# Dysregulated processes in NAFLD (BB103X)

Hong Yang

10/02/2022

### 2.1 Load necessary packages

- Rstudio
- load packages (need each time you run your code when you come back from )

```
library(DESeq2)
library(pheatmap)
library(tidyverse)
library(xlsx)
library(readxl)
library(gplots)
library(ggbiplot)
library(piano)
library(venn)
library(clusterProfiler)
library(GEOquery)
library(openxlsx)
library(GEOquery)
```

### 2.2 Load data you've prepared

-metadata: characteristics of samples

-count: count values of genes fro each sample

-Those 2 data tables need to be merged at end of the pro-processing step

```
metadata = read.xlsx('./data/DGSE135251_metadata.xlsx')
sample2count = read.xlsx('./data/DGSE135251_sample2count_str.xlsx')
```

### 2.3 What the data looks like? (1)

```
## Metadata
metadata[1:6,1:4]
```

```
##                title geo_accession                status submission_date
## 1  Liver patient 97    GSM3998167 Public on Dec 03 2020     Aug 01 2019
## 2  Liver patient 98    GSM3998168 Public on Dec 03 2020     Aug 01 2019
## 3 Liver patient 101    GSM3998169 Public on Dec 03 2020     Aug 01 2019
## 4 Liver patient 102    GSM3998170 Public on Dec 03 2020     Aug 01 2019
## 5 Liver patient 105    GSM3998171 Public on Dec 03 2020     Aug 01 2019
## 6 Liver patient 106    GSM3998172 Public on Dec 03 2020     Aug 01 2019
```

- This table contains all the clinical information of samples in this study

```
sample2count[1:8,1:6]
```

```
##                        ID GSM3998167 GSM3998168 GSM3998169 GSM3998170 GSM3998171
## 1 __alignment_not_unique    3064384    2537331    3007853    3645473    3832511
## 2            __ambiguous     992499     855096    1062611    1225874    1187577
## 3           __no_feature    1544139    1238869    1050137    1345656    1491388
## 4          __not_aligned          0          0          0          0          0
## 5        __too_low_aQual          0          0          0          0          0
## 6        ENSG00000000005          0         14          0          0          0
## 7        ENSG00000000419        605        525        709        671        869
## 8        ENSG00000000457        315        330        329        418        348
```

- This table contains the count of each gene in each sample.

## 2.4 What the data looks like? (2)

- Check all the columns that metadata table contains

```
colnames(metadata)
```

```
##  [1] "title"                   "geo_accession"
##  [3] "status"                  "submission_date"
##  [5] "last_update_date"        "type"
##  [7] "channel_count"           "source_name_ch1"
##  [9] "organism_ch1"            "characteristics_ch1"
## [11] "characteristics_ch1.1"   "characteristics_ch1.2"
## [13] "characteristics_ch1.3"   "characteristics_ch1.4"
## [15] "molecule_ch1"            "extract_protocol_ch1"
## [17] "extract_protocol_ch1.1"  "taxid_ch1"
## [19] "description"             "data_processing"
## [21] "data_processing.1"       "data_processing.2"
## [23] "data_processing.3"       "data_processing.4"
## [25] "platform_id"             "contact_name"
## [27] "contact_email"           "contact_institute"
## [29] "contact_address"         "contact_city"
## [31] "contact_zip/postal_code" "contact_country"
## [33] "data_row_count"          "instrument_model"
## [35] "library_selection"       "library_source"
## [37] "library_strategy"        "relation"
## [39] "relation.1"              "supplementary_file_1"
## [41] "disease:ch1"             "fibrosis.stage:ch1"
## [43] "group.in.paper:ch1"      "nas.score:ch1"
## [45] "Stage:ch1"
```

- Summary of samples in each group

```
table(metadata$`group.in.paper:ch1`)
```

```
##
##    control       NAFL NASH_F0-F1    NASH_F2    NASH_F3    NASH_F4
##         10         51         34         53         54         14
```

