A systems biological approach to identify key transcription factors and their genomic neighborhoods in human sarcomas

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Abstract

Identification of genetic signatures is the main objective for many computational oncology studies. The signature usually consists of numerous genes that are differentially expressed between two clinically distinct groups of samples, such as tumor subtypes. Prospectively, many signatures have been found to generalize poorly to other datasets and, thus, have rarely been accepted into clinical use. Recognizing the limited success of traditionally generated signatures, we developed a systems biology-based framework for robust identification of key transcription factors and their genomic regulatory neighborhoods. Application of the framework to study the differences between gastrointestinal stromal tumor (GIST) and leiomyosarcoma (LMS) resulted in the identification of nine transcription factors (SRF, NKX2-5, CCDC6, LEF1, VDR, ZNF250, TRIM63, MAF, and MYC). Functional annotations of the obtained neighborhoods identified the biological processes which the key transcription factors regulate differently between the tumor types. Analyzing the differences in the expression patterns using our approach resulted in a more robust genetic signature and more biological insight into the diseases compared to a traditional genetic signature.

Key words Systems biology, transcription factor, gene regulation, binding motif, sarcoma

Introduction

Genetic diseases are attributed to changes in the expression pattern of one or more key genes. In cancer, abnormal expression of oncogenes and tumor suppressor genes is often caused by focal genetic events, including gene mutations and copy number changes, as well as epigenetic changes like altered promoter methylation[1-3]. Focal events are then followed by tumorigenic alterations in transcription programs, which further affect downstream gene expression patterns. A well known example of this trickle-down effect is the MDM2-p53 pathway. When MDM2, an inhibitor of the tumor suppressor gene p53, is deactivated by a single nucleotide polymorphism, p53 is overexpressed, resulting in aberrant expression of hundreds of downstream genes[4]. Thus, as gene expression is often controlled by hierarchical regulatory networks, experiments that measure expression patterns at steady-state levels, such as microarray, provide little insight into how gene expression is regulated.

Given the limitations of steady-state experiments, many genomic studies are conducted solely to identify genes that either are differentially expressed between two clinical groups, such as tumors with different clinical characteristics[5-10], or are correlated with clinical parameters like patient survival. The signature genes identified in these studies can be used, for example, as features in computational classifiers for discriminating patients as poor or long survivors, or as responders or non-responders to treatment. Nevertheless, although signature genes for some tumor subclasses have been successfully identified using computational methods, most signature gene lists have been found highly unstable and unreliable in prospective studies. Ill-conceived experiments, lack of clinical validation, biased selection of signature molecules, and overly positive error estimates for classifiers have been deemed downfalls of this approach in practice[10-20]. Further, global

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gene lists fail to shed light on molecular relationships and the biological difference that gene dysregulation conveys to the phenotype. An additional significant problem is that signature genes are often passive bystanders (“passenger genes”) that do not have a major driving function (“driver genes”) in the disease, such as promoting faster relapse or conveying resistance to treatment.

As a result of the shortcomings of gene-level analyses, there has been a transition towards module-level analysis of gene regulation. Several investigators propose that it is imperative to analyze smaller, coherent sets of genes instead of global signatures \([26-32]\). The coherence of a gene set can be defined without knowing the network topology by such parameters as shared biological function, molecule type or localization, such as in Gene Ontology \([33]\); or topologically, by genetic regulatory connections or other physical interactions. Gene regulatory networks include signaling pathways, metabolic pathways, and disease pathways and are made publicly available in several databases \([34,35]\). In oncology, pathways have replaced genes as primary building blocks after the observations that critical pathways, such as the Rb, WNT, PI3K/AKT, and p53 pathways \([36]\), can be activated or deactivated by varied and mutually exclusive single gene mutations. Although we emphasize the gene set approach, information on just one or few dominant genes is sometimes sufficient to provide meaningful clinical information. There are a number of one-gene oncogenic events which define certain expression patterns and, thus, cancer subtypes \([16,19,37,38]\). Indeed, using a mouse model, a single oncogenic activation, such as elevated expression of PDGFB, suffices to induce brain tumor development \([39]\).

Gastrointestinal stromal tumor (GIST) and leiomyosarcoma (LMS), two soft tissue sarcomas of the abdominal cavity, are morphologically remarkably similar, yet clinically and biologically very diverse. Clinicians primarily achieve correct diagnosis of GIST or LMS using histological examination and a number of immunohistochemical markers, including KIT, CD34, desmin, and smooth muscle actin \([40]\). Although some characteristic genetic markers of these tumors, such as gene mutations, have been known for a few years, the upstream causes and downstream effects of these signature mutations are not well understood. Of the greatest clinical relevance are gain-of-function mutations in KIT or PDGFRα genes in GIST. These mutations allow treatment with targeted therapy in patients with GIST but not LMS. Conversely, LMS is effectively treated with chemotherapy, whereas the objective response rate for GIST is negligible with this regimen \([41-44]\). To complement immunohistochemical, computational classifiers, such as the OBSCN/PRUNE2 gene classifier \([45]\), have been previously devised for these tumors. However, like the immunohistochemical markers, the features in this genetic classifier have not been interpreted biologically, mainly due to the very limited knowledge of the marker genes. Although these malignancies are now recognized as two different tumor types as supported by our recent genomic characterization studies \([46,47]\), they also share many common characteristics, like morphology and anatomical sites.

