EMA - Easy Microarray data Analysis

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1 Introduction

The increasing number of methodologies and tools currently available to analyse gene expression microarray data can be confusing for non specialist users. Based on the experience of biostatisticians of Institut Curie, we propose both a clear analysis protocol and the tools to investigate the data. It provides a useful starting point for many microarrays users.
All the functions included in the powerful and free EMA (Easy Microarray data Analysis) package were tested and chosen according to their performance and their suitability.
Most of these functions have also been improved to facilitate their use, the visualization and the interpretation of the results in the specific field of microarray data analysis.
EMA supports entire analysis process from normalisation, unsupervised and supervised analysis to functional analysis. Exploratory analyses are also proposed in the context of censored data.
Below, we present the analysis strategy used in the Institut Curie for a class comparison study. This strategy is applied to the cancer microarrays dataset (Marty *et al.* (2008)) that compares 12 Basal-like breast carcinomas (BLCs) and 11 HER2 positive breast carcinomas (HER2+). The mRNA profiles of these 23 samples were analyzed using U133 plus 2.0 Affymetrix chips that measure the expression values of 54613 probesets (after removal of AFFX probesets).
The aim is to detect differentially expressed genes between the two groups of tumors, in order to discover potential therapeutic targets in human BLCs. A brief application to simulated survival data is also provided.

2 Quality Control

Data quality control is a major concern in microarray analysis. This step aims to detect problematic raw probe-level data (array with spatial artifacts or with poor RNA quality for example) to facilitate the decision of whether to remove this array from further analysis. However, raw probe-level data artefacts can also be removed thanks to normalisation procedures (see section 3.1).
This is why we recommend to combine the results obtained by both the quality control step and the normalisation process to make a decision. For quality assessment, we recommend to use the arrayQualityMetrics package (Kauflmann *et al.* (2009)) which proposes powerful and comprehensive tools and an automatic report generation.

3 Data pre-processing

3.1 Normalisation

Microarray normalisation is a fundamental step in order to remove systematic bias and noise variability caused by technical and experimental artefacts.
MAS5.0 (Affymetrix Inc. (2002)), RMA (Irizarry *et al.* (2003)) and GCRMA (Wu *et al.* (2004)) are the most famous techniques for Affymetrix GeneChip normalisation.
We recommend the use of GCRMA method because it outperforms MAS5.0 by ignoring the MM intensities (observed to detect some specific signal) and it improves RMA by taking into account the sequence information to describe background noise.

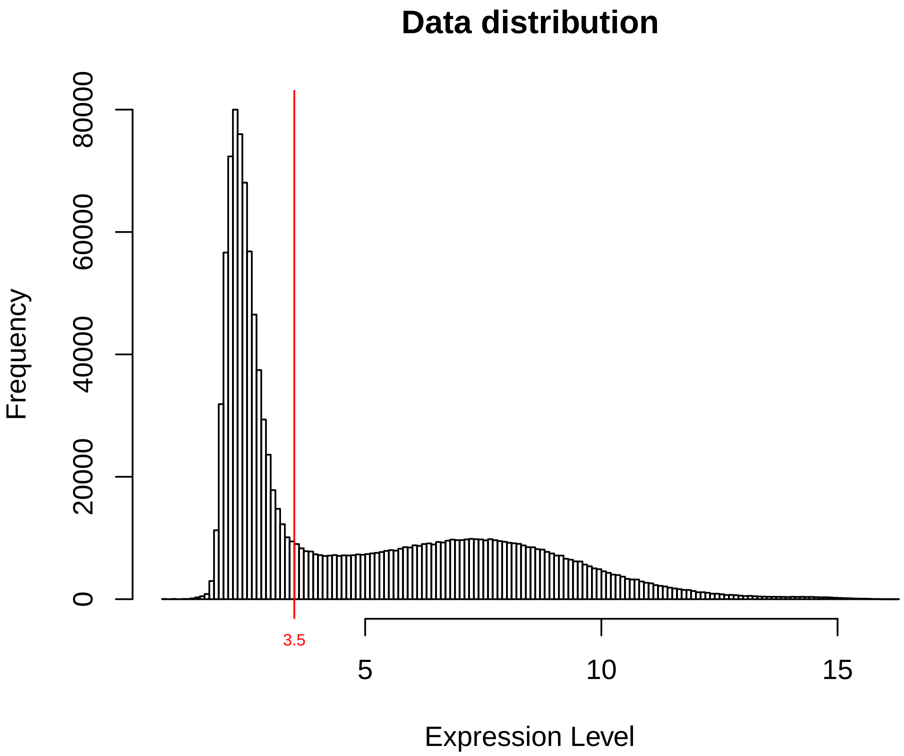
```
> ##Load EMA package
> require(ABCIS)
> ## GCRMA Normalisation
> ## Not run because cel files are not available from this package
> cel.path=paste(getwd(),"/Data/E-GEOD-13787", sep="")
> marty<-normAffy(cel.path, method="GCRMA")
```

3.2 Data filtering

The aim of the filtering step is to discard the probesets with very low expression across the samples (and that provide no biological information) in order to reduce noise in data and to avoid wrong interpretations of the final results.
In differential analysis for example, the number of hypothesis to test is therefore reduced (which is very useful in the multiple testing context).
Another problem also occurs in case of data scaling (for example in PCA) where the biological signal is completely shrinked by the probesets with very low variability.
Most of the probesets with a low variability have either a very low expression level or, on the contrary, have reached their saturation level. However, as shown in the following histogram, those with a low expression level are much more frequent. That's why by discarding the probesets with a low expression level for all the samples, and with a low variability, we expect to easily reduce the problem of noise and data scaling.

```
> ##Load GCRMA Normalised data
> ##marty.type.cl=Her2+ corresponds to Her2+ breast cancer
> ##marty.type.cl=Basal corresponds to Basal-Like carcinoma
> data(marty)
> ##And discard probesets with a maximum log2 expression value below 3.5
> marty.f<-expFilter(marty)
> dim(marty.f)
```

```
[1] 33409    23
```



By displaying a bimodal distribution, this histogram supports the choice to use the threshold value of 3.5 that divides low and high expression values.