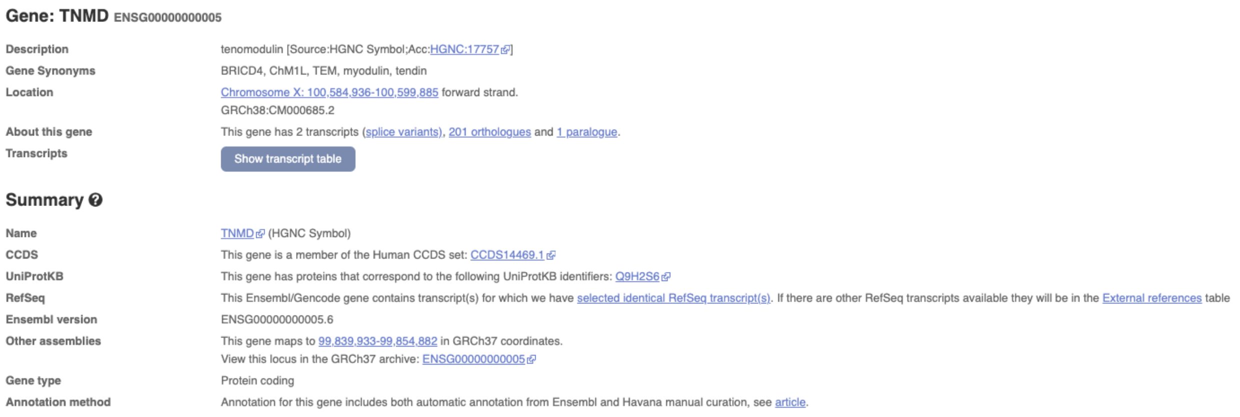
- Summary of samples in each fibrosis stage

```
table(metadata$`fibrosis.stage:ch1`)
```

```
##
##  0  1  2  3  4
## 46 48 54 54 14
```

- Check other columns using the above code (just change the column names behind '$')

## 2.5 Annotate count table by ID conversion (*)

- It is not obvious when we look at the ID of gene, for example, ENSG00000000005. So we need to convert this ID to gene name.
- ENSG00000000005 is identifier of gene TNMN in database Ensembl
- 

**Gene: TNMD** ENSG00000000005

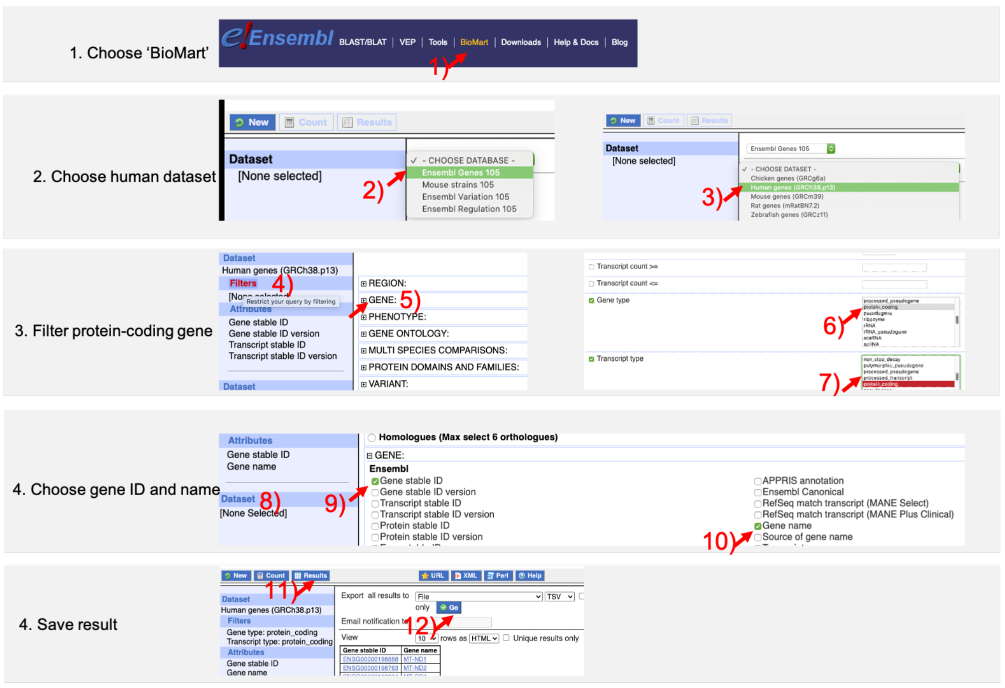
| | |
|---|---|
| Description | tenomodulin [Source:HGNC Symbol;Acc:HGNC:17757] |
| Gene Synonyms | BRICD4, ChM1L, TEM, myodulin, tendin |
| Location | Chromosome X: 100,584,936-100,599,885 forward strand. GRCh38:CM000685.2 |
| About this gene | This gene has 2 transcripts (splice variants), 201 orthologues and 1 paralogue. |
| Transcripts | Show transcript table |

