

MLEXPRESSO: differential expression and methylation analysis

Case study using RTCGA data

Aleksandra Dąbrowska, Alicja Gosiewska

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1 Introduction

The following guide presents the effect of the MLEXPRESSO package on the analysis of changes in expression and methylation of the human genome.

Scientists believe that the result of increased methylation is decreased gene expression. Recent studies suggest that the relationship between methylation and expression is more complex than we previously thought.

MLEXPRESSO is an R package for integrative analyses and visualization of gene expression and DNA methylation data.

Key functions of this package are:

- Identification of genes with affected expression,
- Identification of DMR - differentially methylated regions,
- Identification of regions with changes in expression and methylation,
- Visualization of identified regions.

For both, methylation and expression data, we conduct some statistical tests and present graphically received results. The joint modeling and visualization of genes expression and methylation improve interpretability of identified signals.

The methodology is supplemented with example applications to The Cancer Genome Atlas data.

2 Standard Workflow

In this vignette we will work with the data sets containing information about gene expression and methylation for patients with breast cancer. We will analyze differences in methylation and expression for patients

with different subtypes of BRCA cancer. To run the examples below you should install `MLEXPRESSoData` package (<https://github.com/geneticsMiNIng/MLGenSigdata>). Data sets in this R package are based on the Bioconductor package `RTCGA` (<https://bioconductor.org/packages/release/bioc/html/RTCGA.html>).

```
library(MLEXPRESSo)
library(MLEXPRESSoData)
```

2.1 Identification of genes with affected expression

2.1.1 BRCA_exp

Package `MLEXPRESSoData` contains `BRCA_exp` dataset. This set contains information about gene expression: read counts per-gene, computed for genes for 736 patients with breast cancer. Rows of this data set correspond to samples taken from patients. First column `SUBTYPE` corresponds to a subtype of BRCA cancer, next columns correspond to genes.

```
BRCA_exp[1:5, 1:5]
```

```
##                               SUBTYPE AANAT AARSD1 AATF AATK
## TCGA-A1-AOSB-01A-11R-A144-07   Normal     9   2354 2870  317
## TCGA-A1-AOSD-01A-11R-A115-07    LumA      2   1846 5656  312
## TCGA-A1-AOSE-01A-11R-A084-07    LumA     11   3391 9522  736
## TCGA-A1-AOSF-01A-11R-A144-07    LumA      0   2169 4625  169
## TCGA-A1-AOSG-01A-11R-A144-07    LumA      1   2273 3473   92
```

Before we go to the testing, we need to define condition values for each sample. We would like to test for differences between `LumA` subtype and `other` subtypes of breast cancer, so we create a vector, which each element corresponds to a sample. Our division into this two groups relies on numbers of occurrences of each subtype. The `LumA` subtype is the most common, in case of breast cancer.

```
condition_exp <- ifelse(BRCA_exp$SUBTYPE == "LumA", "LumA", "other")
head(condition_exp, 8)
```