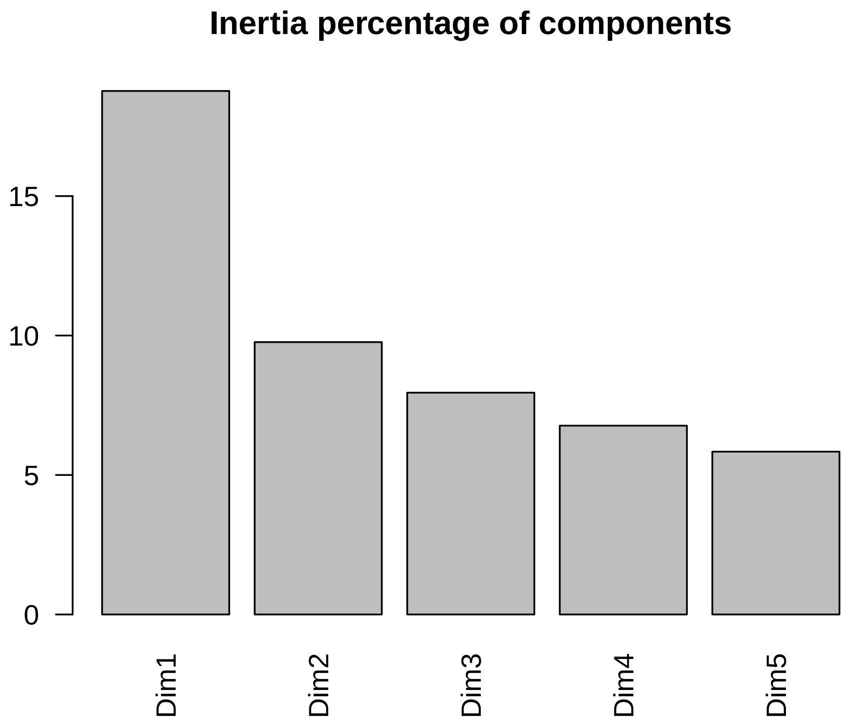
4 Exploratory Analysis

The aim of exploratory analysis is to explore the gene expression data structure. First, it is recommended to look at the distribution of arrays and/or genes to give a simple summary of the data structure. Among exploratory techniques, PCA or clustering are widely used. They make it possible to detect potential outliers and/or bias like batch effect or any non relevant effects. In addition, clusters of similar gene profiles and similar sample profiles can also be identified.

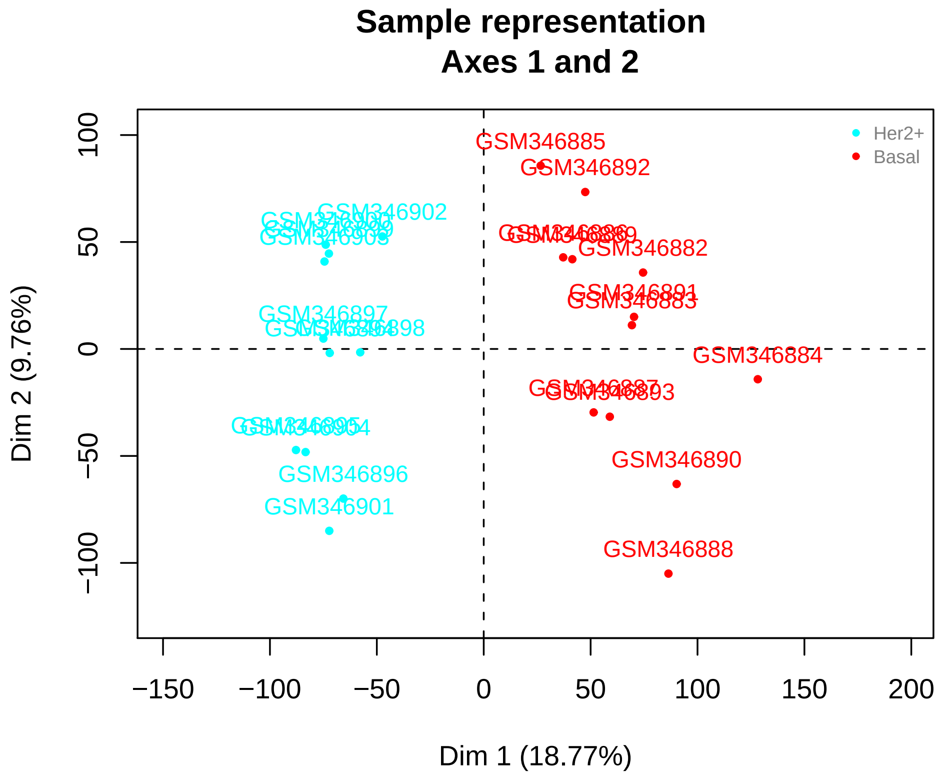
4.1 Principal Component Analysis

The principal component analysis (PCA) is a standard tool for extracting relevant information from a dataset. The goal is to reduce the dataset to a lower dimension and to reveal the underlying structure of the data.

```
> acp<-runPCA(t(marty.f), scale=FALSE,lab.sample=marty.type.cl,
+             plotSample=FALSE, plotInertia=FALSE)
> plotInertia(acp)
```



```
> ## Individual map (axe 1 and 2)
> plotSample(acp,axes=c(1,2),lab=marty.type.cl)
```