**Summary**

| | |
|---|---|
| Name | TNMD (HGNC Symbol) |
| CCDS | This gene is a member of the Human CCDS set: CCDS14469.1 |
| UniProtKB | This gene has proteins that correspond to the following UniProtKB identifiers: Q9H2S6 |
| RefSeq | This Ensembl/Gencode gene contains transcript(s) for which we have selected identical RefSeq transcript(s). If there are other RefSeq transcripts available they will be in the External references table |
| Ensembl version | ENSG00000000005.6 |
| Other assemblies | This gene maps to 99,839,933-99,854,882 in GRCh37 coordinates. View this locus in the GRCh37 archive: ENSG00000000005 |
| Gene type | Protein coding |
| Annotation method | Annotation for this gene includes both automatic annotation from Ensembl and Havana manual curation, see article. |

## 2.6 (*) Download a table for annotation

- This table would contain 2 columns, 1st column is ID, 2nd column is gene name
- Using BioMart in Ensembl
- https://www.ensembl.org/index.html



- save the result under 'data' folder and name as 'ID2genename.database.txt'

### 2.7 Load annotation table into R

```
ID2gene = read.table('./data/ID2genename.database.txt', sep = '\t', header = TRUE) %>% distinct()
head(ID2gene)
```

```
##    Gene.stable.ID Gene.name
## 1 ENSG00000198888    MT-ND1
## 2 ENSG00000198763    MT-ND2
## 3 ENSG00000198804    MT-CO1
## 4 ENSG00000198712    MT-CO2
## 5 ENSG00000228253   MT-ATP8
## 6 ENSG00000198899   MT-ATP6
```

### 2.8 Merge annotation table into count table

```
count_reindex = merge(sample2count, ID2gene, by.x = 'ID', by.y = 'Gene.stable.ID') %>%
  select(Gene.name, everything())

### Check if we mapped data correctly
(count_reindex %>% filter(ID == 'ENSG00000198888'))[,1:6]
```

```
##   Gene.name              ID GSM3998167 GSM3998168 GSM3998169 GSM3998170
## 1    MT-ND1 ENSG00000198888     143159     111171     164564     159035
```

### 2.9 Processing the case with one gene related to multiple ID

- for example: gene 'ABHD16A'

```
(count_reindex %>% filter(Gene.name == 'ABHD16A'))[,1:6]
```

```
##   Gene.name              ID GSM3998167 GSM3998168 GSM3998169 GSM3998170
## 1   ABHD16A ENSG00000204427         12         12         10          7
## 2   ABHD16A ENSG00000206403          0          0          0          0
## 3   ABHD16A ENSG00000224552          0          0          0          0
## 4   ABHD16A ENSG00000230475          0          0          0          0
## 5   ABHD16A ENSG00000231488          0          0          0          0
## 6   ABHD16A ENSG00000235676          0          0          0          0
## 7   ABHD16A ENSG00000236063          0          0          0          0
```

**Solution:** take one row that have high expression value across samples

**First of all, find all the rows need to be processed.**

```
count_multipleIDs = count_reindex %>% select(Gene.name) %>% group_by(Gene.name) %>% tally() %>% filter(
head(count_multipleIDs)
```

```
## # A tibble: 6 x 2
##   Gene.name     n
##   <chr>     <int>
## 1 ""          267
## 2 "AADACL2"     2
## 3 "AATF"        2
## 4 "ABCB11"      2
## 5 "ABCC1"       2
## 6 "ABCC6"       2
```

**n:** represents the number of rows (IDs) for one specific gene.

## The total number of genes with multiple IDs

```
dim(count_multipleIDs)
```

```
## [1] 692   2
```

- The output indicates there are 692 genes with multiple IDs or null in 'Gene.name' columns.