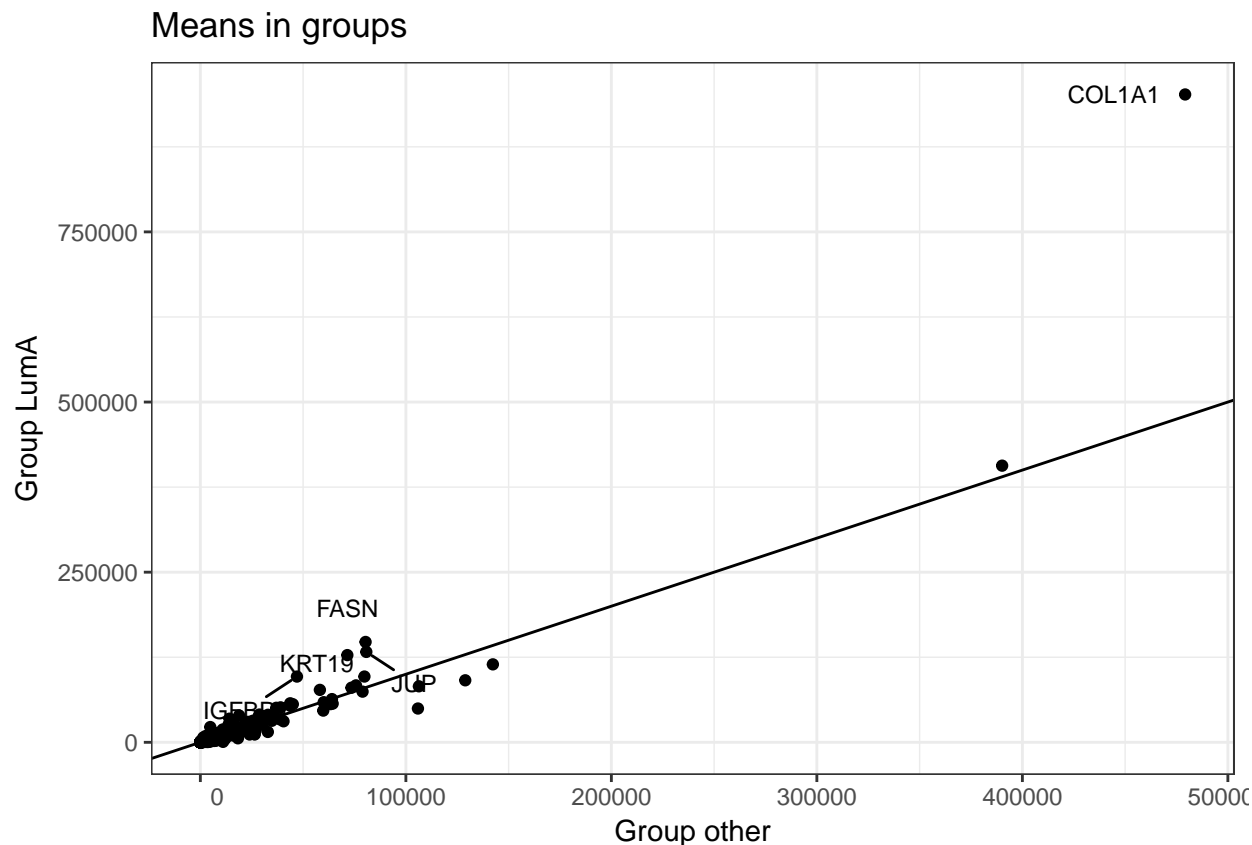
```
## [1] "other" "LumA"  "LumA"  "LumA"  "LumA"  "LumA"  "other" "LumA"
```

Now we can visualize mean expression of each gene in a division of groups `LumA` and `other`. To do this we use the `plot_means()` function.

This function requires parameters such as:

- `data` - data set with information from methylation or expression. In the example below, we use the `BRCA_exp` data without the `SUBTYPE` column.
- `condition` - a factor of levels corresponding to order of samples in data. In our example, we use the `condition_exp` vector defined earlier.
- `names` - number of genes to be labeled. On a plot are marked genes with the biggest difference between the means.

```
plot_means(
  data = BRCA_exp[,!(colnames(BRCA_exp) == "SUBTYPE")],
  condition = condition_exp,
  names = 5
)
```



2.1.2 Testing

In the `MLEXPRESSO` package we carry out the tests for identification of genes with affected expression. To do this we use the `calculate_test()` function.

This function requires parameters such as:

- **data** - object of the class appropriate for the given test. In the example below, we use the `BRCA_exp` data without the `SUBTYPE` column.
- **condition** - the factor of levels corresponding to order of samples in data. In our example, we use the `condition_exp` vector defined earlier.
- **test** - character defining test. Possible values of parameter `test` are described in the documentation of this function.

Tests are based on the methods implemented in Bioconductor packages `Deseq`, `Deseq2` and `edgeR`.

```
res_exp <- calculate_test(
  data = BRCA_exp[,!(colnames(BRCA_exp) == "SUBTYPE")],
  condition = condition_exp,
  test = "lrt"
)
head(res_exp)
```

##	id	log2.fold	pval	mean_LumA	mean_other	mean
## 1	AURKB	2.339920	3.191000e-32	539.0426	2323.8868	1485.01
## 2	CBX2	2.895062	2.834335e-26	632.5106	4296.6038	2574.48

```
## 3 KPNA2 1.447288 8.551812e-24 11547.36 26427.38 19433.77
## 4 PRR11 3.822148 2.286874e-22 396.383 3479.981 2030.69
## 5 BIRC5 1.988998 1.953941e-21 1957.085 6658.358 4448.76
## 6 GSG2 1.405039 3.527773e-21 278.2128 629.3396 464.31
```

As a result we get a data frame with columns describing the log2 of the fold change, p-value and the mean for each gene.