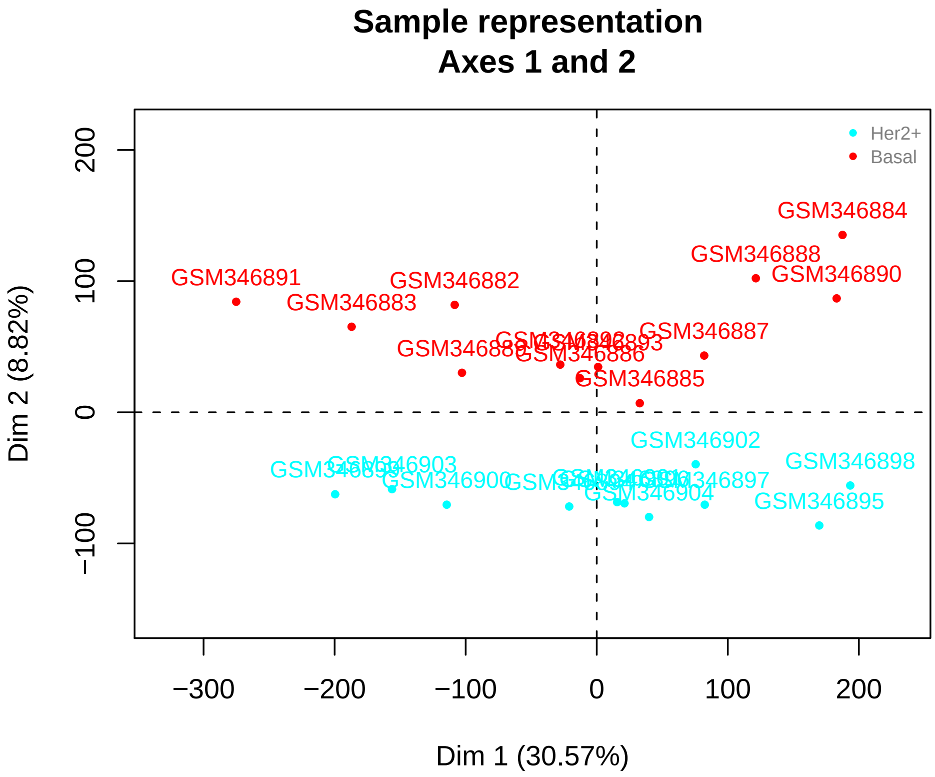


```
> ## Or create a pdf report with selected plots
> runPCA(t(marty.f), scale=FALSE, pdfname="PCA.pdf",lab.sample=marty.type.cl)
```

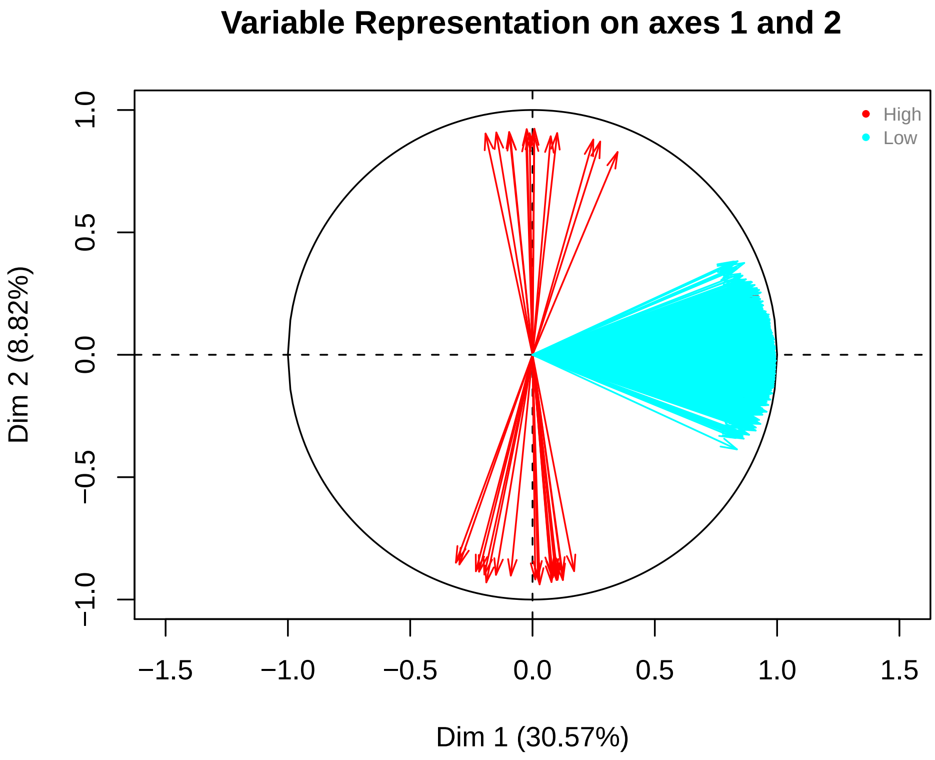
The two groups of tumors (HER2+ in red and BLCs in blue) are clearly separated by their gene expression profile.

