

Analysis of a The Cancer Genome Atlas (TCGA) RNA-seq data set on Uterine Corpus Endometrial Carcinoma (UCEC)

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KEYWORDS The Cancer Genome Atlas; Endometrial Carcinoma; Differential Expression; Functional Enrichment

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Introduction

Endometrial cancer develops in the cells that form the inner lining of the uterus, or the endometrium, and is one of the most common cancers of the female reproductive system. In 2010, approximately 43,000 women in the United States were estimated to have been diagnosed and almost 8,000 to have died of endometrial cancer. This cancer occurs most commonly in women aged 60 years or older. About 69 percent of endometrial cancers are diagnosed at an early stage, and as a result about 83 percent of women will survive five years following the time of diagnosis.

The Cancer Genome Atlas (TCGA) ([The Cancer Genome Atlas 2016](#)) researchers have:

- Identified four subtypes of endometrial cancer: POLE ultramutated, Microsatellite instability hypermutated, Copy number low and Copy number high.
- Uncovered shared genomic features between endometrial cancer and serous ovarian cancer, the Basal-like subtype of breast cancer as well as colorectal cancer.
- Identified three histologic diagnosis: Endometrioid endometrial adenocarcinoma, Mixed serous and endometrioid and Serous endometrial adenocarcinoma

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- Characterized the marked differences between the two types of endometrial tumors (endometrioid and serous), and found that some endometrioid tumors have developed a strikingly similar pattern to serous tumors, suggesting they may benefit from a common treatment.
 - The serous and some of the endometrioid tumors are characterized by frequent mutations in TP53, extensive copy number alterations and few DNA methylation changes.
 - The rest of the endometrioid tumors are characterized by few copy number alterations, scarce mutations in TP53 and frequent mutations in PTEN and KRAS.

Materials and Methods

The Bioconductor project ([Gentleman et al. 2004](#)) is an open-source community effort to develop software packages on top of R for the analysis of molecular data obtained from high-throughput experimental technologies such as microarrays or high-throughput sequencing instruments.

Data Availability

The SummarizedExperiment ([Morgan et al. 2016](#)) class was designed to meet requirements from high-throughput sequencing experiments such as storing molecular data from multiple assays and providing more flexibility to define the profiled features.

The RNA-seq data set on Uterine Corpus Endometrial Carcinoma (UCEC) have 20115 genes and 589 samples. Associated to the row (feature) data, there are 455 sequences (1 circular) from hg38 genome.

From the S4 object, it is possible to extract information about the gender of the patients who donated the samples. As the study is focused on endometrial cancer, all the samples are from female patients (556 samples). There are also 33 'NA' samples which were considered to be discarded, but finally they have been maintained as they provide the project with some normal samples, which are not abundant in the dataset.

Quality assessment and normalization

The fact that each RNA-seq sample may have been ultimately sequenced at slightly different depth and that there may be sample-specific bias related implies it may need to consider two normalization steps:

- Between-sample: adjustments to compare a feature across samples.
 - Sample-specific normalization factors: using the TMM algorithm from the R/Bioconductor package edgeR ([Robinson et al. 2010](#)).
 - Quantile normalization: using the CQN algorithm from the R/Bioconductor package cqn ([Hansen et al. 2012](#)).
- Within-sample: adjustments to compare across features in a sample.
 - Scaling: using counts per million reads (CPM) mapped to the genome. This is already implemented in edgeR ([Robinson et al. 2010](#)) through the function `cpm()` which can take as input a `DGEList` object and can also output the CPM values in logarithmic scale.

It has been considered to discard those samples corresponding to the 10% quartile of the sample depth distribution, as the

quality of the sequentiation of these samples is poorer. After that, the filtered set has 20115 genes and 527 samples.

It is important to work with a subset which is as much representative as the initial set of samples and that contains the samples with higher quality. The paired subsetting offers the advantage that as samples are paired, the posterior analysis of batch effect identification will be performed with a perfectly balanced set, which avoids confusions for not having samples of one of the variables. However, in this dataset there are only 36 paired samples, which is a very small subset of samples.

The distribution of expression levels among samples and among genes in terms of logarithmic CPM units are checked. A cut-off of 1 \log_2 CPM unit is made as minimum value of expression to select genes being expressed across samples in order to filter out lowly-expressed genes. The dataset ends up with 11571 genes.