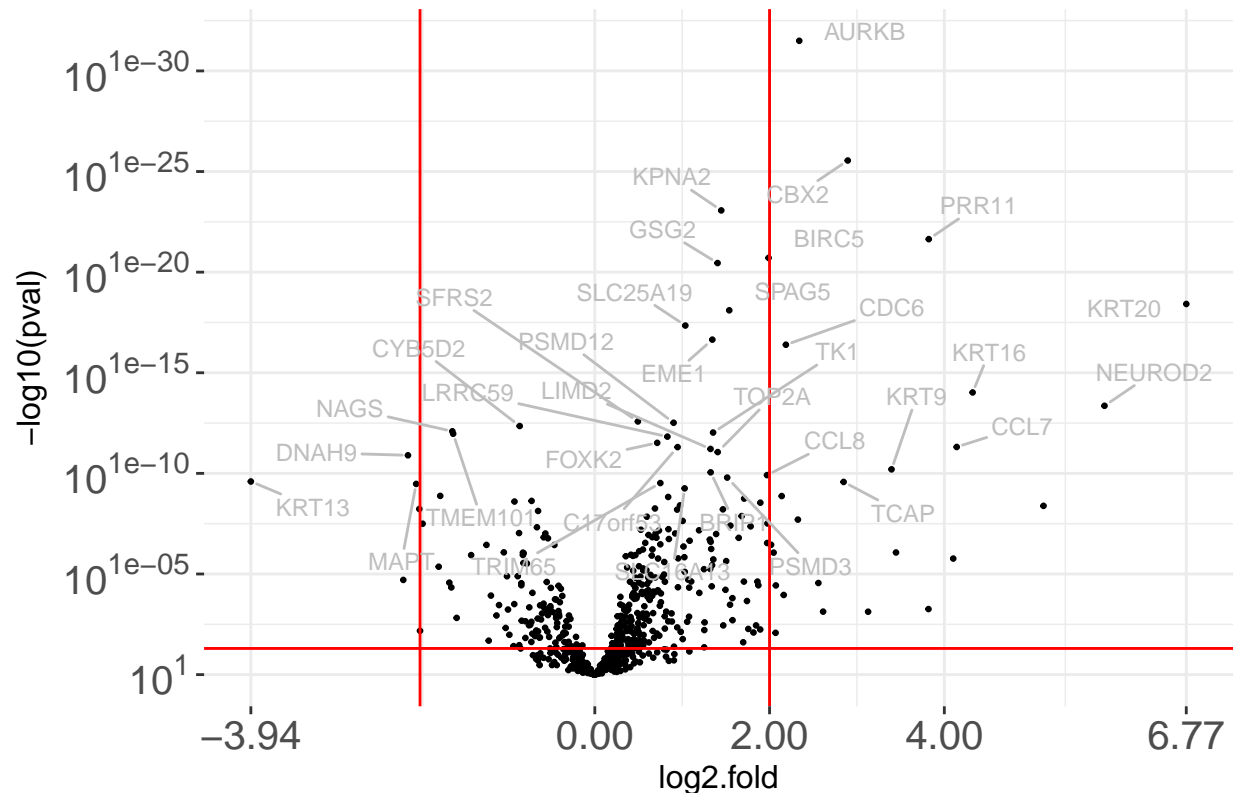
We can visualize the result of `calculate_test()` function by `plot_volcano()` function. It draws a plot with p-values and fold logarithm from methylation or expression data.

This function requires parameters such as:

- **data** - data frame containing result of chosen test from `calculate_test()` function. In the example below, we use the `res_exp` data.frame calculated earlier.
- **line** - p-value on which we draw a horizontal line.
- **names** - p-value below which we want to draw genes names.
- **fold_line** - value on which we want to draw a vertical line on both sides of zero.