In the following example, we show how the filtering step affects the PCA results. In the case of scaled data, the weights associated to genes with low expression accross samples become high and can lead to some wrong interpretations.

```
> ## PCA after normalisation and without filtering, but with scaling
> acp<-runPCA(t(marty), scale=TRUE, lab.sample=marty.type.cl,
+               plotSample=FALSE, plotInertia=FALSE)
> ## Individual map (axe 1 and 2)
> plotSample(acp,axes=c(1,2),lab=marty.type.cl)
```



```
> ##Gene representation (only genes the most correlated to the two first components)
> level.cl<-ifelse(apply(marty,1,max)>3.5,"High","Low")
> col.cl<-ifelse(apply(marty,1,max)>3.5,"orange","green")
> plotVariable(acp,axes=c(1,2),lim.cos2.var=0.8,lab=level.cl,label="", col.lab=col.cl)
```

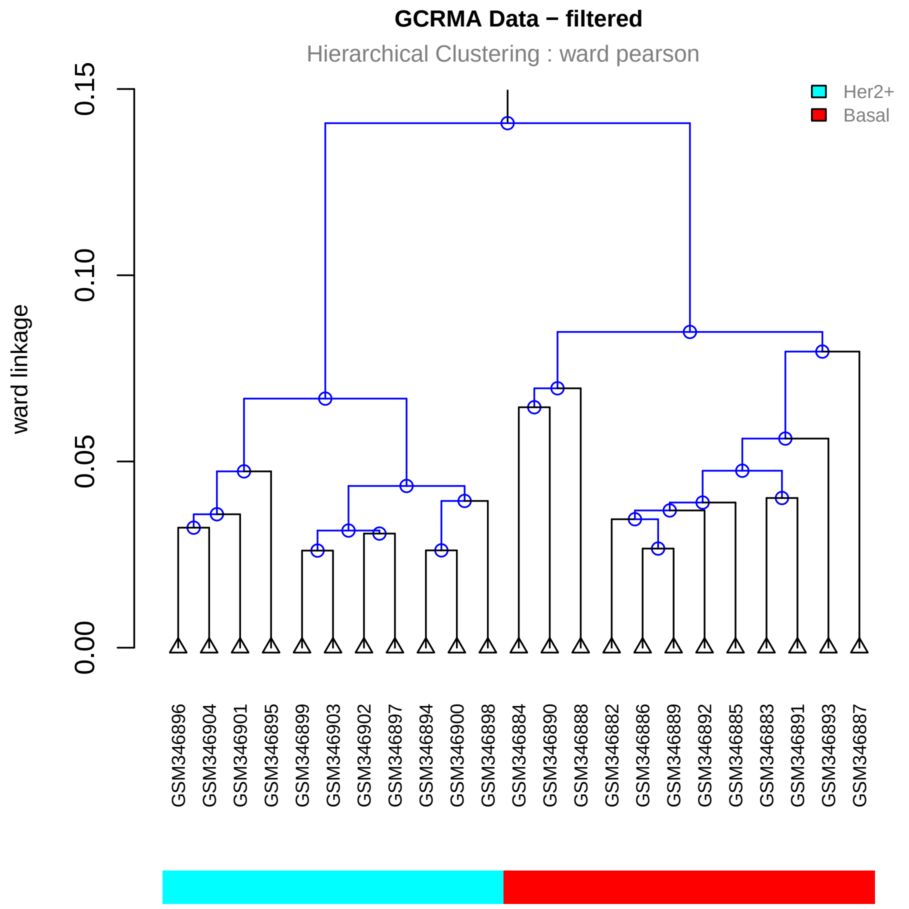


The main information provided by the data (summarized by axe 1) is not associated with the two groups of tumors. Only Axe 2 provides this information. It can be shown that Axe 1 is associated with the low expressed genes (high scaled signal level) rather than biological signal.

4.2 Agglomerative Hierarchical Clustering

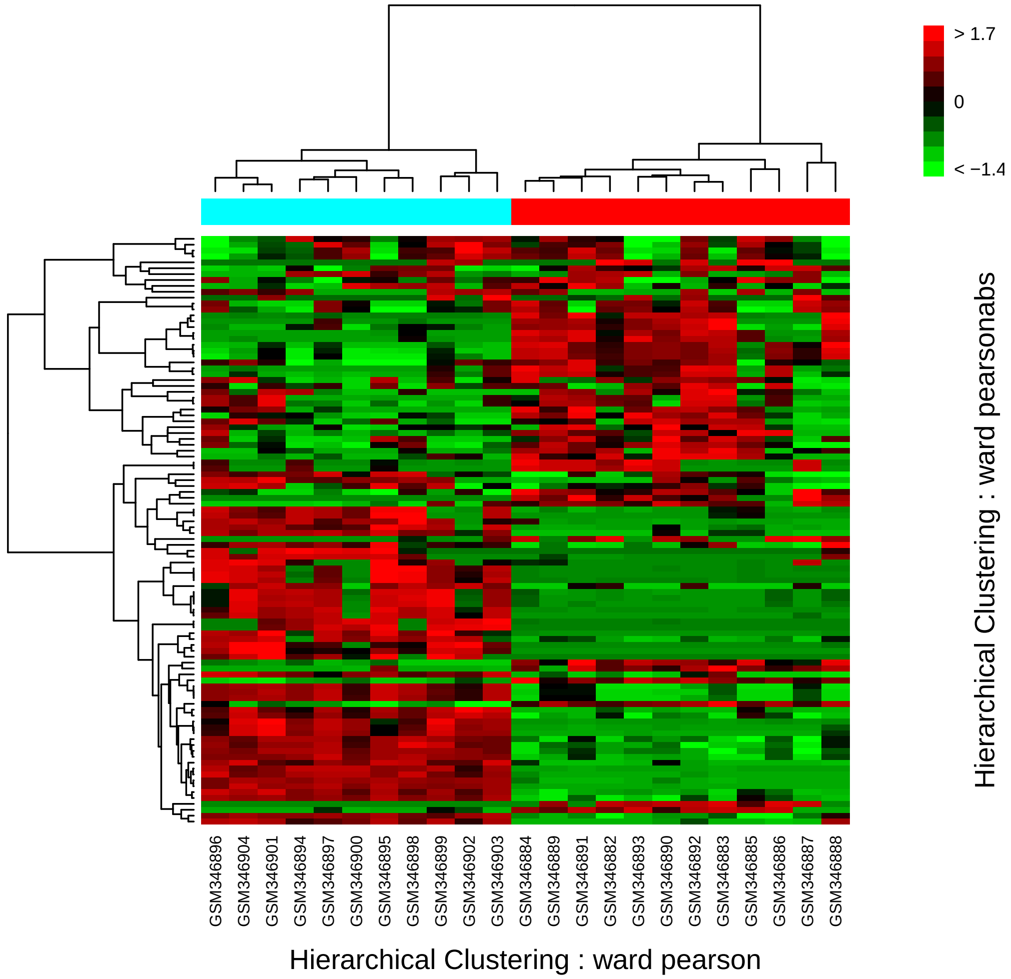
As the PCA, the agglomerative hierachical clustering is a useful method to identify clusters or to highlight experimental bias.
Clustering algorithm is based on the determination of two distances.
The first (called 'metric') defines the similarity between tow elements. The second (called 'linkage criterion') define the similarity between two clusters of elements.
The choice of these distances is very important and depends on the similarities we want to detect (for example, the clustering of genes using the Pearson correlation or the Euclidean distance can lead to very different results).

```
> ## Sample Hierarchical Clustering (Pearson's correlation coefficient and Ward method)
> c.sample<-clustering(data=marty.f, metric="pearson", method="ward")
> clustering.plot(tree=c.sample,  lab=marty.type.cl,
+                 title="GCRMA Data - filtered")
```



