Integration of Gene Expression Data and Methylation Reveals Genetic Networks for Glioblastoma

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ABSTRACT

Motivation: The consistent amount of different types of omics data requires novel methods of analysis and data integration. In this work we describe Regression2Net, a computational approach to analyse gene expression and methylation profiles via regression analysis and network-based techniques.

Results: We identified 284 and 447 unique candidate genes potentially associated to the Glioblastoma pathology from two networks inferred from mixed genetic datasets. In-depth biological analysis of these networks reveals genes that are related to energy metabolism, cell cycle control (AATF), immune system response and several types of cancer. Importantly, we observed significant over-representation of cancer related pathways including glioma especially in the methylation network. This confirms the strong link between methylation and glioblastomas. Potential glioma suppressor genes ACCN3 and ACCN4 linked to NBPF1 neuroblastoma breakpoint family have been identified in our expression network. Numerous ABC transporter genes (ABCA1, ABCB1) present in the expression network suggest drug resistance of glioblastoma tumors.

Availability: The original glioblastoma pre-processed data can be obtained from [28]. Functional analysis source code is available at https://copy.com/KbosQMqijKdwD9Y

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1 INTRODUCTION

Glioblastomas are aggressive brain tumors affecting glial cells of the central nervous system including astrocytes and oligodendrocytes. The exact causes are not fully understood but current experimental evidence suggests that its onset is linked to mutations in gene p53, the essential cell cycle control protein and the neurofibromin 1 - NF1, inhibitor of RAS signalling pathway [30]. In oligodendrocyte tumors the key marker OLIG2 that regulates oligodendrocyte differentiation, is not expressed [29]. Additional studies confirm that the key markers of glioblastoma are related to nerve cell development BMP-BMPR/RAS-MAPK and PI3K-activated signaling pathways. The genetic inheritance component of glioblastomas is thought to be weak based on heterogeneity of genetic alternations of known disease markers amongst subjects [15]. This heterogeneity enormously complicates the development of effective therapies. Nevertheless, availability of high-throughput expression and genotype profiling technologies together with the development of novel computational data analysis approaches help with formulation of targeted biological and clinical hypotheses. As a matter of fact, such hypotheses could speed up research and improve early detection and diagnosis of glioblastomas in clinical settings.

In this work we apply penalised regression analysis and a network-based approach to a mixed dataset of gene expression profiles and methylation of individuals affected by Glioblastoma Multiforme. By comparing the topology of the inferred networks and applying functional analysis, we detect a list of genes that are potentially related to the disease of interest. Moreover, our approach is capable of discovering gene-gene interactions that could carry important information about the gene regulatory mechanisms that control the disease. Although experimental verification is needed to validate the hypotheses formulated hereby, our approach detects significant pathways that are already associated to Glioblastoma. We indicate our approach as an analysis technique that can translate data into usable knowledge.

In Section 2, we describe the method in detail. In Section 3, we provide our findings on real data of gene expression and methylation. A discussion of our findings is provided in Section 4. Conclusion and future developments are drawn in Section 5.

2 METHODS

The data at our disposal are heterogeneous datasets composed of gene expression and methylation profiles of 215 individuals affected by Glioblastoma Multiforme already considered in a study of patient similarity in [28]. DNA methylation probes span across 1491 genes and mRNA expression profiles across 12042 genes. The method we describe hereby - that we call Regression2Net - is a combination of penalised regression analysis and network theory. The main goal of Regression2Net consists in inferring the network topology of genes that are potentially associated to the disease under investigation. Not only the concept of the network makes it easier to visualise highly connected genes as potential regulators of biological activity, but also it facilitates the detection of biological pathways under which those genes operate. The fact that genes act depending on other genes and not as singletons is a well accepted hypothesis in the biology community [24, 5].

Generally speaking, a network model is composed by nodes and edges. In our specific application, each node represents a gene. Two genes are connected by an unweighted edge to indicate conditional dependence between them. If the edge between two genes does not exist, these two genes are considered to be conditionally independent. Biological networks often show sparse topology that usually resembles the structure of a power law network [3, 23]. Specifically, the distribution of the nodes degree in this type of networks follows a power law. This particular shape will ease
our inference procedure, for the reasons we explain below. The inference procedure consists in finding the best sparse set of neighbours associated to each gene. We interpret the biological meaning of genetic associations within the terms specified by regression analysis.

