Supplementary materials

DrGA: cancer driver gene analysis in an automatic manner

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DrGA is an R package can be freely found on Github repository (https://github.com/huynguyen250896/DrGA). The method has been developed based on the idea of our most recent driver gene analysis scheme (Nguyen and Le, 2020). Before your raw data become inputs of DrGA, it requires two following steps: (i) Determine candidate driver genes using advanced driver gene identification tools, and (ii) Do pre-processing procedures to turn your raw data structure into a satisfactory structure (Figure S1).

To show a comprehensive picture of using DrGA, we re-use -omic data used in our prior study (Nguyen and Le, 2020), downloaded from our github repository (https://github.com/huynguyen250896/DrGA/tree/master/data_n_code/breast_cancer) or the cBioPortal for Cancer Genomics (http://www.cbioportal.org) (Cerami, et al., 2012; Gao, et al., 2013), including somatic mutation (MUT; n = 2,369), gene expression (EXP; n = 1,904), and copy number alteration (CNA; n = 2,173), in a cohort of breast cancer patients. Due to as an application example, to simplify all the processes and be convenient to count the time as well as reproduce the results from the previous study (Nguyen and Le, 2020), we decide to preserve the tools at each stage with their selected parameters as the previous study unless otherwise specified.

Next, we apply our tool to mouse metabolic syndrome containing liver EXP from female mice (n =135) (Ghazalpour, et al., 2006). The raw data and R codes for pre-processing processes can be seen in (https://github.com/huynguyen250896/DrGA/tree/master/data_n_code/metabolic_syndrome).

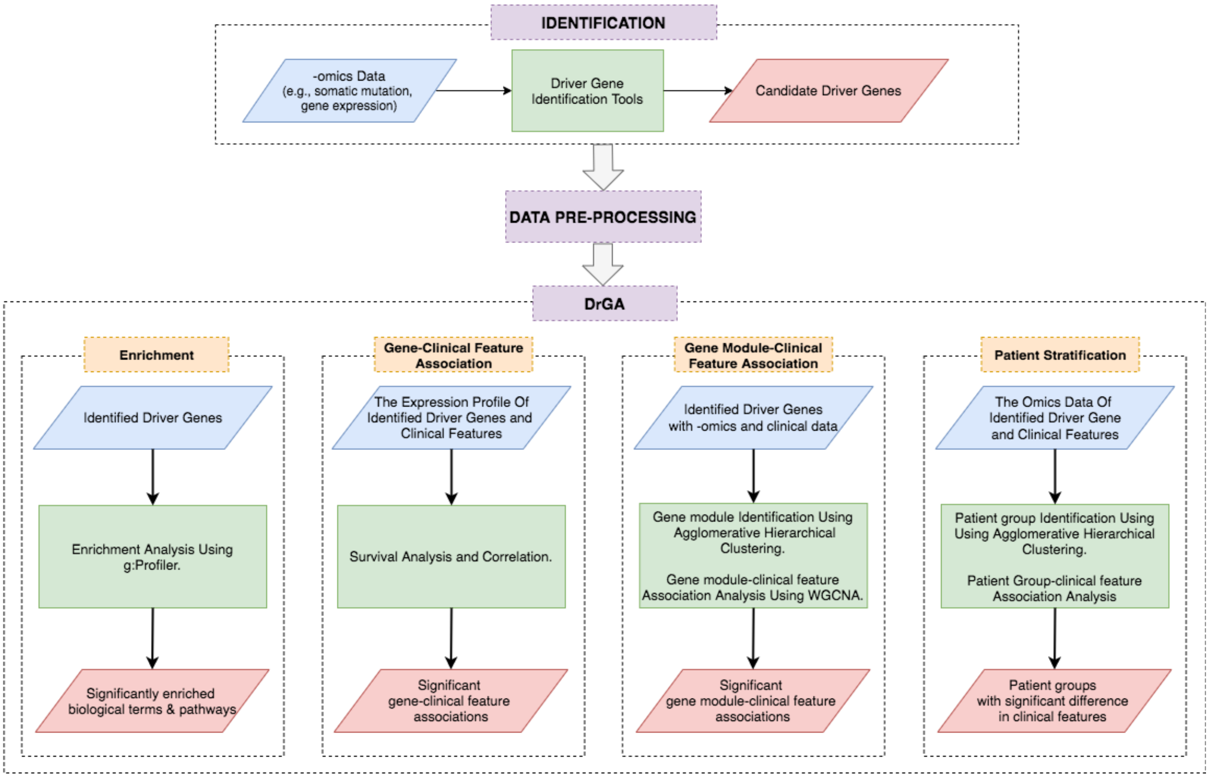

IDENTIFICATION
-omics Data (e.g., somatic mutation, gene expression)
Driver Gene Identification Tools
Candidate Driver Genes
DATA PRE-PROCESSING
DrGA
Enrichment
Identified Driver Genes
Enrichment Analysis Using g:Profiler.
Significantly enriched biological terms & pathways
Gene-Clinical Feature Association
The Expression Profile Of Identified Driver Genes and Clinical Features
Survival Analysis and Correlation.
Significant gene-clinical feature associations
Gene Module-Clinical Feature Association
Identified Driver Genes with -omics and clinical data
Gene module Identification Using Agglomerative Hierarchical Clustering.
Gene module-clinical feature Association Analysis Using WGCNA.
Significant gene module-clinical feature associations
Patient Stratification
The Omics Data Of Identified Driver Gene and Clinical Features
Patient group Identification Using Using Agglomerative Hierarchical Clustering.
Patient Group-clinical feature Association Analysis
Patient groups with significant difference in clinical features

Figure S1. Users first use high-tech driver gene identification tools to predict cancer-related genes. Then, raw data must be processed following the data structure required by the tool DrGA before running the four analysis modules (left to right).

I. Human breast cancer

1. Identification of driver genes.

In this step, we input MUT data into the two web-based tools OncodriveFML (Mularoni, et al., 2016) and OncodriveCLUSTL (Arnedo-Pac, et al., 2019), rendering a total of 35 unique driver genes, in which 30 and 10 driver genes were predicted by the two, respectively. We then match those 35 genes with three gene lists from the Cancer Gene Census database (https://cancer.sanger.ac.uk/census) (Futreal, et al., 2004), Pereira *et al.* reference paper (Pereira, et al., 2016) and Nik Zainal *et al.* paper (Nik-Zainal, et al., 2016), and realize that 31 out of 35 are genuine (Table S1).

MAP2K4, ARID1A, PIK3CA, TBX3, MAP3K1, TP53, AKT1, GATA3, CDH1, RB1, CDKN1B, NCOR1, CDKN2A, ERBB2, KRAS, BRCA2, BAP1, PTEN, CBFB, KMT2C, RUNX1, NF1, PIK3R1, ERBB3, FOXO3, SMAD4, GPS2, AGTR2, ZFP36L1, MEN1, SF3B1.

Table S1. 31 predicted driver genes are shown by the two tools OncodriveFML and OncodriveCLUSTL.

2. Pre-processing procedures & Run DrGA

Here the first module (i.e., enrichment analysis) only needs a list of the driver genes above (Table S1), so we will place it in the two input data (e.g., EXP and CNA) required for the three leftover modules. The second and third modules (i.e., association analyses) need the same input data (usually EXP selected). Finally, input data for the last module can be any data of your choice (e.g., EXP, CNA, methylation, ...). In particular, this work selects EXP for the second and third modules, and CNA for the fourth module as examples, users can replace them with anything else if appropriate.

Firstly, we will set up the working directory, and call necessary R libraries. Set up the working directory is very important due to two reasons: (i) let you be able to import the raw data into R and (ii) the majority of results given by DrGA will move directly into. Secondly, we input the raw data (i.e., assigned as `exp`, `cna`, and `clinical`) into the R environment.

```
#set up the working directory
setwd("~/path/to/your/raw/data")

#library
devtools::install_github("huynguyen250896/DrGA", force = T) #NOTE:
Download all dependencies of the tool!

x=c("DrGA", "dplyr", "survival", "tibble", "tidyr",
"ComplexHeatmap",
     'cluster', 'mclust', 'clValid', 'Biobase', 'annotate', 'GO.db',
     'mygene', "dynamicTreeCut", "flashClust", "Hmisc",
"WGCNA","purrr",
     "gprofiler2", "table1", "compareGroups")
lapply(x, require, character.only = TRUE)

#load raw data
exp = read.table('data_mRNA_median_Zscores.txt', sep = '\t',
check.names = FALSE, header = TRUE, row.names = NULL)
```

```
cna = read.table('data_CNA.txt', sep = '\t', check.names = FALSE,
header = TRUE, row.names = 1)

clinical = read.table('data_clinical_patient.txt', sep = '\t',
check.names = FALSE, header = TRUE, row.names = 1, fill=TRUE)
```