A heatmap is a graphical representation to visualize the level of expression of genes across the samples, and therefore allowing a better understanding of the clusters (genes and/or samples) detected. To facilitate the visualization and the interpretation of the results, we propose to perform a gene selection (keep the genes with the highest interquartile range - IQR values) before performing the heatmap.

```
> ## Heatmap performed on the 100 probesets with the highest IQR values
> mvgenes<-genes.selection(marty.f, thres.num=100)
> c.sample<-clustering(data=marty.f[mvgenes,], metric="pearson", method="ward")
> c.gene<-clustering(data=t(marty.f[mvgenes,]), metric="pearsonabs", method="ward")
> clustering.plot(tree=c.sample, tree.sup=c.gene, data=marty.f[mvgenes,],
+                  names.sup=FALSE, lab= marty.type.cl, trim.heatmap=0.99)
```



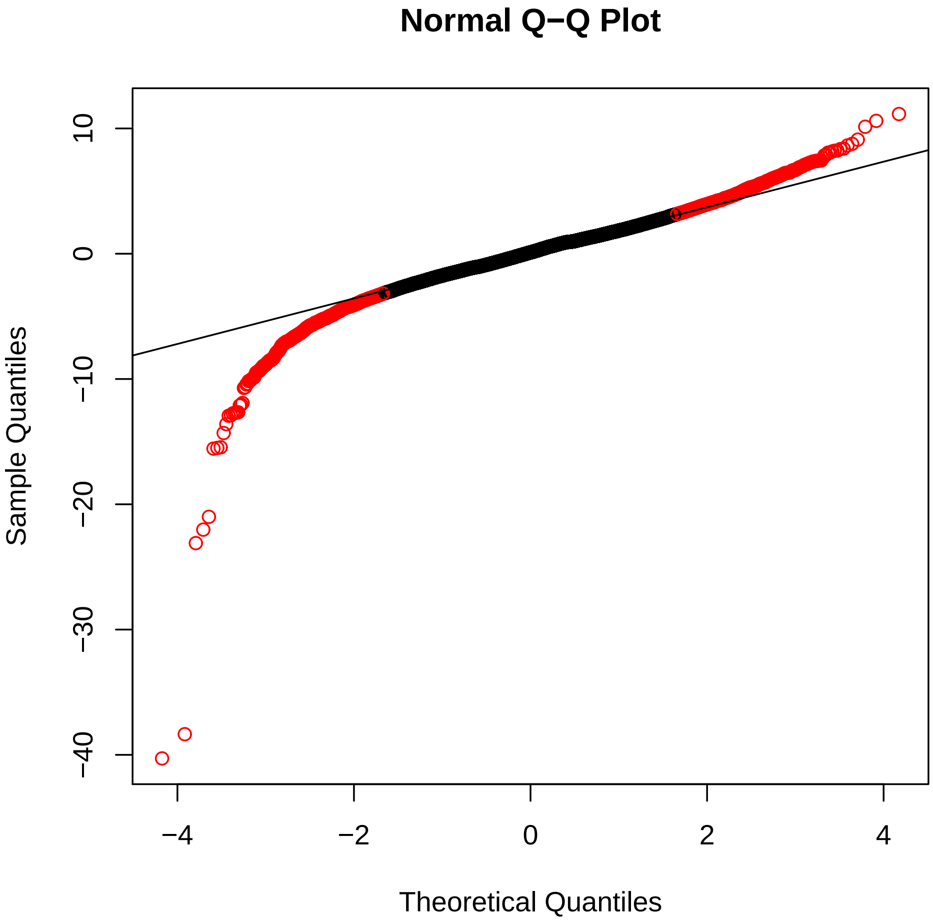
The two groups of tumors (HER2+ in red and BLCs in blue) are clearly separated by their gene expression profile, as also shown by the PCA.

