ENSG00000104313.20 OTTHUMG00000149894.17
## ENSG00000100162.15  OTTHUMG00000151277.3
## ENSG00000197776.8   OTTHUMG00000140295.5
## ENSG00000160808.11  OTTHUMG00000133516.9
## ENSG00000272321.1   OTTHUMG00000185509.1
## ENSG00000163467.12  OTTHUMG00000024060.2
## ENSG00000077152.11  OTTHUMG00000041392.5
## ENSG00000072571.20  OTTHUMG00000130381.8
## ENSG00000143228.13  OTTHUMG00000034275.4
## ENSG00000007908.16  OTTHUMG00000034851.9
## ENSG00000276409.5   OTTHUMG00000188403.3
## ENSG00000185920.16  OTTHUMG00000020280.6
## ENSG00000182397.14  OTTHUMG00000149852.2
## ENSG00000163882.9   OTTHUMG00000156746.2
## ENSG00000258498.8   OTTHUMG00000171682.1
## ENSG00000126947.13  OTTHUMG00000022033.3
## ENSG00000161888.11  OTTHUMG00000180798.2
## ENSG00000111911.7   OTTHUMG00000015514.3
## ENSG00000132514.14  OTTHUMG00000177939.4
## ENSG00000229274.2   OTTHUMG00000086581.3
## ENSG00000101746.15  OTTHUMG00000132291.5
## ENSG00000011304.22 OTTHUMG00000181789.13
## ENSG00000134853.12  OTTHUMG00000128699.6
## ENSG00000164283.13  OTTHUMG00000097010.6
## ENSG00000117724.13  OTTHUMG00000036955.2
```

Step 3 - Visualize

```r
# make a function to generate a scatter plot to show a separation of tumor vs normal points
plot_PCA = function(voomObj, condition_variable){
  # create a factor
  group = factor(voomObj$targets[, condition_variable])
  # perform a principal component analysis
  pca = prcomp(t(voomObj$E))
  # Take PC1 and PC2 for the plot
  plot(pca$x[,1:2],col=group, pch=19)
  # include a legend for points
  legend("bottom", inset=.01, levels(group), pch=19, col=1:length(levels(group)))
  return(pca)
}

# call the plot function with the voom object and the defintion column
res_pca = plot_PCA(limma_res$voomObj, "definition")
```




```
# create a volcano plot
x = limma_res$topGenes$logFC
y = limma_res$topGenes$adj.P.Val
TCGAVisualize_volcano(
  x,
  y,
  xlab = "logFC",
  title = "Volcano plot of top 1000 genes",)
```

```
## Saving file as: volcano.pdf
```

Step 4 - Classification model training, testing, and evaluation

```
# TODO need to redo this whole step using WGCNA

# use the expression data that has been normalized
# Transpose and make it into a matrix object
d_mat = as.matrix(t(limma_res$voomObj$E))

# and the clinical feature to distinguish cases ("definition")
# Make it a factor
d_resp = as.factor(limma_res$voomObj$targets$definition)
```

```r
# Divide data into training and testing set
# 75% of samples for training and 25% for testing

# Set (random-number-generator) seed so that results are consistent between runs
set.seed(42)

# create a vector of booleans to subset the cases
train_ids = createDataPartition(d_resp, p=0.75, list=FALSE)

# x is the matrix with normalized expression data
# y is the vector with the response variable (tumor vs normal)
x_train = d_mat[train_ids, ]
x_test  = d_mat[-train_ids, ]

y_train = d_resp[train_ids]
y_test  = d_resp[-train_ids]

# do an elastic net model - a generalized linear model that
#  combines lasso and ridge regression, it selects the genes or groups of genes
#  that best predict the condition and uses these to build the model
#  that is then used for classification

# Train model on training dataset using cross-validation
#  alpha can be between 0 (ridge regression) and 1 (lasso)
# the res object here is an object that holds the model coeffiecients and the
#  mean error found during training
res = cv.glmnet(
  x = x_train,
  y = y_train,
  alpha = 0.5,
  family = "binomial")

# Test/Make prediction on test dataset
y_pred = predict(res, newx=x_test, type="class", s="lambda.min")

# confusion matrix shows the TP, TN, FP, and FN
confusion_matrix = table(y_pred, y_test)

# Evaluation statistics
print(confusion_matrix)
```

```
##                      y_test
## y_pred                Primary solid Tumor Solid Tissue Normal
##   Primary solid Tumor                 103                   1
##   Solid Tissue Normal                   0                   3
```