We leverage penalised linear regression with $L_1$-norm penalty. Given $X$, the expression of gene $i$ and the expression profiles of the remaining genes (referred to as $X$, for simplicity), the $L_1$-norm penalised estimate consists of providing a solution for Equation 1

$$\hat{\Theta}^{\alpha,\lambda} = \arg\min_{k.t. \hat{\Theta}^{\alpha,\lambda}=0} \frac{1}{n} \|X_i - X\hat{\Theta}^{\alpha,\lambda}\|_2^2 + \lambda\|\hat{\Theta}\|_1$$

The vector of regression coefficients $\Theta$ determines the conditional independence structure among predictors. One important feature of the $L_1$-norm penalty consists in the tendency to shrink many coefficients to zero and to consequently remove them from the set of predictors $X$. This is an effective way to provide sparse solutions, which in turns lowers the variance of the selected regression coefficients. Variance that is lower than the one provided by non-penalised regression approaches is usually associated to higher bias of the prediction, as explained in [25]. However, since our goal is to perform variable selection, we do not consider higher bias a harmful limitation. It would be so if we were interested in predicting the expression value of the response genes. It is known that the crucial parameter that directly determines the rate of false positives and false negatives is the shrinkage factor $\lambda$ in Equation 1. Regardless of a number of methods specifically designed to estimate $\lambda$ reported in [32, 7, 13], we perform 10-fold cross validation on a subset of the dataset, which provides an optimal estimate of the shrinkage factor.

To facilitate the explanation of our approach, we logically split the dataset, which provides an optimal estimate of the shrinkage factor. To facilitate the explanation of our approach, we logically split the entire analytic pipeline in two steps, that we describe below.

**Step 1** In order to integrate diverse datasets we perform regression analysis twice. This leads to the construction of two separate networks from a mixture of the datasets. The first network, which we indicate as $EE_{net}$ (Expression-Expression network) is the network built solely from gene expression profiles (the predictor variable is always a gene expression value). The strategy that we use has been considered in [19] and applied to synthetic data in [10]. Each gene is first treated as response and its value regressed against the remaining ones. At the end of the iterative procedure, all connections are stored within an adjacency matrix $A$ the entries of which $(A_{ij})$ are binary values 0/1 that show if gene$_i$ and gene$_j$ are connected or not.

The second network, that we call $EM_{net}$ (Expression-Methylation network), is built from a mixture of the two datasets. Specifically, the expression value of each gene is regressed against the methylated probes collected from the same individual. Each methylated probe selected by the regression procedure for the current gene is then mapped into gene space to the gene it belongs to, based on its genomic coordinates. After regressing all genes and mapping all predictors to the closest gene, another adjacency matrix is calculated similarly to the first network. Obviously, individuals with no methylation profile have not been considered to construct $EM_{net}$.

**Step 2** In the second part of the pipeline we apply network-theory concepts to select the most significant genes of the networks. A fundamental concept that needs to be clarified is the concept of importance. A simple procedure to select the most significant genes from a network is to consider its degree distribution. The degree of a gene in both $EM_{net}$ or $EE_{net}$ networks, which represents the number of connections to that gene, is a good indicator of the gene’s biological importance [2]. It has been found that highly connected genes are responsible of fundamental biological functions and are potentially involved in many biological processes [2, 5]. A procedure to select significant genes would take into consideration highly connected genes in both networks and would be driven by the degree distribution of the networks. As a matter of fact, the degree distribution completely determines the statistical properties of uncorrelated networks. However, there are cases of real networks in which the degree correlation depends on the degree itself, that is the probability that a node of degree $k$ is connected to another node of degree $k’$ depends on $k$. To better estimate such correlation it is required to compute the conditional probability $P(k’|k)$. The direct evaluation of such a probability for real world networks can be inaccurate due to the finite size $N$ of the network [4]. Moreover, if both the networks were affected by a large number of false positives, considering the degree distribution as a measure to select the most significant genes would not ameliorate the false positive rate. Finally, the arrays themselves present technical and probe-selection biases that normalisation procedures could not fully deal with. This presents challenges in obtaining non ambiguous signal values[21]. We report the degree distributions of the two derived networks in Figure 2 and Figure 3.