5 Differential Analysis

A standard approach to detect Differentially Expressed Genes (DEG) is to test the difference between means of the two groups (with Student or Mann-Whitney tests for example) and then to adjust for multiple testing by applying the Benjamini and Hochberg (BH) procedure (Benjamini and Hochberg, 1995)

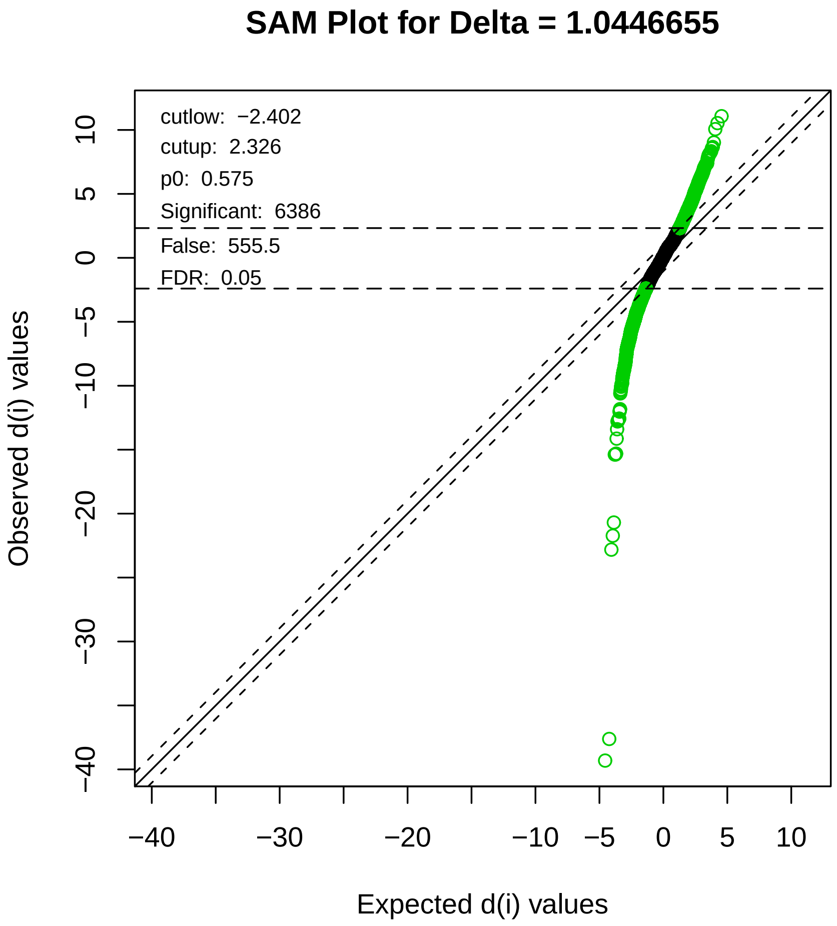
```
> ### Student test with BH correction and qqplot of genes.
> marty.type.num <- ifelse(marty.type.cl=="Her2+",0,1)
> rt<-runTtest(marty.f, labels=marty.type.num,algo="t.equalvar", q=0.05)

[1] "Launch  t.equalvar  test"
[1] "Calculate pval"
[1] "Adjusted pval"
[1] "typeFDR= FDR-BH"
[1] "3292 significant genes."

> head(rt)
```

```
   probeID        Stat  RawpValue AdjpValue
1   117_at  0.05985479 0.95283725 0.9758069
2   121_at  0.75142912 0.46073334 0.6481549
3   177_at  0.94302249 0.35639290 0.5661514
4 243_g_at -1.11306304 0.27826051 0.5100864
5 266_s_at -0.39507776 0.69676987 0.8213388
6   320_at  1.88783936 0.07294414 0.2481408
```



Detection of the DEG can also be performed by the Significance Analysis of Microarrays (SAM) algorithm (Tusher at al., 2001) (with t-test as statistic test for example). This algorithm is less stringent than the Benjamini and Hochberg procedure because it estimates the proportion of trully not differentially exressed genes.

```
> rs<-runSAM(marty.f, labels=marty.type.num)
```



```
> head(rs)

          probeID        Stat  RawpValue FoldChange Significant
117_at     117_at  0.05934444 0.95674615  1.0175331       FALSE
121_at     121_at  0.73334318 0.49933132  1.0790582       FALSE
177_at     177_at  0.93207125 0.38127259  1.2219343       FALSE
243_g_at 243_g_at -1.09205014 0.28482541  0.8654911       FALSE
266_s_at 266_s_at -0.39202276 0.71968278  0.8810822       FALSE
320_at     320_at  1.86467162 0.06180167  1.4614741       FALSE
```