To meet the requirement of the first module, we simply put those 31 driver genes into `exp` and `cna`. As `exp` and `cna` have different dimensions, we will naturally have two corresponding clinical data for each (called `clinicalEXP` and `clinicalCNA`, respectively). Remember that `exp` and `cna` are two matrices whose rows are samples and columns are genes, whereas `clinicalEXP` and `clinicalCNA` include their rows are samples, and their columns are clinical features of the breast cancer patients.

```
#identified driver genes
driver=c("MAP2K4", "ARID1A", "PIK3CA", "TBX3", "MAP3K1", "TP53",
"AKT1", "GATA3", "CDH1", "RB1", "CDKN1B", "NCOR1", "CDKN2A",
"ERBB2", "KRAS", "BRCA2", "BAP1", "PTEN", "CBFB", "KMT2C", "RUNX1",
"NF1", "PIK3R1", "ERBB3", "FOXO3", "SMAD4", "GPS2", "AGTR2",
"ZFP36L1", "MEN1","SF3B1")
length(driver) #31 driver genes

#only keep the 31 driver genes in exp and cna
exp=exp %>%
  dplyr::filter(.$Hugo_Symbol %in% driver) %>%
  tibble::column_to_rownames('Hugo_Symbol') %>%
  dplyr::select(-Entrez_Gene_Id)

cna=cna[driver, ]

#check dimension
dim(exp) # 31 1904
dim(cna) # 31 2173
dim(clinical) # 2509   21

#match patients sharing between exp versus clinical, and cna versus
clinical
#exp and cna are two matrices whose rows are samples and columns are
genes
exp = exp[,intersect(colnames(exp), rownames(clinical))] %>% t()
clinicalEXP = clinical[intersect(rownames(exp), rownames(clinical)),
]

cna = cna[,intersect(colnames(cna), rownames(clinical))] %>% t()
clinicalCNA = clinical[intersect(rownames(cna), rownames(clinical)),
]
```

To meet the requirement of the second and third modules, we use `exp` and `clinicalEXP`. For `exp`, its format is now ready to use. For `clinicalEXP`, its columns must only possess clinical features of your choice. This study re-selects three clinical features like the number of lymph nodes `lymph` (continuous variable), Nottingham prognostic index `npi` (continuous variable), and tumor stage `stage` (ordinal variable). Since the second module performs correlation analyses between the expression levels of individual driver genes and each clinical feature, while the third module performs correlation analyses between the expression levels of individual functional modules and each clinical feature, we will assign variable type for the three clinical features in `clinicalEXP` as ‘numeric’ or ‘integer’ in R. In addition, to run survival analyses between the expression levels of individual driver genes and patient outcome, `clinicalEXP` must have two additional columns like overall survival time `time` (continuous variable) and overall survival status `status` (binary variable; usually coded 1 as death and 0 as alive) of all the subjects.

```
#preprocess clinicalEXP
clinicalEXP = clinicalEXP %>%
  tibble::rownames_to_column('sample') %>%
  dplyr::select(c(sample, LYMPH_NODES_EXAMINED_POSITIVE, NPI, stage,
OS_MONTHS, OS_STATUS)) %>%
  dplyr::mutate(status = ifelse(clinicalEXP$OS_STATUS ==
"DECEASED",1,0)) %>%
  tibble::column_to_rownames('sample') %>%
  dplyr::select(-OS_STATUS)

colnames(clinicalEXP)[1:4] =  c("lymph", "npi", "stage", "time")
str(clinicalEXP)
# 'data.frame':  1904 obs. of  5 variables:
# $ lymph : int  1 5 8 1 0 1 0 2 0 6 ...
# $ npi   : num  4.04 6.03 6.03 5.04 3.05 ...
# $ stage : int  2 2 3 2 2 2 1 2 2 4 ...
# $ time  : num  47 20.4 138.1 119.8 101.2 ...
# $ status: num  1 1 0 0 0 0 0 0 0 1 ...
```

To meet the requirement of the last module, we do exactly the same as the second and third modules for `cna` and `clinicalCNA`. To help DrGA automatically detect which statistical test is appropriate for testing difference between identified patient subgroups in terms of each clinical feature in `clinicalCNA`, users should carefully pre-define variable type of each in R. For example, `lymph` and `npi` are continuous variables should be assigned as a ‘numeric’ or ‘integer’ type in R, whereas `stage` is an ordinal variable should be assigned as a ‘character’ or ‘factor’ type in R.

```
#preprocess clinicalCNA
clinicalCNA = clinicalCNA %>%
  tibble::rownames_to_column('sample') %>%
  dplyr::select(c(sample, LYMPH_NODES_EXAMINED_POSITIVE, NPI, stage,
OS_MONTHS, OS_STATUS)) %>%
  dplyr::mutate(status = ifelse(clinicalCNA$OS_STATUS ==
"DECEASED",1,0)) %>%
  tibble::column_to_rownames('sample') %>%
  dplyr::select(-OS_STATUS)

colnames(clinicalCNA)[1:4] =  c("lymph", "npi", "stage", "time")
clinicalCNA$stage = as.character(clinicalCNA$stage)
str(clinicalCNA)
# 'data.frame':  2173 obs. of  5 variables:
# $ lymph : int  10 0 3 24 1 3 0 0 0 1 ...
# $ npi   : num  6.04 2.04 5.04 6.07 4.05 ...
# $ stage : chr  "2" "1" "2" "2" ...
# $ time  : num  140.5 163.5 164.9 14.1 103.8 ...
# $ status: num  0 0 0 1 0 0 0 0 0 0 ...
```

Now, put all the processed data into the function `DriverGeneAnalysis` in the R package `DrGA` and run.

```
#RUN!!!!
#make sure that patients that share between exp and clinicalEXP are
#included at their rows and in exactly the same order
all(rownames(exp) == rownames(clinicalEXP))
#[1] TRUE

#make sure that patients that share between cna and clinicalCNA are
#included at their rows and in exactly the same order
all(rownames(cna) == rownames(clinicalCNA))
#[1] TRUE

drga = DriverGeneAnalysis(exp = exp, clinicalEXP = clinicalEXP,
timeEXP = clinicalEXP$time, statusEXP = clinicalEXP$status,
                         datMODULE4 = cna,  cliMODULE4 = clinicalCNA,
timeMODULE4 = clinicalCNA$time, statusMODULE4 = clinicalCNA$status)
```

where the argument `exp` is the place where the processed data `exp` are inputted to serve to run the second and third modules of DrGA. Its corresponding clinical data `clinicalEXP`, overall survival time `clinicalEXP$time`, and overall survival status `clinicalEXP$status` of all the patients are inputted into the three arguments `clinicalEXP`, `timeEXP`, and `statusEXP`, respectively, to serve to perform correlation analyses in these modules. Similarly, the argument `datMODULE4` is the place where the processed data `cna` are inputted to serve to run the last module. Its corresponding clinical data `clinicalCNA`, overall survival time `clinicalCNA$time`, and overall survival status `clinicalCNA$status` of all the patients are inputted into the three arguments `cliMODULE4`, `timeMODULE4`, and `statusMODULE4`, respectively, to serve to perform survival analysis as well as observe statistically significant differences between identified patient subgroups in terms of clinical features of your interest.

If the tool runs smoothly and successfully, several results will be printed out in the R environment, and some will be moved directly to the working directory. We show them in the section ‘Understanding the tool and gained results’ below. Users can find the results of this example running at https://github.com/huynguyen250896/DrGA/blob/master/data_n_code/breast_cancer/output_BRCA.zip.

3. Understanding the tool and gained results

Since DrGA integrates state-of-the-art statistical tools for each module; specifically, g:Profiler (Raudvere, et al., 2019) for the first module, computeC (Nguyen and Le, 2020) and geneSA (Nguyen and Le, 2020) for the second module, WGCNA (Langfelder and Horvath, 2008) for the third module, and hierarchical agglomerative clustering (Lance and Williams, 1967) for the last module, we suggest users to refer to their original papers to understand what results each module issues. Alternatively, users can also refer to our study (Nguyen and Le, 2020). Here we outline the interpretation of them.

a. Results from the module 1

The results of the first module will move to the working directory as a txt file named *EnrichmentAnalysis.txt*. This includes the column 'source' reports types of selected biological mechanisms: biological processes (GO:BP) and KEGG pathways (KEGG), while the column 'term_name' points out certain biological mechanisms. g:Profiler then tests the statistical significance among all results and shows at the column 'p_value' (g:SCS multiple testing correction method (Raudvere, et al., 2019); the smaller the P-value, the more significant). Finally, we can know which gene specifically involves which biological mechanism and how many through the 'intersections' and 'query_size' columns, respectively. Note that, `DrGA` may annotate driver genes from other species rather than humans by using the argument `organism`. Please refer to a full list of organisms at (https://biit.cs.ut.ee/gprofiler/page/organism-list). In addition, `sources` helps you choose possible biological mechanisms of driver genes (e.g., Gene Ontology - 'GO:BP', 'GO:MF', 'GO:CC'; 'KEGG'; 'REAC'; 'TF'; 'MIRNA'; 'CORUM'; 'HP'; 'HPA'; 'WP';…).

b. Results from the module 2

The result from successfully performing the association analysis between the expression of individual driver genes and survival rates of all the patients is reported in *gene_SA.txt* in the working directory (Table S1). Roughly, given a driver gene, the median expression of that gene was calculated across the patients, then the patients were classified into two groups based on the expression of the gene. The first group 'up-regulation' includes patients having the expression of the genes was greater than the median; meanwhile, the second group 'down-regulation' includes patients having the expression of the genes was less than the median. The column 'HR' implies the Hazard ratio with its 95% confidence interval (95% CI) that is a measure that helps to determine whether either of two expression levels of each driver gene will result in an increased (i.e., HR > 1) or decreased (i.e., HR < 1) probability of experiencing the defined event (i.e., death), at any time (in this case, the below-median expression level is the reference). P-values are computed by the Cox proportional hazard method to test the statistical difference between the given results. Q-value is computed following the Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995).