### Second, calculate the mean value of each ID and decide which row will take for downstream analysis based on above statistics

**Multiple steps for this process**

**a. filter rows that have the gene names with multiple IDs**

**b. filter out rows that contain nothing in column 'Gene.name'**

**c. convert count value to numeric since it is character now, which cann't be used for calculation**

**d. calculate the mean count value across samples and add a new columns named as 'mean_row'**

**e. put the column 'mean_row' at the begining of this subset table**

```
count_multipleIDs_subset = count_reindex %>%
  filter(Gene.name %in% count_multipleIDs$Gene.name) %>%
  filter(Gene.name != '') %>% mutate(across(starts_with('GSM'), ~as.numeric(.))) %>%
  mutate(mean_row = rowMeans(across(where(is.numeric)))) %>% select(mean_row, everything()) %>%
  arrange(., desc(Gene.name))

###
count_multipleIDs_subset[1:6,1:6]
```

```
##       mean_row Gene.name              ID GSM3998167 GSM3998168 GSM3998169
## 1 376.45833333    ZNHIT3 ENSG00000273611        416        358        506
## 2   0.00000000    ZNHIT3 ENSG00000278574          0          0          0
## 3  11.85185185     ZNF85 ENSG00000105750          4          9         23
## 4   0.00000000     ZNF85 ENSG00000278091          0          0          0
## 5   0.06018519    ZNF729 ENSG00000196350          0          0          0
## 6   0.00000000    ZNF729 ENSG00000279552          0          0          0
```

### Third, subset the dataset based on above calculation

```
count_multipleIDs_subset_selected = count_multipleIDs_subset %>%
  group_by(Gene.name) %>% slice_max(mean_row) %>%
  filter(!(mean_row == 0)) %>% arrange(., desc(Gene.name))

count_multipleIDs_subset_selected[1:6,1:6]
```

```
## # A tibble: 6 x 6
## # Groups:   Gene.name [6]
##   mean_row Gene.name ID              GSM3998167 GSM3998168 GSM3998169
##      <dbl> <chr>     <chr>                <dbl>      <dbl>      <dbl>
## 1 376.     ZNHIT3    ENSG00000273611        416        358        506
## 2  11.9    ZNF85     ENSG00000105750          4          9         23
## 3   0.0602 ZNF729    ENSG00000196350          0          0          0
## 4  54.9    ZNF707    ENSG00000181135         62         52         47
## 5   4.63   ZNF676    ENSG00000196109          2          2          1
## 6   2.41   ZNF66     ENSG00000160229          1          2          3
```

**Finally, combine rows with multiple ID after filtering and rows with unique IDs**

```
count_uniqueIDs = count_reindex %>% filter(!(Gene.name %in% count_multipleIDs$Gene.name))
count_multipleIDs_subset_selected = count_multipleIDs_subset_selected %>% select(-mean_row)

sample2count_processed = rbind(count_uniqueIDs, count_multipleIDs_subset_selected)
dim(sample2count_processed)
```

```
## [1] 19081   218
```

**In summary, the proceed table contains the count data of 19081 protein-coding gene.**

```
sample2count_processed[1:6, 1:6]
```

```
##   Gene.name              ID GSM3998167 GSM3998168 GSM3998169 GSM3998170
## 1      TNMD ENSG00000000005          0         14          0          0
## 2      DPM1 ENSG00000000419        605        525        709        671
## 3     SCYL3 ENSG00000000457        315        330        329        418
## 4  C1orf112 ENSG00000000460         92         89        115         97
## 5       FGR ENSG00000000938         96         91        292        115
## 6       CFH ENSG00000000971      41898      38693      37744      42796
```

## 2.10 The results would be expected to be count matrix

- Gene name would be setted to be row names

```
sample2count_processed = as.matrix(sample2count_processed %>%
  column_to_rownames(., var = 'Gene.name') %>% select(-ID))

mode(sample2count_processed) = 'numeric'
sample2count_processed[1:6,1:6]
```

```
##          GSM3998167 GSM3998168 GSM3998169 GSM3998170 GSM3998171 GSM3998172
## TNMD              0         14          0          0          0          7
## DPM1            605        525        709        671        869        500
## SCYL3           315        330        329        418        348        347
## C1orf112         92         89        115         97        108         65
## FGR              96         91        292        115        144        154
## CFH           41898      38693      37744      42796      44933      62515
```

## 2.11 Differential analysis

- DeSeq package will be used in this step
- Read the website carefully to study the methods implemented in this package
- https://bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html

```
mkdir Figures
```

## 2.12 Generate DESeqDataSet (called dds)

- read document to study DESeqDataSet

```r
rownames(metadata) = metadata$geo_accession
sample2count_processed = sample2count_processed[,rownames(metadata)]

####Perform the Differential Expression Analysis
conds=as.factor(metadata$`group.in.paper:ch1`)
coldata = data.frame(row.names=rownames(metadata),conds)
dds = DESeqDataSetFromMatrix(countData=round(as.matrix(sample2count_processed)),
                              colData=coldata,
                              design=~conds)
```

```
## converting counts to integer mode

##   Note: levels of factors in the design contain characters other than
##   letters, numbers, '_' and '.'. It is recommended (but not required) to use
##   only letters, numbers, and delimiters '_' or '.', as these are safe characters
##   for column names in R. [This is a message, not a warning or an error]
```