More parameters were described in documentation of a `plot_volcano()` function.

```
plot_volcano(res_exp, line = 0.05, names = 0.000000001, fold_line = 2)
```



2.2 Identification of DMR - differentially methylated regions

2.2.1 BRCA_met data set

In this section, we will work with the methylation level data from TCGA database. Package `MLEXPRESSoData` contains `BRCA_met` dataset. This data set contains information about methylation of CpG probes for patients with breast cancer. Rows of this data set correspond to patients, more precisely, to samples taken from patients. First column `SUBTYPE` corresponds to a subtype of BRCA cancer, next columns correspond to CpG probes. Values inside the table indicate the percentage methylation level of CpG probe for specified sample.

```
head(BRCA_met)[1:5, 1:4]
```

```
##              SUBTYPE cg00021527 cg00031162 cg00032227
## TCGA-A1-A0SD-01A-11D-A112-05    LumA 0.03781858 0.7910348 0.006391233
## TCGA-A2-A04N-01A-11D-A112-05    LumA 0.01437552 0.7359370 0.008752293
## TCGA-A2-A04P-01A-31D-A032-05    Basal 0.01360124 0.6967802 0.009442039
## TCGA-A2-A04Q-01A-21D-A032-05    Basal 0.01525656 0.5341244 0.014674247
## TCGA-A2-A04T-01A-21D-A032-05    Basal 0.01167384 0.7378100 0.012251559
```

2.2.2 Data preparation

In this analysis we would like to find genes with different methylation. At first we need to use function `aggregate_probes()`, which generates new data frame with CpG probes aggregated to genes. To this aggregation we use, by default, the Illumina Human Methylation data set from the `TxDb.Hsapiens.UCSC.hg18.knownGene` Bioconductor package.

Function `aggregate_probes()` requires a parameter `data` - data frame containing methylation values for CpG probes.

```
BRCA_met_gen <- aggregate_probes(data = BRCA_met)
head(BRCA_met_gen)[1:5, 1:4]
```

```
##              AANAT    AARSD1    AATF    AATK
## TCGA-A1-A0SD-01A-11D-A112-05 0.7148533 0.8625816 0.24294092 0.7835302
## TCGA-A2-A04N-01A-11D-A112-05 0.5850106 0.8355825 0.21367129 0.8466190
## TCGA-A2-A04P-01A-31D-A032-05 0.4495537 0.8786166 0.03277413 0.3417919
## TCGA-A2-A04Q-01A-21D-A032-05 0.7120650 0.8819490 0.03460160 0.7264985
## TCGA-A2-A04T-01A-21D-A032-05 0.6010397 0.7739978 0.02501599 0.6276399
```

In our example we will test for differential methylation between groups with `LumA` breast cancer subtype and other subtypes of that cancer. Again we will use condition vector, which consist of two values corresponds to a subtype of breast cancer: `LumA` and `other`.

```
condition_met <- ifelse(BRCA_met$SUBTYPE == "LumA", "LumA", "other")
head(condition_met, 8)
```