Gene	HR (95% CI)	P-value	Q-value
AKT1	1.30 (1.15-1.46)	1.48×10^{-05}	2.29×10^{-04}
KMT2C	1.24 (1.10-1.40)	3.47×10^{-04}	2.69×10^{-03}
KRAS	1.20 (1.07-1.35)	2.30×10^{-03}	1.19×10^{-02}
PTEN	0.85 (0.76-0.96)	7.92×10^{-03}	2.73×10^{-02}
TBX3	0.84 (0.75-0.95)	4.91×10^{-03}	1.90×10^{-02}
PIK3R1	0.84 (0.75-0.95)	4.37×10^{-03}	1.93×10^{-02}
MAP3K1	0.82 (0.73-0.93)	1.23×10^{-03}	7.61×10^{-03}
SMAD4	0.78 (0.69-0.88)	4.85×10^{-05}	5.01×10^{-04}
MAP2K4	0.76 (0.67-0.85)	4.57×10^{-06}	1.42×10^{-05}

Table S1. Association between the expression of individual driver genes and the overall survival of BRCA patients. Three genes including *AKT1*, *KMT2C*, and *KRAS* with above-median expression level and six genes including *PIK3R1*, *PTEN*, *SMAD4*, *MAP3K1*, *MAP2K4* and *TBX3* with below-median expression level significantly associated with a shortened lifespan. HR: hazard ratio. 95% CI: 95% confidence interval.

The result from successfully performing the association analysis between the expression of individual driver genes and each clinical feature is reported as a txt file termed *CC_results.txt* in the working directory. The Spearman's rank-order correlation analysis (default in `DrGA`) is computed by DrGA between the expression of individual driver genes and each leftover clinical feature (i.e., numbers of lymph nodes, Nottingham prognostic index, and pathologic stage). Users can use other correlation methods with the argument `methodCC` (e.g., Pearson's correlation - 'pearson' or Kendall's correlation - 'kendall'). Table S2 reports correlation coefficients *r* (column 'CC'), P-values, and Q-values of each driver gene with all the three clinical features. The column 'CC' measures the degree of association between the two variables: the expression levels of each driver gene versus each clinical feature. It takes on values ranging between -1 and +1. When *r* = 0, there is no relationship between the two variables. When *r* closer to 1, there is an increasingly strong positive (uphill) relationship between the two variables, otherwise is an increasingly strong negative (downhill) relationship between the two variables. Q-value is computed following the Benjamini–Hochberg procedure.

Gene	**Number of lymph nodes**			**Nottingham prognostic index**			**Cancer Stage**		
	CC	**P-value**	**Q-value**	**CC**	**P-value**	**Q-value**	**CC**	**P-value**	**Q-value**
ARID1A	-0.06	0.01	0.02	-0.13	1.31×10^{-8}	3.20×10^{-8}	-0.10	1.13×10^{-4}	4.73×10^{-4}
RUNX1	-0.14	1.65×10^{-9}	3.63×10^{-8}	-0.25	2.20×10^{-16}	2.42×10^{-15}	-0.11	2.97×10^{-5}	3.12×10^{-4}
GATA3	-0.01	1.27×10^{-5}	9.31×10^{-5}	-0.28	2.20×10^{-16}	4.84×10^{-15}	-0.12	3.83×10^{-5}	2.68×10^{-4}
TBX3	-0.10	9.10×10^{-6}	1.00×10^{-4}	-0.18	1.44×10^{-15}	6.31×10^{-15}	-0.12	6.12×10^{-5}	3.21×10^{-4}
NF1	-0.09	5.77×10^{-5}	3.17×10^{-4}	-0.08	2.23×10^{-4}	3.07×10^{-4}	-0.07	6.36×10^{-3}	0.02
MAP2K4	-0.08	4.61×10^{-4}	1.69×10^{-3}	-0.22	2.20×10^{-16}	1.21×10^{-15}	-0.07	6.99×10^{-3}	0.02
PTEN	-0.08	6.02×10^{-4}	1.89×10^{-3}	-0.23	2.20×10^{-16}	1.61×10^{-15}	-0.11	2.93×10^{-5}	6.15×10^{-4}
SMAD4	-0.06	0.01	0.03	-0.10	1.79×10^{-5}	3.29×10^{-5}	-0.08	1.48×10^{-3}	3.89×10^{-3}
MAP3K1	-0.06	0.01	0.03	-0.16	6.35×10^{-13}	2.00×10^{-12}	-0.09	7.16×10^{-4}	2.15×10^{-3}
SF3B1	0.09	7.83×10^{-5}	1.02×10^{-3}	0.07	1.78×10^{-3}	3.31×10^{-3}	0.10	8.48×10^{-5}	1.19×10^{-3}

Table S2. Association between the expression of driver genes and the other clinical features. *ARID1A, RUNX1, GATA3, TBX3, NF1, MAP2K4, PTEN, SMAD4, MAP3K1* and *SF3B1* significantly associated with all of the three clinical features. CC: correlation coefficient.

Alternatively, users also can see specifically the results in the txt file *CC_results.txt* by R command

```
View(drga$CC_module2)
```

c. Results from the module 3

When DrGA goes to the third module, it will ask users to choose a value of soft-thresholding power based on the Figure S2 (printed out in the R environment). The idea behind soft thresholding is to emphasize more on stronger associations (larger correlation coefficients). Based on a recommendation from the previous study (Nguyen and Le, 2020), you should choose a point at which R^2 reaches the peak for the first time (y-axis). Namely, we select six for this case.

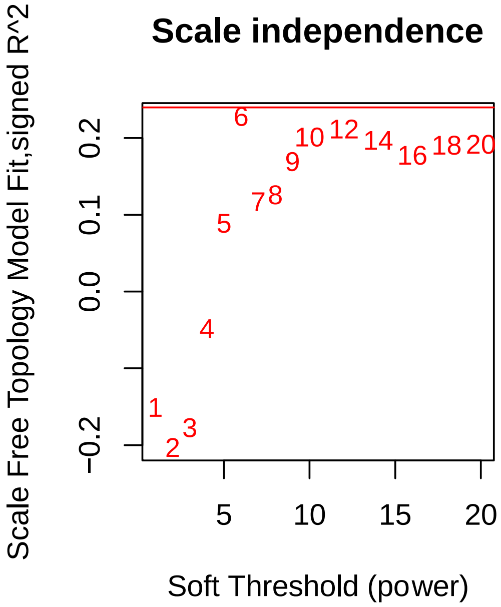
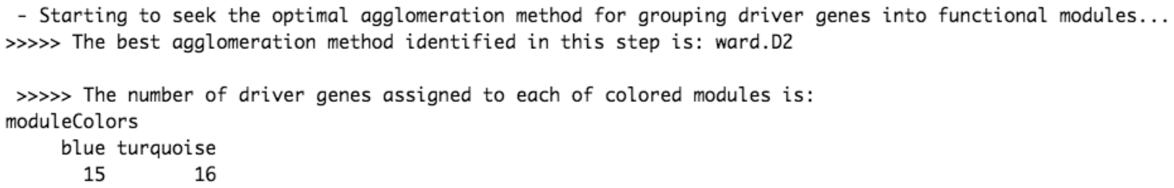


Figure S2. The graph printed out in the R environment shows the scale-free fit index (y-axis) as a function of the soft-thresholding power β (x-axis).

In the middle of the analysis, we will see that DrGA automatically recognizes the best agglomeration method for grouping driver genes into functional modules (Figure S3; printed out in the R environment). More specifically, a set of the agglomeration methods, including ‘complete’, ‘average’, ‘single’, or ‘ward’, will be reviewed and picked the best one by DrGA.

- Complete/average/single-linkage (‘complete’/ ‘average’ / ‘single’): pairwise dissimilarities of the objects in group 1 and group 2 are calculated, and then each method treats the maximum/mean/minimum value of these dissimilarities as the distance between the involved groups, respectively.
- Ward’s minimum variance method: This method first minimizes the total within-cluster error sum of squares, and then, at each stage, iteratively identifies pairs of groups with minimum between-group distance and do the fusion of those two.


```
 - Starting to seek the optimal agglomeration method for grouping driver genes into functional modules...
>>>>> The best agglomeration method identified in this step is: ward.D2

 >>>>> The number of driver genes assigned to each of colored modules is:
moduleColors
     blue turquoise
       15        16
```

Figure S3. DrGA automatically detects the `ward`’s hierarchical clustering is the best method, and then indicates two functional modules exists (marked as blue and turquoise) along with the number of driver genes distributed into each.