The normalization factors are calculated on the filtered expression data set. The Trimmed Mean of M-values (TMM) method addresses the issue of the different RNA composition of the samples by estimating a scaling factor for each library. This is implemented in the edgeR package ([Robinson et al. 2010](#)) through the function `calcNormFactors()`.

The MA-plots of the normalized expression profiles are performed. In general, there are not tumor samples with major expression-level dependent biases, although some of them show variations in low-expressed values. However, there are slightly expression-level dependent biases for some normal samples. The most suspicious cases are TCGA-AJ-A3NH, TCGA-AX-A2HC, TCGA-BK-A13C and TCGA-DI-A2QY, showing sizable dependency between M and A values.

Tissue Source Site (TSS) is used as surrogate of batch effect indicator variable. It is examined how samples group together by hierarchical clustering and multidimensional scaling by Spearman correlation, annotating the outcome of interest and the surrogate of batch indicator.

In the multidimensional plot (MDS) and the hierarchical clustering are shown that TCGA.AX.A2HC.01A and TCGA.DI.A2QY.11A samples are problematic samples as seen in the MA-plots. Therefore, both samples and its paired are removed. The dataset ends up with 32 samples.

Moreover, the sva ([Leek et al. 2016](#)) R/Bioconductor package provides a function called `ComBat()`. A better stratification of the tumor and normal samples are shown when `ComBat` is applied. `ComBat` is an empirical Bayes method robust to outliers in small sample sizes which removes batch effect.

Differential expression

We perform a simple examination of expression changes and their associated p-values using the R/Bioconductor package sva ([Leek et al. 2016](#)). Surrogate variable analysis (sva) is a technique that tries to capture sources of heterogeneity in high-throughput profiling data, such as non-biological variability introduced by batch effects. The output of SVA is an estimation of the number of so-called "surrogate variables" and their continuous values, which can be used later on to adjust for these unmeasured and unwanted effects. The SVA algorithm are used to assess the extent of differential expression this time adjusting for these surrogate variables.

After that, different types of linear regression models are built in order to assess differential expression. The conceptual purpose of a linear regression model is to represent, as accurately as possible, something complex, the data denoted by y , which is

n-dimensional, in terms of something much simpler, the model, which is p -dimensional. Thus, if the model is successful, the structure in the data should be captured in those p dimensions, leaving just random variation in the residuals which lie in an (n-p)-dimensional space. In the context of DE analysis, linear regression models can be written in matrix form, design matrices. The design matrix contains as many rows as samples and as many columns as coefficients to be estimated. The limma (Ritchie *et al.* 2015) R/Bioconductor package has been used to calculate DE analysis.

Functional enrichment

Functional enrichment analyses constitute a straightforward way to approach the question of what pathways may be differentially expressed (DE) between normal and tumor genes in our data.

The Gene Ontology (GO) database project provides a controlled vocabulary to describe gene and gene product attributes in any organism. It consists of so-called GO terms, which are pairs of term identifier (GO ID) and description. The GOSTats (Falcon and Gentleman 2007) R/Bioconductor package performs a functional enrichment analysis on the entire collection of GO gene sets. A parameter object with information specifying the gene universe, the set of DE genes and the annotation packages org.Hs.eg.db (Carlson 2016) to use are built. The functional enrichment analysis is turned by a conditional test which takes into account the hierarchical structure of GO terms.

Results and Discussion

A. Differential expression analysis

From the differential expression analysis, four different models were created in order to assess differential expression, all of them using a False Discovery Rate of 5%. The first was the simplest one, as **the linear model was fitted directly in the design matrix to every gene**. 6457 genes were differentially expressed using this approach. The second model consisted in **fitting the model adjusting for the mean-variance relationship**. This is done in order to correct the variability of RNA-seq counts from sample to sample for the same gene and different depths of different samples. The results obtained were 6441 differentially expressed genes. The third model was **adjusting the model for known covariates**, known sources of unwanted variation. The source of variation used was the *Tissue Source Site (TSS)*, obtained from the patient barcode. The results were 6491 differentially expressed genes. The fourth analysis was to complement the previous model of known covariates with a **surrogate variable analysis (SVA)**. There were 9 surrogate variables found, which were added to the design matrix of the third model. From this analysis, 7845 differentially expressed genes were found. In all the models created the chromosome with higher number of differentially expressed genes was the chromosome 1.