6 Gene annotation

Working with probesets name is often not the easiest way to interpret the data in term of biology. Annotations, as gene symbol are often very useful to understand the results. BioMart (http://www.biomart.org/) is a very powerful tool and gives direct access to several annotations databases (Ensembl, Uniprot, etc). A R package dedicated to interface R and the BioMart databases was created by Durinck S, Huber W, and Davis S.
EMA allows to query BioMart in a easiest way, without any knowledge of the biomaRt object.

```
> ##Sort by SAM significance
> rs.sorted <- rs[order(abs(rs$Stat), decreasing=TRUE),]
> rs.sign <- rs.sorted[which(rs.sorted[,"Significant"]),]
> rs.annot<-bioMartAnnot(rs.sign, inputTypeId ="affy_hg_u133_plus_2",outputTypeId =c("entrezgen

Checking attributes ... ok
Checking filters ... ok

> head(rs.annot)

     affy_hg_u133_plus_2     probeID      Stat RawpValue  FoldChange
2197           204667_at   204667_at -39.31048         0 0.017095646
9393           237086_at   237086_at -37.62050         0 0.007155071
2184           204623_at   204623_at -22.81831         0 0.009191687
2185           204623_at   204623_at -22.81831         0 0.009191687
8653         230323_s_at 230323_s_at -21.72791         0 0.017358886
7818           226226_at   226226_at -20.69646         0 0.028651862
     Significant entrezgene hgnc_symbol
2197        TRUE         NA        <NA>
9393        TRUE       3169       FOXA1
2184        TRUE         NA        TFF3
2185        TRUE       7033        TFF3
8653        TRUE     120224     TMEM45B
7818        TRUE     120224     TMEM45B
```

Annotations are an important issu of the microarray analysis. The probes used for the design of the array relied on earlier genome and transcriptome annotation and can be different from current knowledge. The first consequence of this problem is that many conclusions derived from analyses may be significantly flawed.

Here, all the analyses were done with the Affymetrix annotation package, but the users can also used their own probe set definition for their analysis.

EMA proposes a simple function to check the signal of the 11 probes summarized in each probeset.

```
> ## Not run because cel files are not available from this package
> filenames <- list.files("Data/E-GEOD-13787", pattern=".CEL", ignore.case=TRUE)
> rawdata <- ReadAffy(filenames=filenames, celfile.path="Data/E-GEOD-13787", cdfname=NULL)
> probePlots(rawdata, pbsList=rownames(rs.annot)[1:10])
```

7 GeneSet analysis

Instead of working at the gene level, several recent studies discussed the problem of identifying differentially expressed groups of genes from a set of microarray experiments.

One of the most famous approach is the Gene Set Enrichment Analysis (GSEA) procedure of Subramanian et al. (Subramanian *et al.* (2006)). Here we decided to use the GSA package, presented in the paper as an improvement of the GSEA approach. It differs from a Gene Set Enrichment Analysis (Subramanian et al 2006) in its use of the "maxmean" statistic: this is the mean of the positive or negative part of gene scores in the gene set, whichever is larger in absolute value. Moreover, GSA does "restandardization" of the genes, in addition of the permutation of columns, to estimate the false discovery rate.

```
> ## http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html
> ## You have to register first and then download the gmt file from their site
> gsaOUT <- runGSA(marty.f, marty.type.num ,
+          gmtfile="c2.kegg.v2.5.symbols.gmt",
+          chip="hgu133plus2")
```

8 Functional enrichment assessment

The goal is to interpret the results provided by the differential analysis. One of the most classical way to answer this question, is to assess if the DEG list is enriched for biologically relevant genesets compared to a reference geneset (called 'universe', typically all the genes of the array). Functional enrichment is based on the GeneOntology and KEGG pathways annotation terms.
The hyper-geometric test of the GOstats package (Falcon *et al.* (2007)) is used to test the over-representation of the functional terms in the gene list.
The choice of the universe could have a significant impact on the results. It is well discussed in the vignette of the GOstats package. Here, we decided to apply a non-specific filtering procedure different from the one proposed by Falcon and Gentleman. Since not all genes will be expressed under all conditions in our data, we can ask the question of defining the universe only with the expressed genes or with all the genes of the array. Actually, we are not able to distinguish the genes which are biologically non expressed, from the ones of low quality. That's why we think that the non-expressed probesets could be biologically relevant, as well as the ones with a little variation accross samples, and we decided to first defined the universe with all the genes of the array. Then, we just remove probe sets that have no Entrez Gene identifier in our annotation data or no GO annotation. Finally, the Hypergeometric test is performed on the unique EntrezId of the gene list, and the unique EntrezId of the universe. The pvalues in output are not corrected from multiple testing. Note that because of the existing dependence structure (between genes, and GO terms) it is difficult to do any multiple testing correction. Moreover the most insteresting genesets are not necessarily the ones with the smallest pvalues. Nodes that are interesting are typically those with a reasonable number of genes (10 or more) and small pvalues.

```
> ## GO and KEGG analysis on the DEG by the SAM procedure
> runHyperGO(list=rownames(rs.sign), pack.annot="hgu133plus2.db", name="HyperGO_type")
> runHyperKEGG(list=rownames(rs.sign), pack.annot="hgu133plus2.db", name="HyperKEGG_type")
```

Two types of reports are generated. The first one is an HTML report (see GOStats package) which describes the significant genesets (Id, pvalue, Odd Ratio, Expected Count, Count, Size and GO/KEGG term) The second (text format) describes the description of the content of each significant genesets, with the associated genes.