Note that, as a recommendation from the previous study (Nguyen and Le, 2020), we recommend choosing the ten number of genes existing in each module minimally (default in `DrGA`) but users can set a different number with the argument `minClusterSize` (e.g., `minClusterSize = 100`). Besides, DrGA currently constructs a ‘signed’ network (default) but users can construct a ‘unsigned’ or ‘signed hybrid’ network with the argument

`NetworkType` (e.g., `NetworkType = 'unsigned '`). In addition, users also may see specifically which genes are assigned to which module by command:

```
View(drga$moduleColors_module3)
```

Figure S4 (*Dendro-MolduColor.pdf* in the working directory) illustrates the co-expressed modules highlighted with distinct colors, and the dendrogram height computed by one minus Pearson's correlation values (1-*r*) represents dissimilarity of two driver genes, in which low dissimilarities indicate that two driver genes are close (similar), whereas the high dissimilarities imply two driver genes are far apart (dissimilar). When it comes to the color of modules, the grey color reserved for genes that do not belong to any identified modules and are not co-expressed as well, whereas the others dedicated to genes that do have their own functional module and are co-expressed.

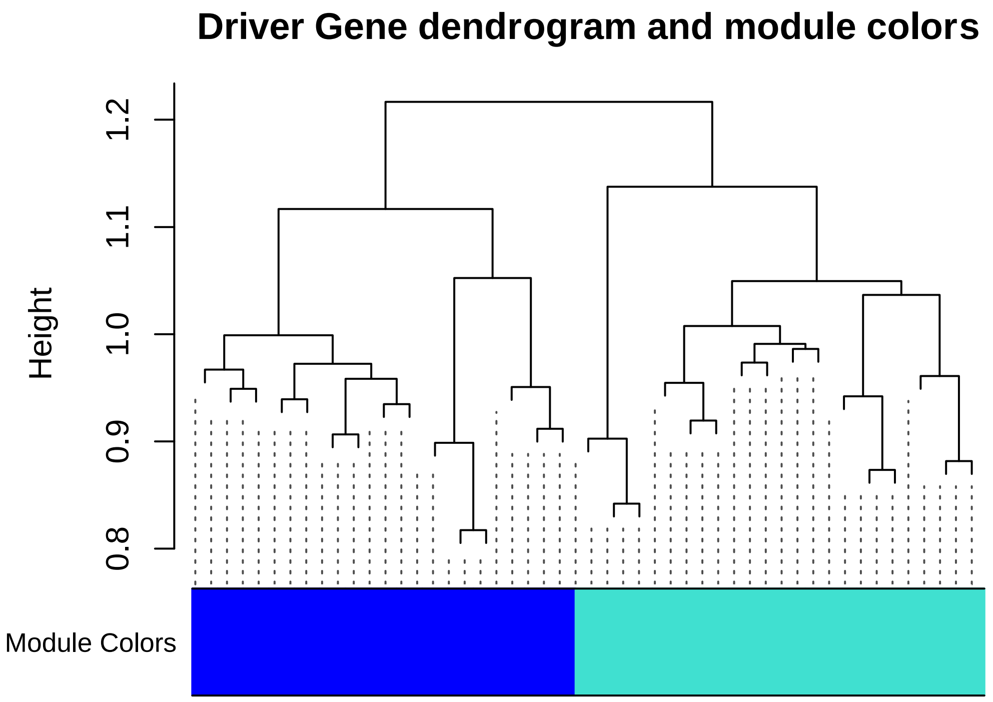


Figure S4. Dendrogram of the identified driver genes on Topology Overlap Matrix-based dissimilarity**.** The dendrogram height corresponds to the coefficient of dissimilarity matrix that is for every pair of the 31 driver genes, in which the low dissimilarities indicate two driver genes are close, whereas the high dissimilarities imply two driver genes are distant apart. Two co-expressed modules were detected and are shown in different colors.

Figure S5 (*Assoc-CliModul.pdf* in the working directory) shows the module-trait heatmap that represents the correlation of the modules with clinical features. The correlation coefficients *r* are computed following Pearson's correlation with an interpretation that can be found in the above sub-section 'Results from the module 2'.

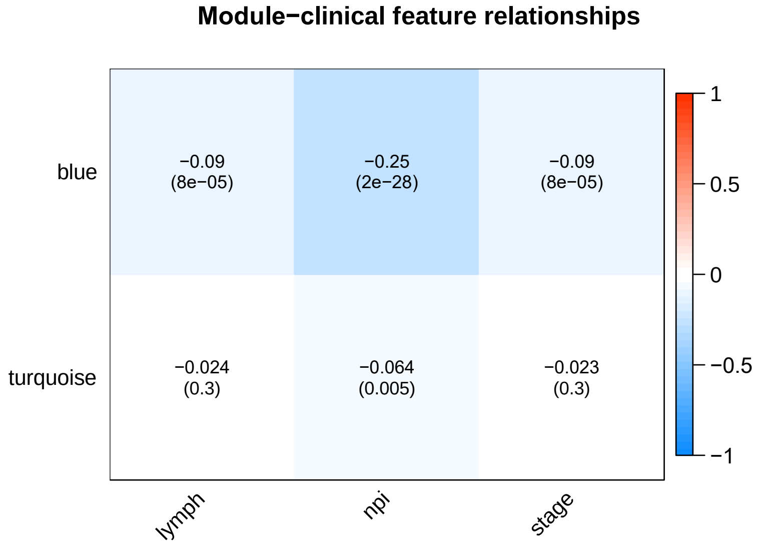
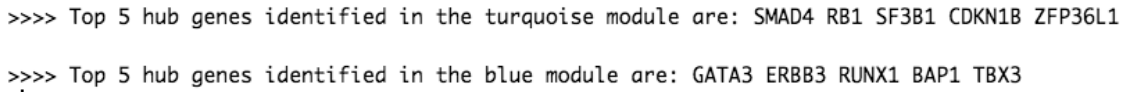


Figure S5. Module–feature associations. Each row corresponds to a module eigengene (ME), column to a feature. Each cell contains the corresponding correlation coefficient *r* and P-value. lymph: number of positive lymph nodes, and npi: the Nottingham prognostic index.

DrGA automatically detects the top five intramodular hub genes in each co-expressed module (Figure S6; printed out in the R environment), indicating possession of a vast range of interactions with other genes as well as playing a crucial role in the co-expression network of those genes.



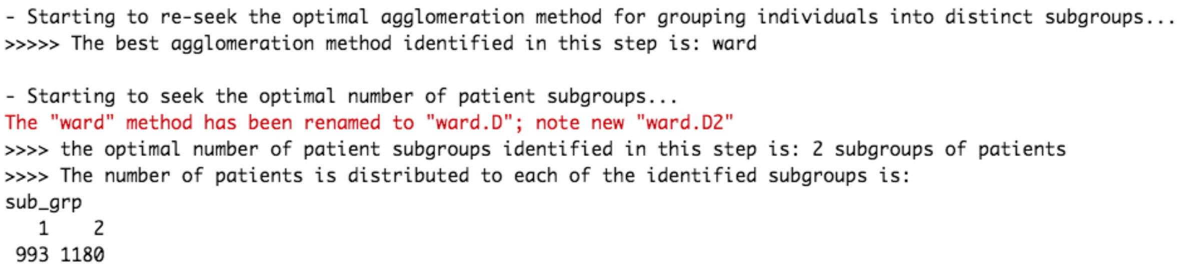
```
>>>> Top 5 hub genes identified in the turquoise module are: SMAD4 RB1 SF3B1 CDKN1B ZFP36L1

>>>> Top 5 hub genes identified in the blue module are: GATA3 ERBB3 RUNX1 BAP1 TBX3
```

Figure S6. Top five hub genes in each identified module are printed out in the R environment.

d. Results from the module 4

Here, DrGA automatically re-seeks the best agglomeration method for grouping breast cancer patients into distinct subgroups (Figure S7; printed out in the R environment).



```
- Starting to re-seek the optimal agglomeration method for grouping individuals into distinct subgroups...
>>>>> The best agglomeration method identified in this step is: ward

- Starting to seek the optimal number of patient subgroups...
The "ward" method has been renamed to "ward.D"; note new "ward.D2"
>>>> the optimal number of patient subgroups identified in this step is: 2 subgroups of patients
>>>> The number of patients is distributed to each of the identified subgroups is:
sub_grp
   1    2
 993 1180
```

Figure S7. DrGA automatically indicates the `ward`'s hierarchical clustering is the best method once again, and then indicates two patient subgroups exists along with the number of breast cancer patients distributed into each.

Here users may see specifically which samples are assigned to which subgroup by R command:

```
View(drga$subgroups_module4)
```

To automatically determine the optimal number of patient subgroups, DrGA performs three common indices like the connectivity (Figure S8), the Dunn's index (Figure S9) and the average Silhouette width (Figure S10), rendering *optimal-group-number.pdf* in the working directory. Ideally, the optimal number of subgroups will be the number indicated by the three indices. If not, the optimal number will be the one indicated by two out of the three indices; otherwise, DrGA will report that it does not seek any optimal number (an extremely rare case).