```r
print(paste0("Sensitivity: ",sensitivity(confusion_matrix)))
```

```
## [1] "Sensitivity: 1"
```

```r
print(paste0("Specificity: ",specificity(confusion_matrix)))
```

```
## [1] "Specificity: 0.75"
```

```
print(paste0("Precision: ",precision(confusion_matrix)))
```

```
## [1] "Precision: 0.990384615384615"
```

```
# now we can look at the genes that most contribute for the prediction
res_coef = coef(res, s="lambda.min") # the "coef" function returns a sparse matrix

# ignore zero value coefficients
res_coef = res_coef[res_coef[,1] != 0,]

# remove first coefficient as this is the intercept, a variable of the model itself
res_coef = res_coef[-1]

relevant_genes = names(res_coef) # get names of the (non-zero) variables.
length(relevant_genes) # number of selected genes
```

```
## [1] 83
```

```
# get the Ensembl gene names
head(relevant_genes) # few select genes
```

```
## [1] "ENSG00000034971.17" "ENSG00000078804.13" "ENSG00000081181.8"
## [4] "ENSG00000086991.13" "ENSG00000101057.16" "ENSG00000102683.8"
```

```
# get the common gene names
head(limma_res$voomObj$genes)
```

```
##                     source type score phase            gene_id      gene_type
## ENSG00000000003.15 HAVANA gene    NA    NA ENSG00000000003.15 protein_coding
## ENSG00000000005.6  HAVANA gene    NA    NA  ENSG00000000005.6 protein_coding
## ENSG00000000419.13 HAVANA gene    NA    NA ENSG00000000419.13 protein_coding
## ENSG00000000457.14 HAVANA gene    NA    NA ENSG00000000457.14 protein_coding
## ENSG00000000460.17 HAVANA gene    NA    NA ENSG00000000460.17 protein_coding
## ENSG00000000938.13 HAVANA gene    NA    NA ENSG00000000938.13 protein_coding
##                     gene_name level    hgnc_id          havana_gene
## ENSG00000000003.15    TSPAN6     2 HGNC:11858 OTTHUMG00000022002.2
## ENSG00000000005.6       TNMD     2 HGNC:17757 OTTHUMG00000022001.2
## ENSG00000000419.13      DPM1     2  HGNC:3005 OTTHUMG00000032742.2
## ENSG00000000457.14     SCYL3     2 HGNC:19285 OTTHUMG00000035941.6
## ENSG00000000460.17  C1orf112     2 HGNC:25565 OTTHUMG00000035821.9
## ENSG00000000938.13       FGR     2  HGNC:3697 OTTHUMG00000003516.3
```

```
relevant_gene_names = limma_res$voomObj$genes[relevant_genes,"gene_name"]
head(relevant_gene_names) # few select genes (with readable names now)
```

```
## [1] "MYOC"     "TP53INP2" "ARG2"     "NOX4"     "MYBL2"    "SGCG"
```

```r
# did elastic net find the same genes originally found by the limma pipeline?
#  "Of note, we do not expect a high overlap between genes selected by limma and Elastic net.
#   The reason for this is the fact Elastic Net criteria bias the selection of genes,
#   which are not highly correlated against each other, while not such bias is
#   present in limma."
print(intersect(limma_res$topGenes$ensembl_gene_id, relevant_genes))
```