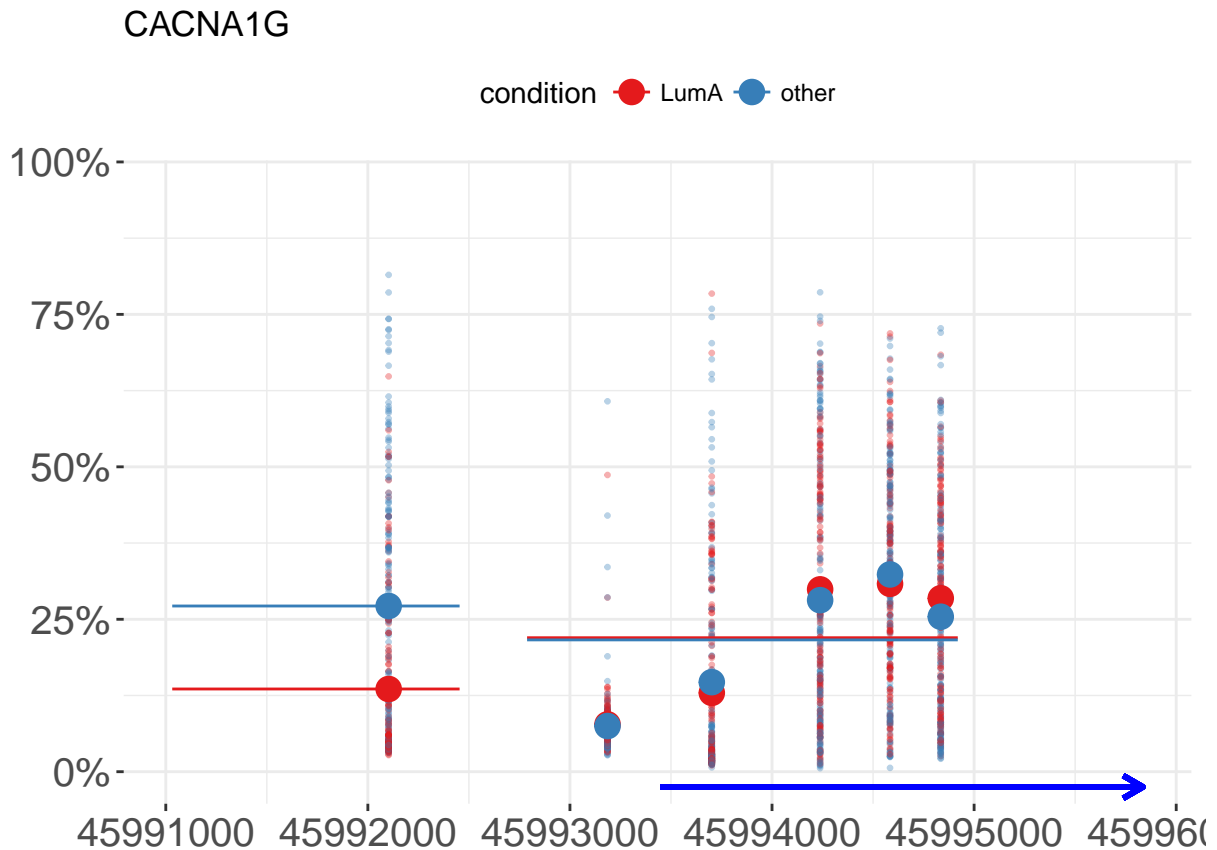
```
## [1] "LumA" "LumA" "other" "other" "other" "other" "LumA" "other"
```

Before we go to the testing, we can visualize a chosen gene with marked CpG probes. Function `plot_methylation_path` visualize placement of probes near the gene with a marked percentage of methylation for each probe in division into groups. It requires parameters such as:

- `data` - data frame containing values from methylation: columns correspond to CpG probes, rows to samples. In the example below, we use the `BRCA_met`.
- `condition` - a vector of levels corresponding to order of samples in data.
- `gene` - name of chosen gene.

- `show.gene` - logical. If TRUE arrows corresponding to gene will be drawn.
- `observ` - logical. If TRUE dots corresponding to CpG probes will be drawn.

```
plot_methylation_path(BRCA_met, condition_met, 'CACNA1G', show.gene = TRUE, observ = TRUE)
```



2.2.3 Testing

In the `MLEXPRESSO` package we carry out the tests for identification of differentially methylated regions. To do this we use the `calculate_test()` function.

This function requires parameters such as:

- `data` - object of the class appropriate for the given test. In the example below, we use the `BRCA_met_gen` methylation data aggregated to probes.
- `condition` - a factor of levels corresponding to order of samples in data. In our example, we use the `condition_met` vector defined earlier.
- `test` - character defining test. Possible values of parameter `test` are described in the documentation of this function.