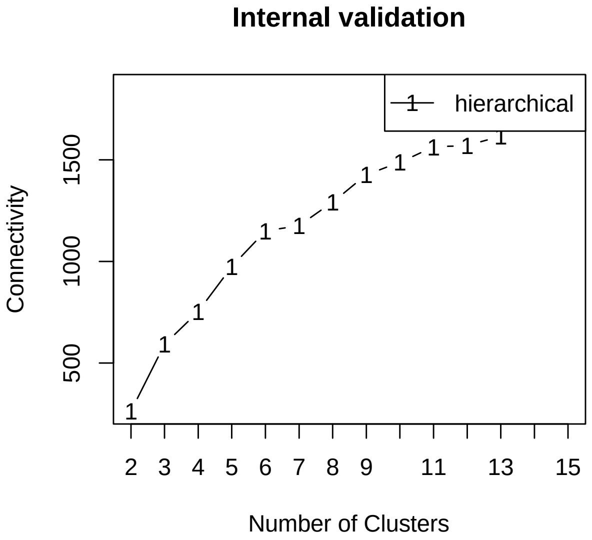


Figure S8. Two optimal groups were determined by the connectivity. The connectivity computes the degree of connectedness of a given group partitioning. The connectivity shows the connectedness of a given cluster partitioning and has a value between 0 and infinity. The user should choose a point reaching the most minimized value (y-axis).

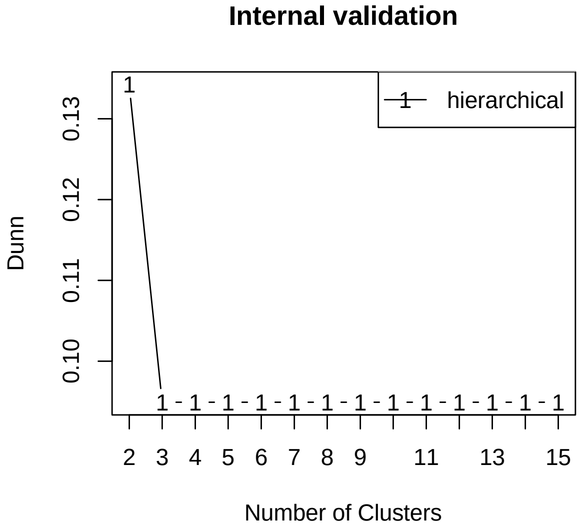


Figure S9. Two optimal groups were also determined by the Dunn's index. The Dunn's index (y-axis) has a value between zero (poorly clustered observations) and infinity (well clustered

observations), and the place where the black line of Dunn's index plot peaks at, which implies that that group number is optimal.

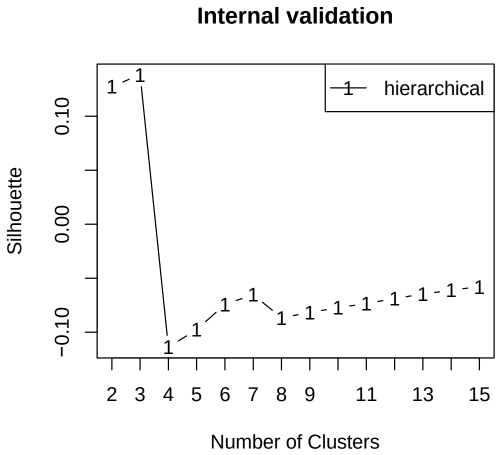


Figure S10. Three optimal groups were determined by the Silhouette width. The average Silhouette has a value between -1 (poorly clustered observations) and 1 (well clustered observations), and the place where the black line of the Silhouette plot peaks at, which implies that that group number is optimal.

The heatmap (Figure S11; *heatmap.pdf* in the working directory) shows the difference in the CNV events between the identified patient groups. If due to the large number of driver genes leading to impossibly showing gene names in rows of the heatmap, users can turn them off using the logical argument `hm_row_names` (`hm_row_names = F`).

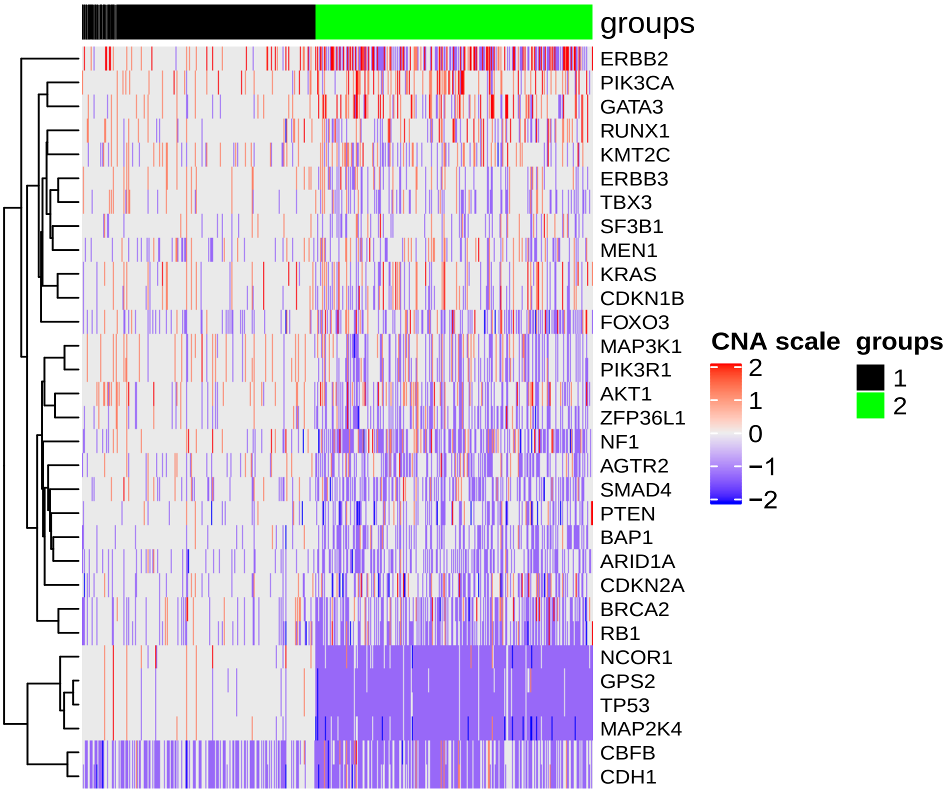
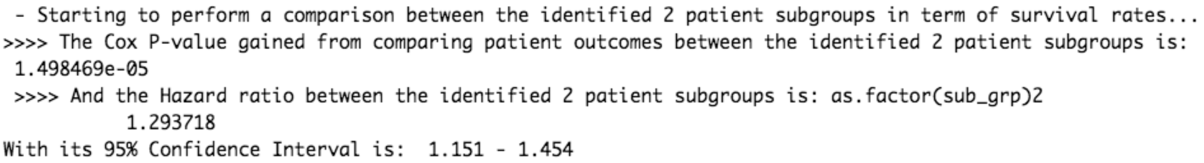


Figure S11. `ward`'s hierarchical clustering of breast cancer patients based on the 31 driver genes. Two distinct groups are found (marked as black and green). For CNA scale, Dark red, red, grey, blue and dark blue represent high-level amplification, amplification, copy-neutral, deletion and high-level deletion, respectively.

DrGA next automatically implements survival analysis between the identified subgroups, and prints Cox P-value and the HR with its 95% CI out in the R environment (Figure S12). Interpretation of the HR can be found above. As shown in Figure S12, we can say that the tumors in the second group exhibit significantly worse outcomes (HR is 1.294 with 95% CI (1.151–1.454), P-value < 0.01; group 1 is the reference).



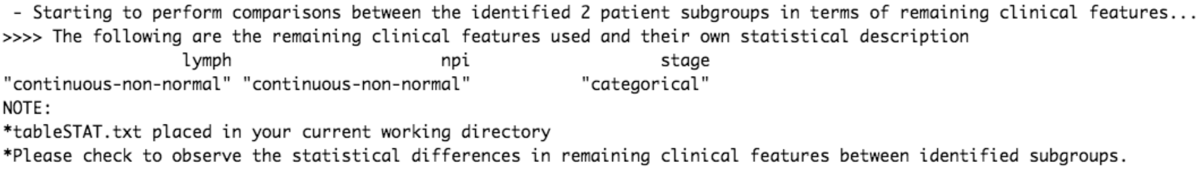
```
 - Starting to perform a comparison between the identified 2 patient subgroups in term of survival rates...
>>>> The Cox P-value gained from comparing patient outcomes between the identified 2 patient subgroups is:
 1.498469e-05
 >>>> And the Hazard ratio between the identified 2 patient subgroups is: as.factor(sub_grp)2
          1.293718
With its 95% Confidence Interval is:  1.151 - 1.454
```

Figure S12. Cox P-value and HR with 95% CI are shown in R.

At last, DrGA automatically performs comparisons between the identified subgroups in terms of the three remaining clinical features (i.e., numbers of lymph nodes, Nottingham prognostic index, and pathologic stage) to test whether there have statistically significant differences. The results are moved into the working directory as a xlsx file termed *tableSTAT.xlsx* (Table S3). Noticeably, depending on whether these clinical features are automatically defined by DrGA as continuous normal-distributed (i), continuous non-normal distributed (ii) or categorical (iii), the following descriptives and tests are performed.