```
## NULL
```

Step 5 - Hierarchical clustering

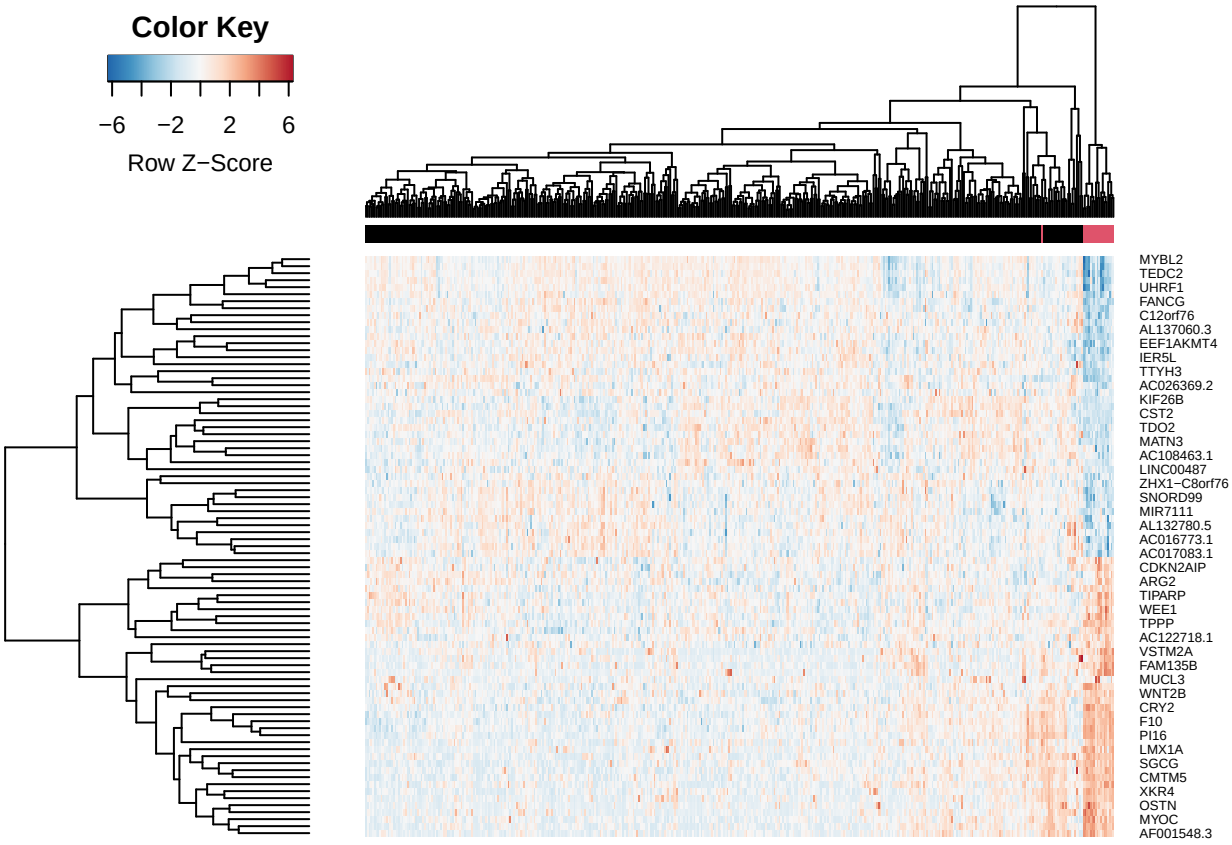
```r
# we are only considering the elastic net results to cluster genes together
# genes in green are original limma results
# genes in red are normal tissue from the elastic net results
# genes in black are tumor tissue from the elastic net results

 # define the color palette for the plot
hmcol = colorRampPalette(rev(brewer.pal(9, "RdBu")))(256)

# perform complete linkage clustering
clust = function(x) hclust(x, method="complete")
# use the inverse of correlation as distance.
dist = function(x) as.dist((1-cor(t(x)))/2)

# Show green color for genes that also show up in DE analysis
colorLimmaGenes = ifelse(
  # Given a vector of boolean values
  (relevant_genes %in% limma_res$topGenes$ensembl_gene_id),
  "green", # if true, return green for that value
  "white" # if false, return white for that value
)

# generate the heatmap
gene_heatmap = heatmap.2(
  t(d_mat[,relevant_genes]),
  scale="row",          # scale the values for each gene (row)
  density.info="none",  # turns off density plot inside color legend
  trace="none",         # turns off trace lines inside the heat map
  col=hmcol,            # define the color map
  labRow=relevant_gene_names, # use gene names instead of ensembl annotation
  RowSideColors=colorLimmaGenes,
  labCol=FALSE,         # Not showing column labels
  ColSideColors=as.character(as.numeric(d_resp)), # Show colors for each response class
  dendrogram="both",    # Show dendrograms for both axis
  hclust = clust,       # Define hierarchical clustering method
  distfun = dist,       # Using correlation coefficient for distance function
  cexRow=.6,            # Resize row labels
  margins=c(1,5)        # Define margin spaces
)
```


Color Key
−6
−2
2
6
Row Z−Score
MYBL2
TEDC2
UHRF1
FANCG
C12orf76
AL137060.3
EEF1AKMT4
IER5L
TTYH3
AC026369.2
KIF26B
CST2
TDO2
MATN3
AC108463.1
LINC00487
ZHX1−C8orf76
SNORD99
MIR7111
AL132780.5
AC016773.1
AC017083.1
CDKN2AIP
ARG2
TIPARP
WEE1
TPPP
AC122718.1
VSTM2A
FAM135B
MUCL3
WNT2B
CRY2
F10
PI16
LMX1A
SGCG
CMTM5
XKR4
OSTN
MYOC
AF001548.3