### 2.1 Beyond degree correlation: $AND_{net}$ and $XOR_{net}$ networks

For the reasons explained in the previous section and in order to overcome the limitations of the degree correlation between the two inferred networks, we hereby consider another strategy. We derive two more networks from the original $EE_{net}$ and $EM_{net}$.

The first network, which we call $AND_{net}$ is given by all the edges that exist in both $EE_{net}$ and $EM_{net}$. The second network, which we call $XOR_{net}$ is built by all the edges that are present in the $EM_{net}$ but are not in the $EE_{net}$.

The reason to derive such networks is due to the fact that we want to separate expression-based from methylation-based gene interactions. Two genes connected in the $EM_{net}$ but not in the $EE_{net}$ can be interpreted as being conditionally dependent according to the methylation measure exclusively.

We subsequently select all connected genes from both $AND_{net}$ and $XOR_{net}$ derived networks, and we build two lists of genes that will enter the last phase of the pipeline. In order to assess the significance of the selected genes, we perform an annotation and pathway enrichment procedures, coupled to literature evidence as explained in the sections below.

### 2.2 Annotation protocol and pathway enrichment analysis

We use R package `biomaRt` to do most of the gene annotations. The selected annotation criteria included gene full name, chromosome name, ensemble gene and transcript IDs and others (see Table S1 and S3). In case of methylation probe annotation, we use R packages `GGHumanMethCancerPanelv1.db` and `GenomicFeatures`.

The KEGG pathway enrichment analysis has been performed with the R package `KEGGpathway` [31]. From the two lists of genes derived from the two networks, we selected only those that do not overlap. The minimum threshold to accept a significant pathway has
been set to p-value <0.05, calculated via hypergeometric test that checks wether the pathway gene counts of the set of genes given as input are significant with respect to the gene counts obtained from KEGG pathways. Moreover, a Bonferroni correction has been applied to deal with multiple hypotheses.

In order to further characterise the gene sets from ANDnet and XORnet networks (see Results) and to better understand the gene regulatory mechanisms behind, we mapped 1305 methylation probes with respect to their genomic position and Coding Domain Sequence (CDS) gene regions with R package GenomicFeatures. The methylation probes are compiled by Illumina into a Golden Gate Human Methylation Cancer Panel Version 1 [17], measuring methylation levels at cancer-related CpG loci.

Based on genomic location and coordinates of the associated transcription starting site (TSS) of the gene and its coding domain sequence (CDS), each methylation probe has been classified into three categories:

- 5'UTR - occurring before the gene CDS;
- CODING - occurring within the gene CDS;
- 3'UTR - occurring after the CDS.

We take into account this additional information to better understand the common regulatory mechanisms governing the genes of the ANDnet and XORnet networks.

3 RESULTS

We identified 284 and 730 genes from the ANDnet and XORnet networks respectively. After filtering out repeated gene names, the two lists have been shrunk to 284 and 447 genes respectively. The two aforementioned lists have been given as input to an annotation procedure that we describe in the following section.

3.1 Annotation of the ANDnet and XORnet genes

In addition to the analysis of the topology of the ANDnet and XORnet networks, we perform in-depth biological analysis. The 284 and 447 genes have been annotated to biological functions and pathways in order to provide key biological context and to validate our findings in the context of the Glioblastoma pathology. Tables S1 and S3 contain the complete list of gene annotations. Results of KEGG pathway enrichment analysis are reported in Table 1.

Overall, the ANDnet network is mainly composed by genes related to energy metabolism, while the XORnet network is formed by genes related to various types of cancer, cell cycle control and immune system responses. An important Glioma pathway (KEGG:hsa05214) is enriched only in the XORnet network. Not surprisingly, the metabolic pathway in cancer (KEGG:hsa05200) is enriched in both the ANDnet and XORnet networks (Table S2 and S4). The following genes are linked to this KEGG pathway:

- AXIN1 axin 1
- FGFR fibroblast growth factor 7
- FZD9 frizzled class receptor 9
- NKX3-1 NK3 homeobox 1
- TGFBI transforming growth factor, beta 1

The relevance of some genes belonging to the aforementioned pathways is supported by literature, specifically regarding gene NKX3-1, which is known to be implicated in prostate cancer development in adult mice [1], and gene FGFR7, implicated in brain tumors [9].