- (i) is applied t-test or ANOVA
- (ii) is applied Kruskall-Wallis test
- (iii) is applied Chi-square or Fisher's exact test

For instance, DrGA tells us about the clinical features in terms of these in the R environment (Figure S13).



```
 - Starting to perform comparisons between the identified 2 patient subgroups in terms of remaining clinical features...
>>>> The following are the remaining clinical features used and their own statistical description
                 lymph                     npi                   stage
"continuous-non-normal" "continuous-non-normal"           "categorical"
NOTE:
*tableSTAT.txt placed in your current working directory
*Please check to observe the statistical differences in remaining clinical features between identified subgroups.
```

Figure S13. The statistical description of the clinical features. In this case, we understand that DrGA applies Kruskall-Wallis test to the first two variables `lymph` and `npi`, whereas Chi-square test is applied to `stage`.

	1 (N=954)	**2 (N=1002)**	**p-value**
lymph	0.00 [0.00;2.00]	0.00 [0.00;2.00]	0.031
npi	4.03 [3.03;4.11]	4.05 [3.08;5.05]	<0.001
stage:			0.016
0	8 (1.11%)	8 (0.96%)	
1	270 (37.4%)	258 (31.1%)	
2	393 (54.4%)	479 (57.7%)	
3	45 (6.23%)	81 (9.76%)	
4	6 (0.83%)	4 (0.48%)	

Table S3. Comparision between involved sub-groups in terms of chosen clinical features. For the first two continuous variables `lymph` and `npi`, median [percentiles 25%; percentiles 75%] are calculated in corresponding cells at the second and third columns. For the ordinal variable `stage`, the number of cases and the percentage of cases in each tumor stage are shown. lymph: numbers of lymph nodes, npi: Nottingham prognostic index, stage: tumor stages.

II. Mouse metabolic syndrome

1. Description of the data

The data is about female mice of a specific F2 intercross with metabolic syndrome (obesity, insulin resistance, dyslipidemia) that EXP measurements from livers. In the original paper (Ghazalpour, et al., 2006), the authors have filtered from over physiologically related 20,000 genes by retaining only the most variant and most connected probes, rendering 3600 ones.

2. Pre-processing procedures & Run DrGA

Firstly, we load the raw data including EXP and its clinical data (`exp` and `cli`)

```
#load raw file
exp = read.table("LiverFemale3600.csv", header = T, check.names = F,
sep=",")
cli = read.table("ClinicalTraits.csv", header = T, check.names = F,
sep=",", row.names = 1)
```

We remove missing genes (i.e., coded as 0) and duplicated genes due to insufficient information to retain them.

```
#remove missing gene names and duplicated genes in exp
exp = exp[which(exp$gene_symbol != "0"),]

dup = duplicated(exp$gene_symbol)
exp = exp[which(dup == FALSE),]

#turn exp1 into satisfactory format of DrGA
#DrGA requires data whose rows are samples and columns are genes.
exp = exp %>%
  dplyr::select(-c(substanceBXH, LocusLinkID, ProteomeID,
cytogeneticLoc,
            CHROMOSOME, StartPosition, EndPosition)) %>%
  tibble::remove_rownames() %>%
  tibble::column_to_rownames('gene_symbol') %>%
  drop_na() %>% t()
```

Since WGCNA is very sensitive to outliers, we find potential ones by using the hierarchical clustering method. Finally, we exclude a mouse named F2_221 (Figure S14).

```
#detect outliers
sampleTree = hclust(dist(exp), method = "average")
par(cex = 0.6);par(mar = c(0,4,2,0))
plot(sampleTree, main = "Sample clustering to detect outliers",
sub="", xlab="",
     cex.lab = 1.5,cex.axis = 1.5, cex.main = 2)

# Plot a line to show the cut
abline(h = 12.2, col = "red");
# Determine cluster under the line
clust = cutreeStatic(sampleTree, cutHeight = 12.2, minSize = 10)
table(clust)
# clust 1 contains the samples we want to keep.
```

```
keepSamples = (clust==1)
exp = exp[keepSamples, ]
```

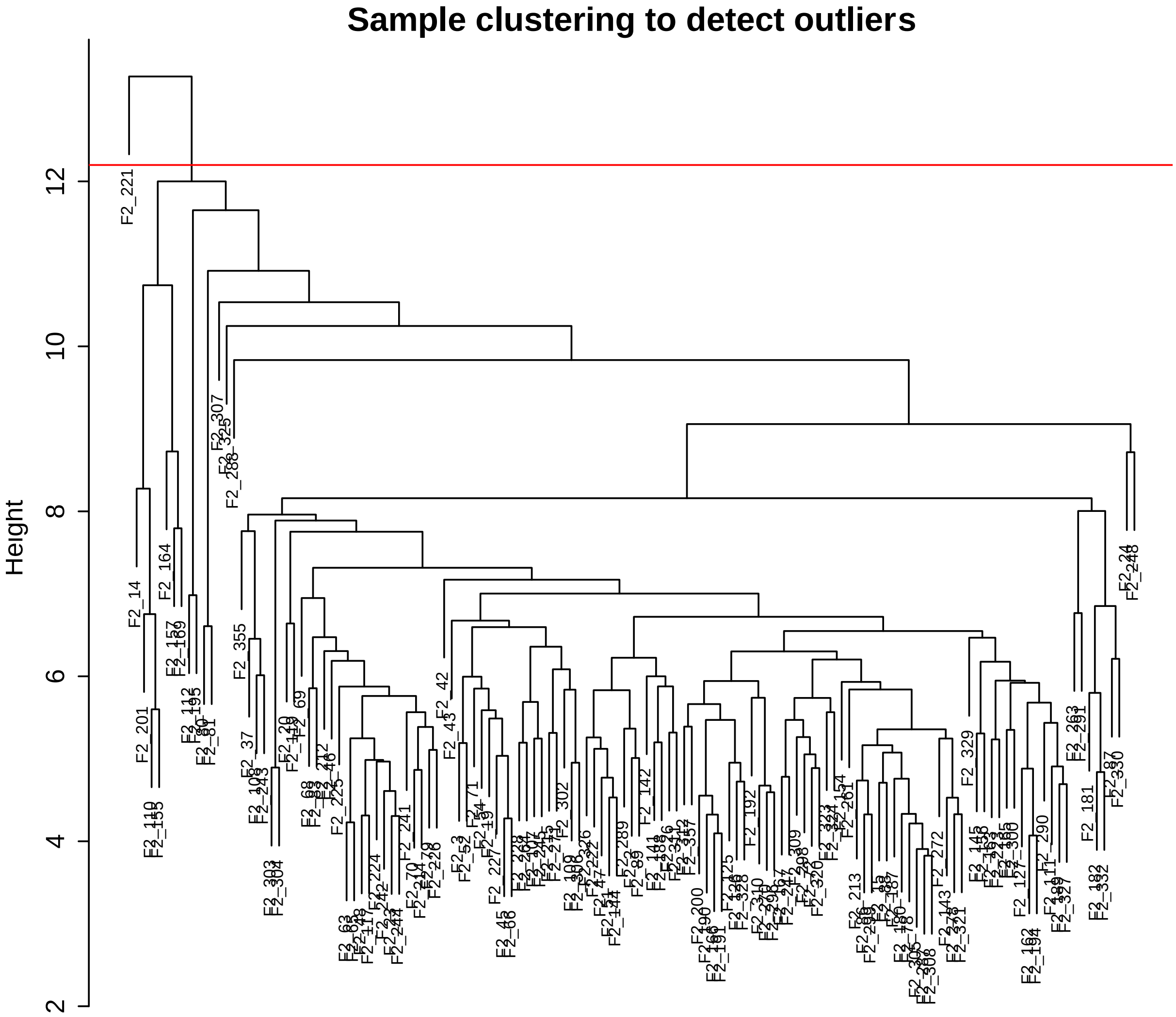


Figure S14. Detect and remove outliers.

We keep mice that share between `exp` and `cli` at their rows and in exactly the same order. We also only keep eight nescessary clinical features among 20 physiological features; i.e., body weight `weight_g`, body length `length_cm`, abdominal fat `ab_fat`, total fat `total_fat`, ulcerative colitis `UC`, free fatty acids `FFA`, glycemic index `Glucose`, two LDL and VLDL cholesterol levels `LDL_plus_VLDL` (Figure S15).

```
#match mouses that share between cli versus exp
cli = cli[cli$Mice %in% rownames(exp),]
cli = cli %>%
  remove_rownames() %>%
  tibble::column_to_rownames('Mice') %>%
  dplyr::select(-c(Number, sex, Mouse_ID, Strain, DOB, parents,
Western_Diet,
            Sac_Date, comments, Note))

#how the clinical traitsrelate to the sample dendrogram.
# Re-cluster samples
```

```
sampleTree2 = hclust(dist(exp), method = "average")
# Convert traits to a color representation: white means low, red
means high, grey means missing entry
traitColors = numbers2colors(cli, signed = FALSE);
# Plot the sample dendrogram and the colors underneath.
plotDendroAndColors(sampleTree2, traitColors,groupLabels =
names(cli),
                    main = "Sample dendrogram and trait heatmap")
#white means a low value, red a high value, and grey a missing
entry.
```