Methylation tests are based on the methods implemented in packages `limma` and `MethyAnalysis`.

```
res_met <- calculate_test(
  data = BRCA_met_gen,
  condition = condition_met,
  test = "ttest"
```

```
)
head(res_met)
```

```
##      id  log2.fold      pval mean_LumA mean_other      mean
## 1  ICAM2 -0.15151320 3.754116e-17 0.2547275 0.4062407 0.3330801
## 2   RILP -0.05073691 2.575168e-13 0.3079069 0.3586438 0.3341447
## 3  PIPOX 0.11505558 5.360053e-12 0.4242804 0.3092248 0.3647812
## 4 TNFSF12 -0.13412855 5.867083e-12 0.1791401 0.3132686 0.2485025
## 5   CD7 0.09822690 1.641919e-11 0.8635077 0.7652808 0.8127112
## 6   KSR1 0.19973400 2.054467e-11 0.658270 0.458536 0.5549808
```

As a result we get a data frame with columns describing the log2 of the fold change, p-value and the mean for each gene.

2.3 Identification of regions with changes in expression and methylation

We can also create a comparison table with results of `calculate_test()` function for methylation and expression data.

Function `calculate_comparison_table()` requires parameters such as:

- `data1`, `data2` - objects of the class appropriate for the given tests.
- `condition1`, `condition2` - factors of levels corresponding to order of samples in `data1` and `data2` respectively.
- `test1`, `test2` - characters defining tests corresponding to order of samples in `data1` and `data2` respectively. Possible values of parameter `test` are described in `calculate_test()` function documentation.

```
comparison <- calculate_comparison_table(
  data1 = BRCA_exp[,!(colnames(BRCA_exp)=="SUBTYPE")],
  data2 = BRCA_met_gen,
  condition1 = condition_exp,
  condition2 = condition_met,
  test1 = "nbinom2",
  test2 = "ttest"
)
head(comparison)
```

```
##      id nbinom2.log2.fold nbinom2.pval ttest.log2.fold  ttest.pval
## 59  AURKB      2.360714 1.704243e-37 0.0017389592 2.077252e-01
## 102 CBX2      2.905397 5.402147e-31 0.0584687549 1.214043e-06
## 327 KPNA2      1.466181 3.396674e-26 0.0012105971 7.505750e-01
## 277 GSG2      1.426569 3.325659e-25 -0.0018566938 2.411495e-01
## 66  BIRC5      2.004989 9.482155e-24 -0.0005444811 5.330216e-01
## 334 KRT16      4.333332 4.102956e-19 0.0486814033 1.606151e-05
##      geom.mean.rank no.probes
## 59  1.881527e-19      2
## 102 8.098418e-19      2
## 327 1.596702e-13      1
## 277 2.831926e-13      2
## 66  2.248153e-12      1
## 334 2.567093e-12      2
```