## 2.13 Gene-level exploratory analysis

- PCA: principle component analyis, read here
- Here is an example for PCA analysis based on vst transformation method,

```r
# PCA analysis
vsd = vst(dds, blind = FALSE)
```

```
##   Note: levels of factors in the design contain characters other than
##   letters, numbers, '_' and '.'. It is recommended (but not required) to use
##   only letters, numbers, and delimiters '_' or '.', as these are safe characters
##   for column names in R. [This is a message, not a warning or an error]
```

```r
pcadata = plotPCA(vsd,intgroup=c("conds"), returnData = TRUE)
percentVar = round(100 * attr(pcadata, "percentVar"))

pcadata_p = ggplot(pcadata, aes(x = PC1, y = PC2, color = factor(conds))) +
  geom_point(size =3, aes(fill=factor(conds),shape=factor(conds))) +
  scale_shape_manual(values=c(16,18,17,15,14,13)) +
  xlab(paste0("PC1: ", percentVar[1], "% variance")) + ylab(paste0("PC2: ", percentVar[2], "% variance"
  theme(axis.text.x = element_blank(), axis.title = element_text(size = 16), legend.text = element_text

ggsave(pcadata_p, filename = "./Figures/00-pca_group.pdf", height = 3.5, width = 5)
```

**please try other method (such as log2, rlog) and generate the graphs for the analyses.**

## 2.14 Perform differential analysis

- First of all, define the groups that you want to compare

```r
table(metadata$`group.in.paper:ch1`)
```

```
##
##     control        NAFL NASH_F0-F1     NASH_F2     NASH_F3     NASH_F4
##          10          51          34          53          54          14
```

- for example, difference between control group and NAFL group

```r
dds_re = DESeq(dds)
```

```
## estimating size factors
##   Note: levels of factors in the design contain characters other than
##   letters, numbers, '_' and '.'. It is recommended (but not required) to use
##   only letters, numbers, and delimiters '_' or '.', as these are safe characters
##   for column names in R. [This is a message, not a warning or an error]

## estimating dispersions

## gene-wise dispersion estimates

## mean-dispersion relationship

##   Note: levels of factors in the design contain characters other than
##   letters, numbers, '_' and '.'. It is recommended (but not required) to use
##   only letters, numbers, and delimiters '_' or '.', as these are safe characters
##   for column names in R. [This is a message, not a warning or an error]

## final dispersion estimates

##   Note: levels of factors in the design contain characters other than
##   letters, numbers, '_' and '.'. It is recommended (but not required) to use
##   only letters, numbers, and delimiters '_' or '.', as these are safe characters
##   for column names in R. [This is a message, not a warning or an error]

## fitting model and testing

##   Note: levels of factors in the design contain characters other than
##   letters, numbers, '_' and '.'. It is recommended (but not required) to use
##   only letters, numbers, and delimiters '_' or '.', as these are safe characters
##   for column names in R. [This is a message, not a warning or an error]

## -- replacing outliers and refitting for 131 genes
## -- DESeq argument 'minReplicatesForReplace' = 7
## -- original counts are preserved in counts(dds)

## estimating dispersions

##   Note: levels of factors in the design contain characters other than
##   letters, numbers, '_' and '.'. It is recommended (but not required) to use
##   only letters, numbers, and delimiters '_' or '.', as these are safe characters
##   for column names in R. [This is a message, not a warning or an error]

## fitting model and testing

##   Note: levels of factors in the design contain characters other than
##   letters, numbers, '_' and '.'. It is recommended (but not required) to use
##   only letters, numbers, and delimiters '_' or '.', as these are safe characters
##   for column names in R. [This is a message, not a warning or an error]
```

```r
cond1 = 'NAFL' #First Condition
cond2 = 'control' #Reference Condition
res=results(dds_re,contrast=c('conds',cond1,cond2))
res$Gene.name = res@rownames
write.xlsx(res,file='./data/DEseq_results_NAFLvscontrol.xlsx')
```

**please complete all the comparisons that you want to look at the difference.**