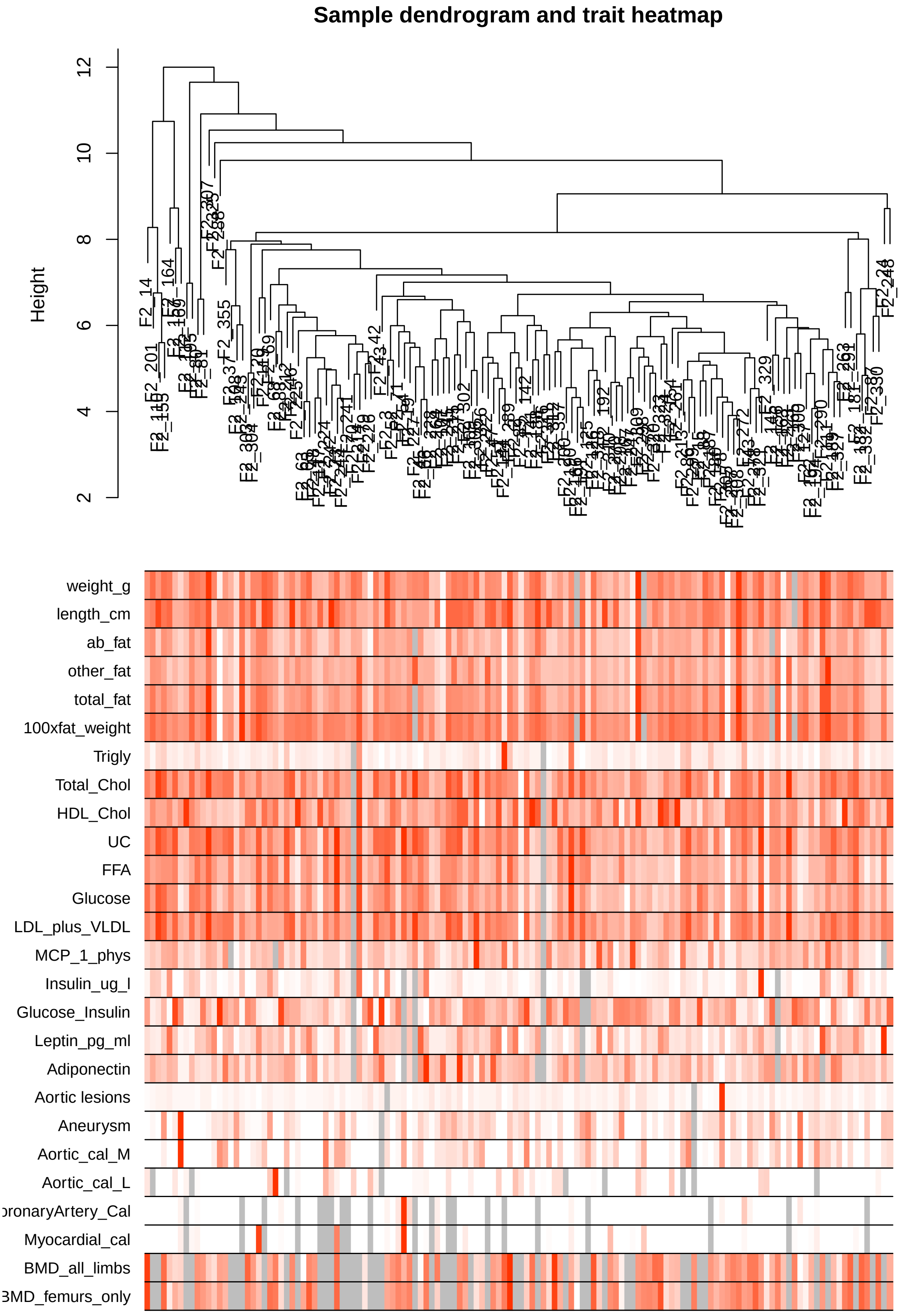


Figure S15. How the clinical features relate to the sample dendrogram. White means a low value, red a high value, and grey a missing entry

```
#Only keep several clinical features with red color
cli = cli %>%
  dplyr::select(weight_g, length_cm, ab_fat,
                total_fat, UC, FFA, Glucose,
                LDL_plus_VLDL)

#make sure that mice that share between exp and cli are included at
their rows and in exactly the same order
all(rownames(exp) == rownames(cli))
#[1] FALSE
exp = exp[rownames(cli), ]

#check dimension
dim(exp)
#[1] 134 2281
dim(cli)
#[1] 134   8

#RUN!!!!
drga = DriverGeneAnalysis(exp = exp, clinicalEXP = cli, datMODULE4 =
exp,  cliMODULE4 = cli, organism = 'mmusculus', hm_row_names = F)
```

3. Discussions about the gained results

The interpretation of all the gained results is the same. Users can find the results of this example running at https://github.com/huynguyen250896/DrGA/blob/master/data_n_code/metabolic_syndrome/output_MS.zip. Here we wish to discuss more the most interesting results compared to the results from the original paper. In the third module, we now choose the soft-thresholding value ß = 5 (Figure S16).

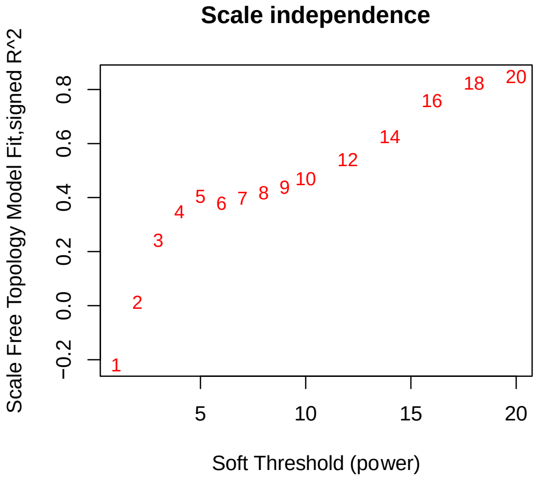


Figure S16. The graph printed out in the R environment shows the scale-free fit index (y-axis) as a function of the soft-thresholding power β (x-axis).

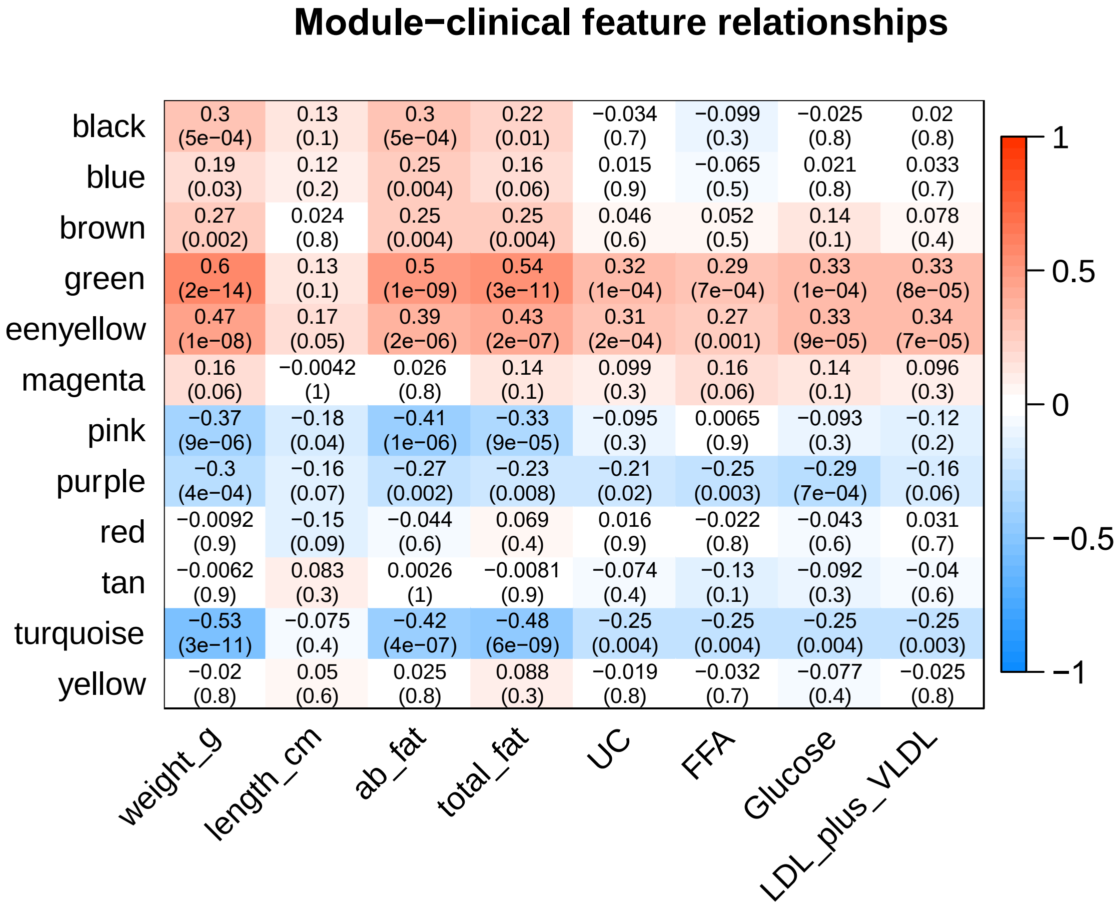
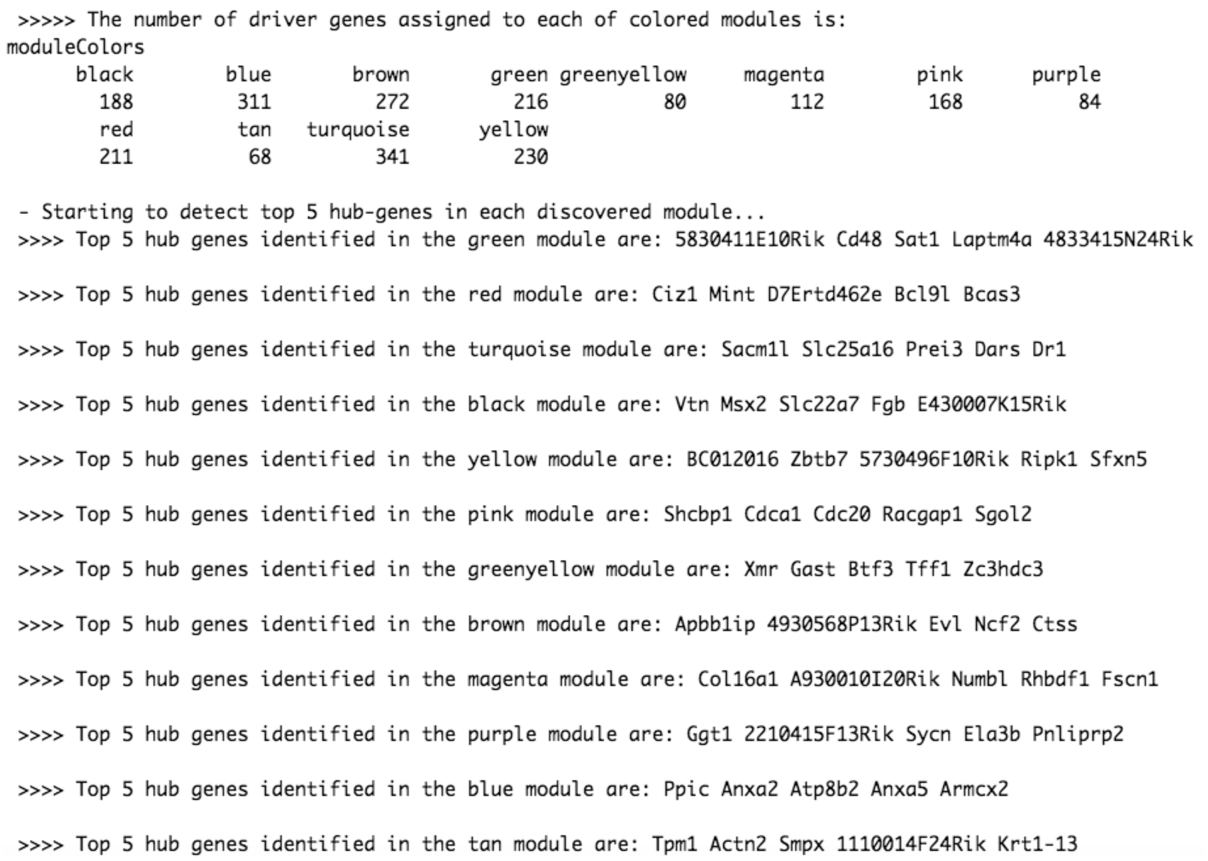