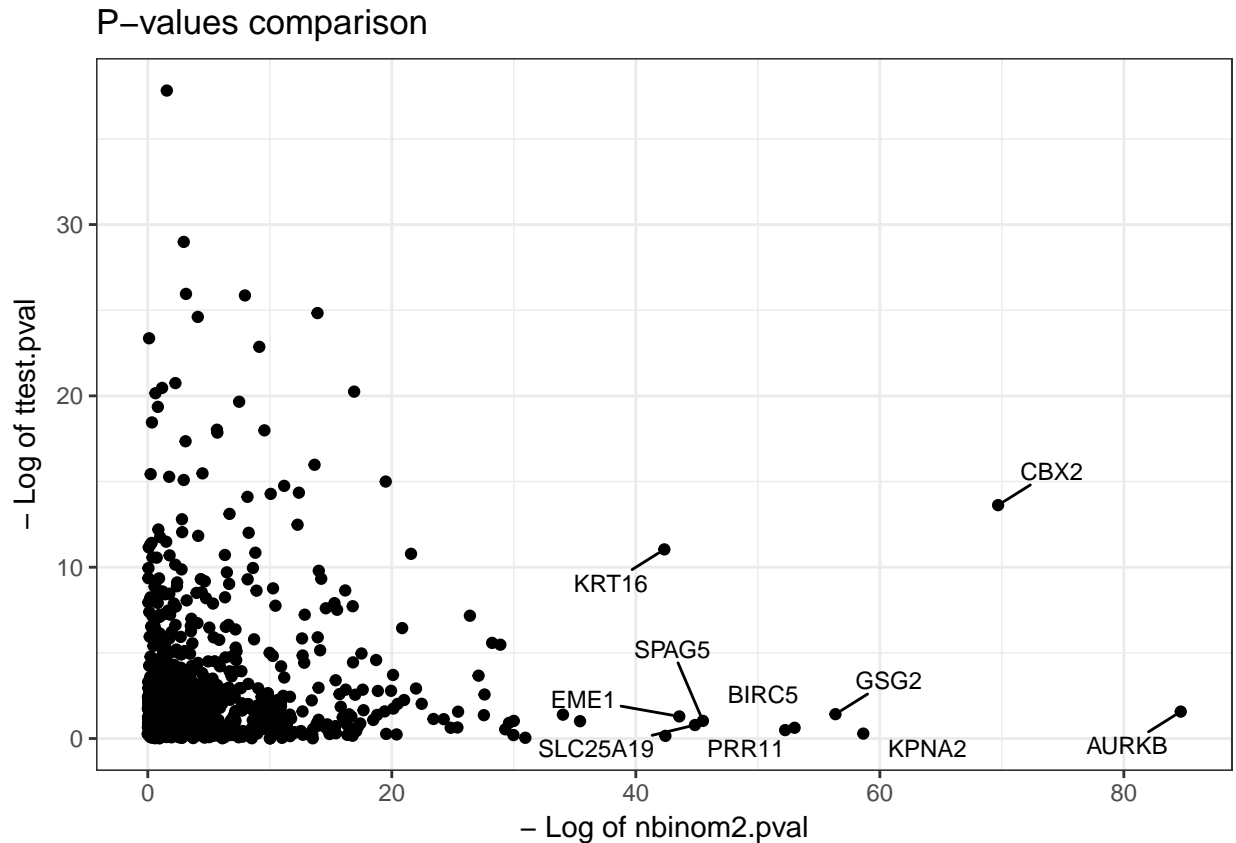
As a result, we get a data frame with columns describing the log2 of the fold change, p-value and the mean for each gene for two tests. With this two test results, we compute the ranking of the most significant changed

genes in terms of both methylation and expression. The created column contains the geometric mean of p-values for expression and methylation.

We can visualize results of `calculate_comparison_table()` by `plot_pvalues()` function. This function requires parameters such as:

- **data** - data.frame - result of `calculate_comparison_table()` function.
- **names** - number of genes to be labeled. Gens are selected based on the ranking of the most significant changed genes in terms of both methylation and expression - `geom.mean.rank` column.

```
plot_pvalues(comparison, names = 10)
```



2.4 Visualization of identified regions

The great advantage of MLExpResso package is the ability to perform a variety of visualizations for expression and methylation. All plots in our package are based on the `ggplot2` package. We use also the `scales` and `ggrepel` packages for mathematical axes and `repel` overlapping text labels.

```
test_exp <- comparison[, c("id", "nbinom2.log2.fold", "nbinom2.pval")]
test_met <- comparison[, c("id", "ttest.log2.fold", "ttest.pval")]
```

For both, methylation and expression data, we can visualise the volcano plots for results of chosen tests and simple statistics for chosen gene.

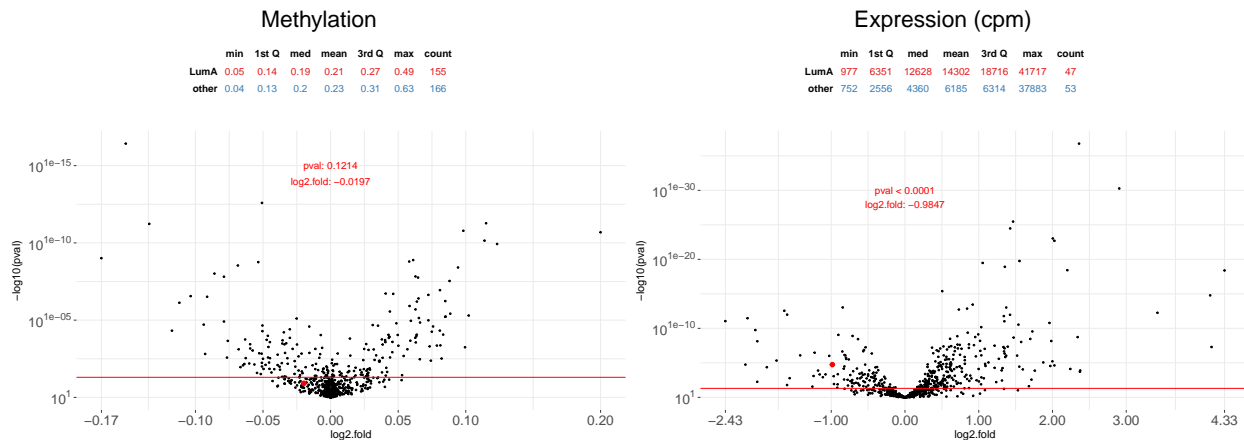
Function `plot_volcanoes()` requires parameters such that:

- **data.m**, **data.e** - data sets with information from methylation and expression respectively. In the example below, we use the `BRCA_met` and `BRCA_exp` data without the `SUBTYPE` columns.

- `condition.m`, `condition.e` - factors of levels corresponding to the order of samples in `data.m` and `data.e` respectively. In our example, we use the `condition_met` and `condition_exp` vectors defined earlier.
- `gene` - character defining the gene name. In the example, we visualize results for `CACNA1G` gene
- `test.m`, `test.e` - results from `calculate_test()` function for methylation and expression data.
- `values` - if TRUE p-values and log fold for the chosen gene will be added to the plot.

```
plot_volcanoes(
  data.m = BRCA_met[,!(colnames(BRCA_met) == "SUBTYPE")],
  data.e = BRCA_exp[,!(colnames(BRCA_exp) == "SUBTYPE")],
  condition.m = condition_met,
  condition.e = condition_exp,
  gene = "CACNA1G",
  test.m = test_met,
  test.e = test_exp,
  values=TRUE
)
```

CACNA1G



Other function `plot_gene()` allow us to visualize the **methylation path** - placement of probes near the gene with a marked percentage of methylation for each probe in division into groups.

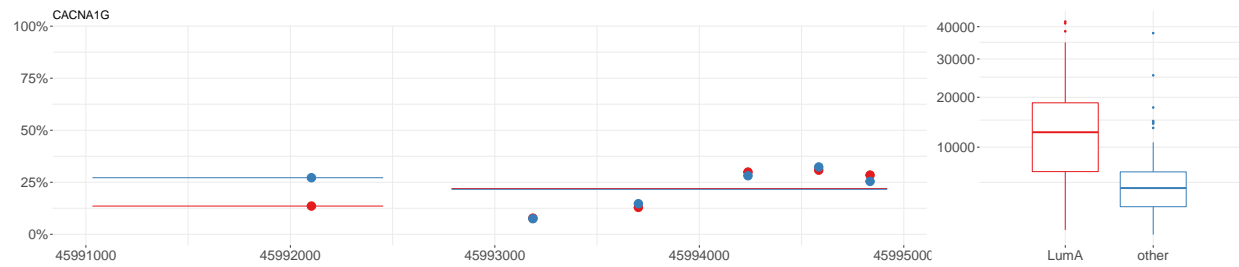
Function `plot_gene()` requires parameters such that:

- `data.m`, `data.e` - data sets with informations from methylation and expression respectively. Note that `plot_gene()` methylation requires data frame with CpG probes, not genes. In the example below, we use the `BRCA_met` and `BRCA_exp`.
- `condition.m`, `condition.e` - factors of levels corresponding to order of samples in `data.m` and `data.e` respectively. In our example, we use the `condition_met` and `condition_exp` vectors defined earlier.
- `gene` - character defining the gene name. In the example, we visualize results for `CACNA1G` gene.
- ... additional parameters. Below we use parameters `show.gene` - If TRUE line corresponding to the gene will be drawn, `observ` - If TRUE dots corresponding to CpG probes will be drawn and `islands` - If TRUE line corresponding to islands should be drawn.

```

plot_gene(
  data.m = BRCA_met,
  data.e = BRCA_exp,
  condition.m = condition_met,
  condition.e = condition_exp,
  gene = "CACNA1G",
  show.gene = TRUE,
  observ = TRUE,
  islands = TRUE
)

```



Using this function we also get boxplots containing values of expression in division from `condition_exp` vector for chosen gene.