For better understanding the context of gene regulation, the number of methylation probes associated to a given gene has been determined via probe mapping. The number of probes associated to a given gene ranges between 0 and 4. We report the distribution of the probe counts across all genes in Figure 1. No distinct pattern between degrees and relative number of methylation probes per gene has been found. We found strong predominance of methylation probes from the the Golden Gate Human Methylation Cancer Panel 1 [17] located in the 5’UTR regions of the genes. This and other panel biases further complicates the analysis of the regulatory mechanisms of genes belonging to the ANDnet and XORnet networks.

4 DISCUSSION

The method described thus far has been performed on a number of methylated probes the mapping of which has not been normalised. Specifically to the mapping procedure from methylation to gene space, a connection between two genes is created whenever at least one methylated probe has been selected (Step 1 of algorithm). We consider this an acceptable strategy due to the fact that the
mapped network of interest is unweighted and a different number of methylated probes (regressed as predictor variables) would not bring additional information to the analysis. Moreover, the methylated probes of the second dataset are not uniformly distributed with respect to all the 12042 genes. We show this in Figure 1 in which all the gaps indicate a missing methylated probe for the gene in the x axis. We expect a funded biological reason behind the choice to measure methylation for only 7% of the genes that appear on the microarray (12042 genes in total). We direct our question to the designers of the experiment on the Illumina array [17].

In order to make our method applicable to studies in which genome wide coverage methylated probes are available we suggest to normalise the number of methylated probes to be mapped into gene space, according to the size of the gene itself. As a matter of fact, larger genes might have more chances to get connected to the networks as they might include a higher number of methylated probes. In the case of normalisation, the threshold of acceptance can be computed from $L_s$, the length of the smallest gene coding sequence (CDS) in the dataset as $t_{acc} = \frac{L_s}{s_{mp}}$. Therefore, a larger gene would be added to EMnet if $\frac{s_{mp}}{L_{gene}} > t_{acc}$, where $s_{mp}$ is the number of selected methylated probes mapped to the gene to be added and $L_{gene}$ is the length of the protein coding region measured in bp.

4.1 Comparing network layers in the context of Glioblastoma pathology

In order to validate and to provide biological context to our EEnet and EMnet networks, we annotated the ANDnet and XORnet genes to biological functions and performed KEGG pathway enrichment analysis as described previously.

![Fig. 1. Distribution of methylation probes across the whole set of genes](image1)

![Fig. 2. Degree distribution of the ANDnet network of edges present in both EEnet and EMnet](image2)

![Fig. 3. Degree distribution of the XORnet network of edges present in EMnet but not in EEnet](image3)
Functional and pathway analysis of the 284 ANDnet genes revealed significant pathway enrichment in cancer-related genes, energy metabolism, ATP-binding membrane transporters, transcription regulation, cell cycle control proteins and other biological functions (Tables S1 and S2). Amongst these are the two Amiloride-Sensitive Brain Sodium Channels encoded by ACCN3 and ACCN4 genes (Table S1). These genes were shown to be linked to the neuroblastoma breakpoint family NBPF1 genes related to the development of Glioblastoma [26]. The NBPF1 genes are thought to be involved in brain development and the neuroblastoma onset [14].

In addition, we looked for the presence of transcription factors (TFs) amongst the genes of the ANDnet network. We identified genes AATF and ABT1. They also play an important role in the context of Glioblastoma due to the fact that gene AATF controls crucial apoptotic cell death processes and gene ABT1 is responsible for basal transcription control via interaction with class II promoter sequences and onset of schizophrenia [22, 11]. The complete annotation of the 284 unique genes of the ANDnet network is provided in Table S1.

Genes belonging to the ATP-binding cassette (ABC) are numerous in the ANDnet network. These transporter proteins are often involved in drug resistance [16]. Their strong presence amongst ANDnet genes suggests a complex gene regulatory mechanism that both involves synergetic methylation and expression components. The complex regulation of the ABC genes has been confirmed in [6].

KEGG pathway enrichment analysis performed on the genes of the ANDnet network shows that the selected network hubs are exclusively enriched in genes related to energy metabolism and ABC transporters (see Table S2) based on 5 non-overlapping pathways (existing only in the ANDnet network). This shows that these biological processes could have both expression and methylation regulatory roles [20].