From the last model, a factorial design was performed in order to analyse the interaction between *type* (tumor or normal) and *TSS*, concretely with BG and BK. It was not successful, as there were not genes which were clearly differentially expressed in only one of them. For future analyses, it could be interesting to analyse the interaction between other possible sources of unwanted variation such as *histological diagnosis*, as it has been seen in several studies that there are genes clearly differentially expressed between serous and endometrioid tumors (Getz *et al.* 2013).

Table 1 Ten most significant gene ontologies found in the Functional Enrichment analysis

| GOBPID | Pvalue | OddsRatio | Excount | Count |
|--|--------|-----------|---------|-------|
| GO:0010762 | 0.0068 | Inf | 8.8567 | 13 |
| regulation of fibroblast migration | | | | |
| GO:1903543 | 0.0100 | Inf | 8.1754 | 12 |
| positive regulation of exosomal secretion | | | | |
| GO:0007213 | 0.0146 | Inf | 7.4941 | 11 |
| G-protein coupled acetylcholine receptor signaling pathway | | | | |
| GO:0032735 | 0.0146 | Inf | 7.4941 | 11 |
| positive regulation of interleukin-12 production | | | | |
| GO:0046636 | 0.0146 | Inf | 7.4941 | 11 |
| negative regulation of alpha-beta T cell activation | | | | |
| GO:0071380 | 0.0146 | Inf | 7.4941 | 11 |
| cellular response to prostaglandin E stimulus | | | | |
| GO:0010842 | 0.0215 | Inf | 6.8128 | 10 |
| retina layer formation | | | | |
| GO:0036092 | 0.0215 | Inf | 6.8128 | 10 |
| phosphatidylinositol-3-phosphate biosynthetic process | | | | |
| GO:0045986 | 0.0215 | Inf | 6.8128 | 10 |
| negative regulation of smooth muscle contraction | | | | |
| GO:0007063 | 0.0316 | Inf | 6.1316 | 9 |
| regulation of sister chromatid cohesion | | | | |

The more complete model is the last one, as it includes to an adjusted model for mean-variance relationship the adjustment to variability of known and unknown covariates. The number of differentially expressed genes increases importantly in this model with respect to the others. But as in previous steps we removed any possible sources of variation, the more variables that we put in our model, the fewer degrees of freedom it has, and thus the less statistical power that it has. Therefore, this model is the one used in the posterior Functional Enrichment analysis.

B. Functional Enrichment: The Gene Ontology analysis

In the *table X* we can see the list of the 10 most significantly differentially expressed pathways in uterine tumour tissue, taking the ordered goresults obtained from the final set of DEgenes.

Taking those 10 top GO terms, our analysis identified nine functional groups which could be, directly or indirectly, involved in endometrial cancer. One of them seems to be a functional group specific from our cancer type, while the others don't. Another one, related to the retina formation, does not seem to be biochemically related to the UCEC.

Between the eight groups that can be related to cancer we find:

- **Regulation of fibroblast migration**, which is directly re-

lated with tissue damage, and therefore, with tumors. Fibroblasts are considered to have a key role in the malignant progression of cancer and represent an important target in endometrial cancer research, as it has been demonstrated in some current articles (Subramaniam *et al.* 2013; Teng *et al.* 2016; Turner and Grose 2010).

- **Regulation of sister chromatid cohesion**, as it has been proved that aberrant sister chromatid cohesion causes instability and contributes to the development of cancer (Le Gallo *et al.* 2012). Several studies have targeted candidate chromosome instability genes in order to treat endometrial cancer (Price *et al.* 2013).
- **Positive regulation of exosomal secretion**, which is fundamental due to the increasing evidence that tumor cells release excessive amount of exosomes (Zhang *et al.* 2015).
- **Regulation of interleukin-12 production and alpha-beta T cell activation** are clearly part of the development of the immune response against the tumor (Colombo and Trinchieri 2002; Martin-Orozco *et al.* 2009), and therefore, they are differentially expressed in comparison with normal samples.
- **G-protein coupled receptor pathway or phosphatidylinositol-3-phosphate biosynthesis** are two cases belonging to signaling pathways, also affected by cancer development (Li *et al.* 2005; Wang and Dubois 2006).