Figure S17. Module–feature associations

Based on Figure S17, DrGA discovers 12 co-expressed gene modules, consistent with the module number from the original paper, in which genes belonging to the green module are jointly expressed that result in the most positive correlation with the syndrome. The opposite is seen in the turquoise module. Correspondingly, the top five hub-genes in the green module is *5830411E10Rik*, *Cd48*, *Sat1*, *Laptm4a*, and *4833415N24Rik*, while the top five hub-genes in the turquoise module is *Sacm1l*, *Slc25a16*, *Prei3*, *Dars*, and *Dr1 (*Figure S18*).* These genes are extremely interesting since it is evident that genes with very high connectivity in lower organisms are confirmedly associated with lethal phenotypes (Carter, et al., 2004; Han, et al., 2004; Jeong, et al., 2001).



```
 >>>>> The number of driver genes assigned to each of colored modules is:
moduleColors
      black        blue       brown       green greenyellow     magenta        pink      purple 
        188         311         272         216          80         112         168          84 
        red         tan   turquoise      yellow 
        211          68         341         230 

 - Starting to detect top 5 hub-genes in each discovered module...
 >>>> Top 5 hub genes identified in the green module are: 5830411E10Rik Cd48 Sat1 Laptm4a 4833415N24Rik

 >>>> Top 5 hub genes identified in the red module are: Ciz1 Mint D7Ertd462e Bcl9l Bcas3

 >>>> Top 5 hub genes identified in the turquoise module are: Sacm1l Slc25a16 Prei3 Dars Dr1

 >>>> Top 5 hub genes identified in the black module are: Vtn Msx2 Slc22a7 Fgb E430007K15Rik

 >>>> Top 5 hub genes identified in the yellow module are: BC012016 Zbtb7 5730496F10Rik Ripk1 Sfxn5

 >>>> Top 5 hub genes identified in the pink module are: Shcbp1 Cdca1 Cdc20 Racgap1 Sgol2

 >>>> Top 5 hub genes identified in the greenyellow module are: Xmr Gast Btf3 Tff1 Zc3hdc3

 >>>> Top 5 hub genes identified in the brown module are: Apbb1ip 4930568P13Rik Evl Ncf2 Ctss

 >>>> Top 5 hub genes identified in the magenta module are: Col16a1 A930010I20Rik Numbl Rhbdf1 Fscn1

 >>>> Top 5 hub genes identified in the purple module are: Ggt1 2210415F13Rik Sycn Ela3b Pnliprp2

 >>>> Top 5 hub genes identified in the blue module are: Ppic Anxa2 Atp8b2 Anxa5 Armcx2

 >>>> Top 5 hub genes identified in the tan module are: Tpm1 Actn2 Smpx 1110014F24Rik Krt1-13
```

Figure S18. Top five hub genes identified in each module.

Moreover, DrGA tries stratifying the mice using the same directions above. As a result, the two out of the three indices indicate the two number of subgroups is optimal (Figure S19).

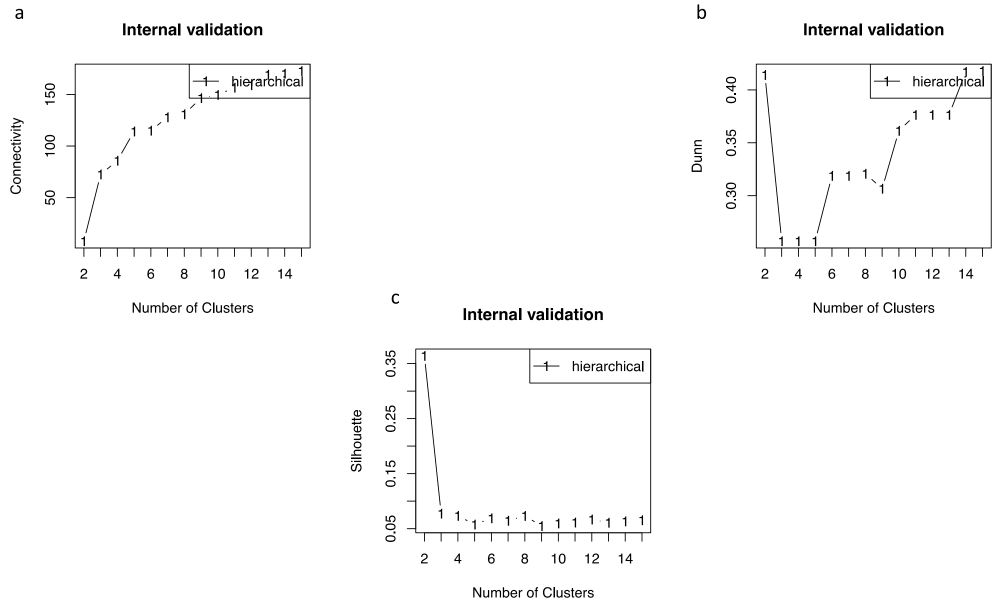


Figure S19. Identification of the optimal number of subgroups. (a) Connectivity index selects two subgroups. (b) Dunn index selects three subgroups. (c) Silhouette index selects two subgroups.

Unfortunately, we do not see any statistically significant differences in the selected clinical features between the two subgroups, possibly due to the small number of samples (Table S4). However, we still can see that mice assigned to the first subgroup have partially significantly worse traits than their counterparts in the second subgroup (higher weight, higher total fat, higher free fatty acids levels, and higher glycemic index).

	1 (N=125)	**2 (N=7)**	**p-value**
weight_g	38.2 (6.21)	36.5 (2.24)	0.110
length_cm	10.2 (0.34)	10.2 (0.36)	1.000
ab_fat	2.53 [1.74;3.20]	2.04 [1.86;2.27]	0.268
total_fat	4.91 [3.97;5.86]	3.96 [3.55;4.19]	0.059
UC	460 (122)	417 (122)	0.401
FFA	109 (29.0)	86.0 (28.7)	0.079
Glucose	432 (97.4)	375 (71.9)	0.086
LDL_plus_VLDL	1196 (315)	1103 (246)	0.371

Table S4. Comparision between involved subgroups in terms of chosen clinical features.

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