The unique historical and clinical relationship between GIST and LMS makes it interesting to compare the differences in their expression patterns. In this study, we developed a systems biological approach for identifying the genomic difference between tumors in more detail than can be attained with a straightforward list of signature genes. We applied this approach to investigate the key transcriptional regulators and their genomic neighborhoods that may cause the clinical differences between the two tumor types. The distinctive gene regulatory information that the results describe should prove more robust and biologically relevant than a signature gene list.

### Materials and Methods

**Gene expression measurements of GIST and LMS**

We acquired 68 surgical specimens of primary tumors at the University of Texas MD Anderson Cancer Center under an Institutional Review Board-approved protocol with patient consent. Of these tumors, 37 were classified as GIST and 31 as LMS based on both clinicopathologic evaluation and molecular marker studies. Specifically, clinicopathologic observations included the site of the primary tumor, the pattern of metastatic spreading, and the efficacy of systemic therapy; molecular marker studies included immunostaining for KIT, CD34, desmin, and smooth muscle actin. All specimens were snap-frozen within 20 min of surgical resection and were verified by histopathologic examination to be composed of a minimum of 90% neoplastic cells. The gene expression profiles of these samples were measured with whole human genome oligo arrays with 44 000 60-mer probes (Agilent Technologies, Palo Alto, CA, USA) according to manufacturer’s protocol. Arrays were scanned with Agilent’s dual laser-based scanner and intensity values were read and processed with Agilent’s Feature Extraction software version 8.0 with default parameters. The intensity values were quantile normalized in Matlab version R2009b (The MathWorks, Natick, MA, USA). All the data analysis was implemented in Matlab. The gene
expression data are publicly available at http://www.cs.tut.fi/sgn/csb/GISTLMS/.

Finding master regulators

To gain confidence towards the generalization properties of the identified master regulators, we created 100 resampled sets of data, each excluding a randomly chosen 15% of the total samples. We computed the differentially expressed genes for each of the sample sets by first applying the two-sided Wilcoxon rank sum test to compute a $P$ value for differential expression for each gene. To correct for multiple comparisons, we computed false discovery rates (FDR) based on the $P$ value distribution. We used a $q$ value of 0.005 as the threshold of significance.

Promoter analysis was applied to predict which transcription factors can regulate each gene by binding to its promoter region. To identify those promoters with binding sites corresponding to binding motifs obtained from the Biobase Transfac database release 2009.3 (BIOBASE GmbH, Wolfenbuettel, Germany), we used the MotifLocator algorithm with a first order background model. Significant $P$ value for binding was estimated from randomly permuted promoter sequences. For promoter scanning, the DNA sequence 1 kb upstream from the transcription start site of each differentially expressed gene were downloaded from UCSC Genome Browser hg19 genome build.

For each gene, we determined a set of transcription factors whose binding sites lie up to 1 kb upstream of the gene’s transcription start site ($P < 0.001$). Using these data, we tested which transcription factor binding motifs occur more often than expected at random at the upstream regions of differentially expressed genes. We used a hypergeometric distribution with a $P$ value threshold of 0.05 to test for statistical significance. We repeated the process of finding the enriched transcription factor binding motifs for each of the 100 resampled sets to obtain the most frequently identified binding motifs and the corresponding transcription factors, which we termed the “master regulators”.

Constructing genomic neighborhoods

Construction of a genomic neighborhood around a master regulator began downstream with the inclusion of the predicted targets using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, http://www.ingenuity.com). Regulatory relationships were then established between the master regulator and its predicted targets in IPA. A $q$ value cutoff of 0.005 was set to identify molecules whose expression was differentially regulated between the sample sets. The connections to molecules that were not differentially expressed were consequently removed from the network. Taking advantage of the global molecular network developed from information contained in Ingenuity’s Knowledge Base, known targets of the master regulator were included in the network, whereas the non-differentially expressed molecules were excluded.

Genomic neighborhoods were then algorithmically generated based on the connectivity of the master regulator and its targets. Further downstream, we added the molecules that are established targets for genes regulated by the master regulator excluding those that are not differentially expressed. We continued adding differentially expressed target genes hierarchically until there were no more target genes to add or we reached a major hub, such as an extensively studied transcription factor that plays a role in many different biological processes and regulates numerous genes under varying conditions. The rationale for excluding the downstream targets of central transcription factors was to limit the size of the neighborhood. We then proceeded similarly upstream from the master regulator by adding the differentially expressed known regulators of the master regulator until we reached the desired depth of the regulatory pathway. A cartoon presentation of neighborhood generation is shown in Figure 1.