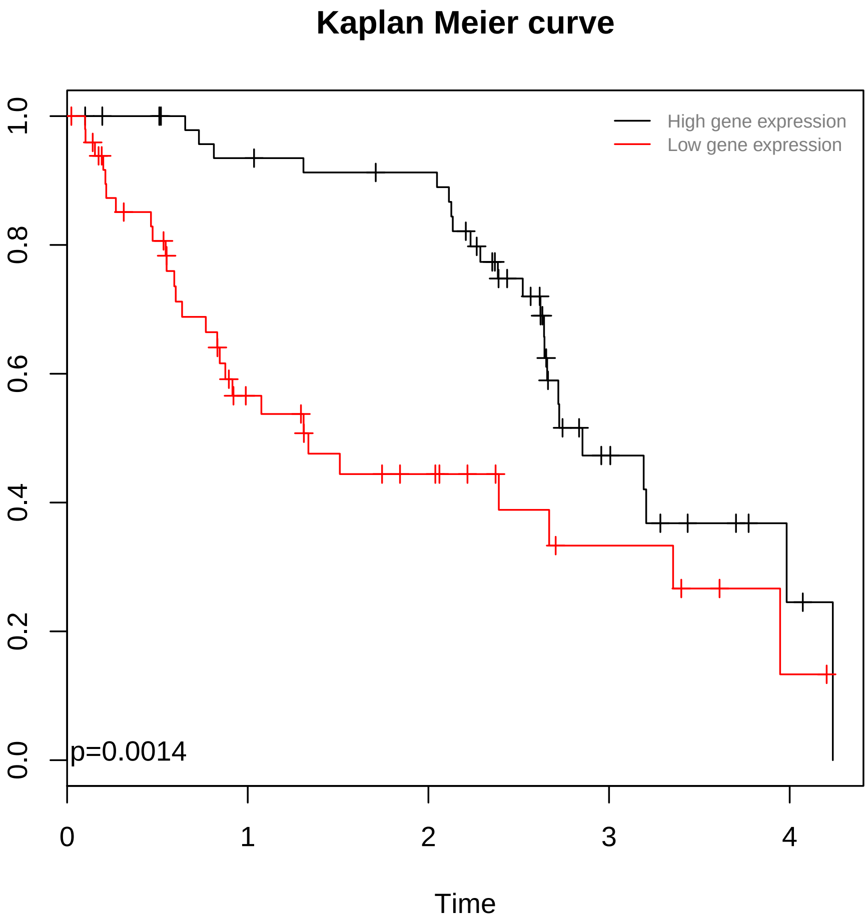
9 Supervised Classification

The classification is a prediction or learning problem from which each object is characterized by a response variable (class label) and a set of measurements (genes expression). One of the main goal of the supervised classification, especially in microarray experiments, is to find the best rule to classify/distinguish the objects.
One of the most famous application of this type of method is the gene signature published by Van't veer as a predictor for metastasis (Van't Veer *et al.* (2002)). For all these approches, we suggest to use the CMA package (Slawski *et al* (2008)) including the most popular machine learning and gene selection algorithms.

10 Survival analysis

Kaplan Meier and log-rank test analyses proposed by the EMA package helps for example to compare survival between patients with low expression value and patients with high expression value of a given gene.

```
> set.seed(5000)
> gene<-rnorm(100)
> gene[51:100]<-gene[51:100]+2
> group<-ifelse(gene<=median(gene),"Low gene expression","High gene expression")
> time<-abs(rnorm(100))
> time[51:100]<-time[51:100]+2
> status<-sample(c(0,1),size=100,replace=TRUE)
> res<-km(time,status,group,title="Kaplan Meier curve")
> res$fit.km

Call: survfit(formula = Surv(time, status) ~ group, na.action = na.omit)

                           records n.max n.start events median 0.95LCL 0.95UCL
group=High gene expression      50    50      50     23   2.85   2.643      NA
group=Low gene expression       50    50      50     27   1.34   0.845      NA

> res$lr

Call:
survdiff(formula = Surv(time, status) ~ group, na.action = na.omit)

                            N Observed Expected (O-E)^2/E (O-E)^2/V
group=High gene expression 50       23     33.4      3.22      10.2
group=Low gene expression  50       27     16.6      6.45      10.2

 Chisq= 10.2  on 1 degrees of freedom, p= 0.00142

> res$p.lr

[1] 0.001424340
```



11 Session Info

- R version 2.10.0 (2009-10-26), `x86_64-unknown-linux-gnu`
- Locale: `LC_CTYPE=fr_FR.UTF-8`, `LC_NUMERIC=C`, `LC_TIME=fr_FR.UTF-8`, `LC_COLLATE=fr_FR.UTF-8`, `LC_MONETARY=C`, `LC_MESSAGES=fr_FR.UTF-8`, `LC_PAPER=fr_FR.UTF-8`, `LC_NAME=C`, `LC_ADDRESS=C`, `LC_TELEPHONE=C`, `LC_MEASUREMENT=fr_FR.UTF-8`, `LC_IDENTIFICATION=C`
- Base packages: base, datasets, graphics, grDevices, methods, splines, stats, utils
- Other packages: ABCIS 1.0, affy 1.24.2, AnnotationDbi 1.8.1, Biobase 2.6.1, biomaRt 2.2.0, Category 2.12.0, cluster 1.12.1, DBI 0.2-4, ellipse 0.3-5, FactoMineR 1.12, gcrma 2.18.0, GOstats 2.12.0, graph 1.24.1, GSA 1.0, heatmap.plus 1.3, hgu133plus2.db 2.3.5, Hmisc 3.7-0, lattice 0.17-26, MASS 7.3-4, multtest 2.2.0, org.Hs.eg.db 2.3.6, RankProd 2.18.0, rgl 0.87, RSQLite 0.7-3, scatterplot3d 0.3-29, siggenes 1.20.0, survival 2.35-7, xtable 1.5-6
- Loaded via a namespace (and not attached): affyio 1.14.0, annotate 1.24.0, Biostrings 2.14.8, genefilter 1.28.2, GO.db 2.3.5, grid 2.10.0, GSEABase 1.8.0, IRanges 1.4.9, preprocessCore 1.8.0, RBGL 1.22.0, RCurl 1.3-0, tools 2.10.0, XML 2.6-0

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Wu,Z., Irizarry R.A., et al. (2004) A Model-Based Background Adjustment for Oligonucleotide Expression Arrays, *Journal of the American Statistical Association*, 99, 909.