An interpretation of the topology of the ANDnet network suggests that the 284 genes could be controlled by both an expression and methylation component, due to the fact that two genes are connected in both the EE and EM networks. Thus the integration of common topological regions of methylation and expression networks is justified from biological and data analysis perspectives.

Interestingly, the KEGG pathway enrichment analysis performed on genes of the ANDnet network identified 4 pathways that are common to both the ANDnet and XORnet network, including cytokine-cytokine receptor interaction, pathways in cancer, malaria and haematopoietic cell lineage pathways. This suggests that genes in these subsets display complex regulation with and without synergetic effects between expression and methylation gene regulatory components. Importantly, a total of 25 out of 284 genes of the ANDnet network and 79 out of 447 genes of the XORnet network have been matched as members of the KEGG metabolic pathway in cancer (KEGG:hsa05200).

We consider this to be a significant result as it suggests that Glioblastoma cancers seem to be strongly linked to the methylation component, which in turn perturbs the expression component [18]. This is directly reflected within the topology of the EEnet and EMnet networks.

The analysis of the 447 genes of the XORnet network (see Table S3 and S4) shows consistent presence of pathways related to cancer and biological processes including various types of carcinomas, cell signalling and immune system responses. This
supports evidence that cancers have a very strong methylation component, confirmed by many studies [8, 18]. We identified a total of 10 pathways amongst the 38 XORnet KEGG pathways linked to cancer. Numerous studies support our finding, suggesting that aberrations in methylation are strongly correlated with different types of cancer onset [18, 20].

The most interesting functional links amongst candidate genes of the XORnet network are those between genes NCAM - neural cell adhesion molecule and FGF7 - fibroblast growth factor 7. The FGF competes with NCAM for FGF receptor binding (FGFR) that results in alteration of FGFR signalling [9]. Aberration in the expression levels of gene NCAM and excessive FGFR signalling were correlated with tumor onset [12, 27]. Thus FGF family of proteins also plays an important role in neurological disorders through alteration of FGFR signalling. The transcription regulation functions of the XORnet network is represented by transcription factors FOSL2 and SIN3B (see Table S3).

4.2 Methylation and gene regulation context

Next, we looked at the genomic location of the 1305 methylation probes associated to the genes of ANDnet and XORnet networks. We checked whether there is an enrichment in relative location of methylation probes from the Golden Gate Human Methylation Cancer Panel 1 [17]. No strong correlation can be drawn with regards to the methylation component of the gene regulatory mechanism due to biases of the Golden Gate Human Methylation Cancer Panel 1, one of which is related to 807 genes linked to cancer [17]. A total of 80% of the probes are supported by ten publications related to cancer that select those genes under active research. Moreover, this panel provides coverage of only 6.7% (807/12042) of the entire gene set of Glioblastoma. A total of 65% of the panel contains probes that are in 5'UTR region. This gives rise to a non uniform distribution of probes across the aforementioned three categories (5' UTR, CODING and 3'UTR). We report the distribution of probes relative to the transcription start sites of the 807 genes in Figure 1. The heavily skewed-to-left tail clearly shows predominance of 5' UTR probes. No significant enrichment in neither of the 3 probe categories has been observed both in ANDnet and XORnet networks.

A biased methylation panel severely affects the analysis of location-dependent CpG sites as a function of gene regulation. We are confident that a more comprehensive methylation probe panel with genome-wide coverage would provide a better assessment of the gene regulatory mechanisms within the epigenetic context.

5 CONCLUSION

In this work we describe a computational method based on penalised regression and graph theory to select relevant genes associated to the Glioblastoma pathology from heterogeneous data of gene expression and methylation. We biologically validated our findings by means of annotations. We show how our method can process multi omics data by mixing sources during regression, which is performed before network inference. We address the strength of our approach in the integrative power that combines diverse and complementary information about the same observations.

Our method produces biologically relevant results that reveal a reliable correspondence between the inferred networks and evidence supported by the literature. We confirm the strong methylation component in Glioblastoma pathologies as a result of different regulatory mechanisms performed by predicted genes (in both ANDnet and XORnet).

Functional biological analysis of these genes shows enrichment in cancer-related genes, energy metabolism, ATP-binding membrane transporters and cell cycle control functions. The evidence provided by our findings strongly suggests the reliability of our approach which we consider as a framework to adopt for the analysis and integration of genetic data.

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