There is one group identified between the top ten differentially expressed functional groups which has been directly related with endometrial cancer: **negative regulation of smooth muscle contraction**. This is because fibroids, which are benign tumours of smooth muscle, are believed to alter muscular contraction of uterus (Georgetown University Hospital 2015). However, there is not much investigation in this direction.

It is also important to remark that there are two differentially expressed genes known to have important roles in endometrial cancer which are part of two of the top ten differentially expressed functional groups. The first is PIK3C2A, which is part of the Phosphatidylinositol-3-phosphate biosynthetic process, and has an established role in the pathogenesis of serous endometrial cancer (Le Gallo *et al.* 2012). The second is CTNNB1, in regulation of sister chromatid cohesion, a gene with an unusually high frequency of mutations in endometroid tumors (52%) (Getz *et al.* 2013).

The differential expression and functional enrichment analysis have provided an interesting perspective of the endometrial cancer, remarking the most important genes and pathways which suffer changes during the disease, and raising hypotheses about how they could affect to its advance.

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Numbers

In the text, write out numbers nine or less except as part of a date, a fraction or decimal, a percentage, or a unit of measurement. Use Arabic numbers for those larger than nine, except as the first word of a sentence; however, try to avoid starting a sentence with such a number.

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Tables numbers are given in Arabic numerals. Tables should not be numbered 1A, 1B, etc., but if necessary, interior parts of the table can be labeled A, B, etc. for easy reference in the text.

Table 2 Students and their grades

| Student | Grade ^a | Rank | Notes |
|---------|--------------------|------|-------------------------------|
| Alice | 82% | 1 | Performed very well. |
| Bob | 65% | 3 | Not up to his usual standard. |
| Charlie | 73% | 2 | A good attempt. |

^a This is an example of a footnote in a table. Lowercase, superscript italic letters (a, b, c, etc.) are used by default. You can also use *, **, and *** to indicate conventional levels of statistical significance, explained below the table.

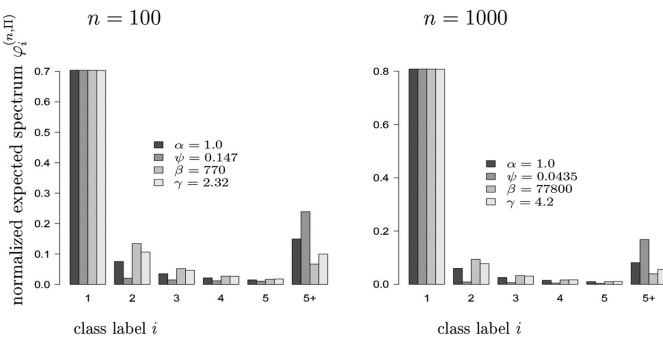


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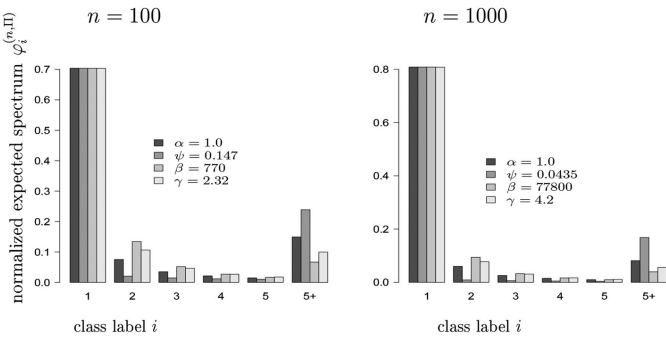


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Let X_1, X_2, \dots, X_n be a sequence of independent and identically distributed random variables with $E[X_i] = \mu$ and $\text{Var}[X_i] = \sigma^2 < \infty$, and let

$$S_n = \frac{X_1 + X_2 + \dots + X_n}{n} = \frac{1}{n} \sum_i X_i \quad (1)$$

denote their mean. Then as n approaches infinity, the random variables $\sqrt{n}(S_n - \mu)$ converge in distribution to a normal $\mathcal{N}(0, \sigma^2)$.

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