Rather than analyzing the biological functions of all the differentially expressed genes simultaneously, we applied enrichment analysis to identify the most significant cancer-critical functions for each genomic neighborhood and master regulator. Molecules that were associated with pathways in Ingenuity’s Knowledge Base were considered for the analysis. Right-tailed Fisher’s exact test was used to calculate a $P$ value to determine the statistical significance for each biological function. A $P$ value threshold of 0.05 was chosen as significance level for functional enrichments. All steps in the analysis are schematically summarized in Figure 2.

ChIP-sequencing validation

To show the regulatory potential of the most prominent master regulator, SRF, in inducing expression differences in the established markers as well as its neighborhood molecules, we validated some of the SRF protein-DNA interactions using publicly available ChIP-seq data. In addition to a library from ChIPs against SRF, we used a negative control library of reversed cross-links and no immunoprecipitation (RX-noIP) as a reference. Both data were measured from a human Jurkat cell line and sequenced using the Solexa platform. We re-aligned the sequenced reads against the most recent human genome build (hg19) using Bowtie algorithm. The sequence alignment of SRF and RX-noIP libraries yielded 8.6 and 17.3 million mapped tags, respectively. Regions of significantly high
read density ("peaks") were identified by MACS\(^{[54]}\) and QuEST\(^{[52]}\) software and smoothed read density profiles were illustrated in Integrative Genomic Browser (http://www.bioviz.org/igb/).

Results

Promoter enrichment analysis revealed nine transcription factors that distinguish GIST from LMS

Promoter analysis for 261 transcription factors identified 433 binding motifs that were located up to 1 kb from the transcription start sites of genes throughout the genome (\(P < 0.001\)). The total number of genes which harbor a particular binding motif in their promoter sequence ranged from 3 to 5951 genes per motif. Iteratively leveraging the promoter analysis data and the GIST and LMS gene expression data, we found 74 different transcription factor binding motifs that were enriched in at least 1 of the 100 resampled sets of differentially expressed genes. The motifs corresponded to 58 differentially expressed (Wilcoxon rank sum; \(P < 0.05\)) transcription factors. The use of resampling

![Figure 1. Cartoon illustration of creating genomic neighborhoods. First, the master regulator and predicted targets are added. Second, known targets of the master regulator are added. Third, to complete the downstream signaling network, genes are hierarchically added according to regulatory relationships. Fourth, direct and indirect regulators of the master regulator are added. Non-differentially expressed genes (red) are removed after each step.](image-url)
methodology in uncovering the enriched binding motifs revealed that most of the 58 transcription factors would probably not generalize well in other data sets because their binding motifs were enriched in only small portion of the resampled sample sets. Only 9 transcription factors had an enriched binding motif in more than 30% of the resampled sets (Table 1). We held the enrichment frequency (a measure of robustness) in greater importance than the $P$ value for differential expression and other statistical or biological parameters.

Serum response factor (SRF) was the only factor with an enriched motif in every signature (100/100 enrichment frequency). In fact, SRF had 6 different robust SRF-binding motifs, whereas the other transcription factors only had one robust motif with the exception of MYC (Figure 3). The next most robust binding motifs corresponded to 2 transcription factors, NK2 transcription factor-related locus 5 (NKX2-5) and coiled-coil domain containing 6 (CCDC6), which had an enriched motif in over 90% of the resampled gene sets (99/100 and 91/100 enrichment frequency, respectively). Six transcription factors [lymphoid enhancer-binding factor 1 (LEF1), vitamin D receptor (VDR), zinc finger protein 350 (ZNF350), tripartite motif-containing 63 (TRIM63), v-maf musculoaponeurotic fibrosarcoma oncogene homolog (MAF), and v-myc myelocytomatosis viral oncogene homolog (MYC)] fell between 30% and 50% in motif enrichment frequency. We termed these 9 genes the master regulators. The remaining 49 transcription factors with enrichment frequency below 30% were regarded as not robust enough and, thus, were excluded from subsequent analyses. The correlation between the expressions of 4 master regulators, SRF, CCDC6, MAF, and NKX2-5, and their predicted targets is shown in Figure 4. We clearly observed an expression-promoting effect for the 3 first master regulators and an inhibitory effect for NKX2-5, particularly in the samples where NKX2-5 was highly expressed (far right in Figure 4D).
Table 1. Summary of top master regulators and their genomic neighborhoods

| Symbol | Gene name                                      | Frequency | P value | Size | Major biological functions                                                                 |
|--------|-----------------------------------------------|-----------|---------|------|---------------------------------------------------------------------------------------------|
| SRF    | Serum response factor                         | 100/100   | < 0.001 | 159  | Cell movement; cell death; cell growth and proliferation; cell development; cell morphology |
| NKX2-5 | NK2 transcription factor related, locus 5     | 99/100    | < 0.001 | 24   | Cell morphology; cell growth and proliferation; cell death; cell development; carbohydrate metabolism |
| CCDC6  | Colled-coil domain containing 6               | 91/100    | < 0.001 | 38   | Cell movement; cell-to-cell signaling and interaction; cell morphology; cell assembly and organization; cell development |
| LEF1   | Lymphoid enhancer-binding factor 1            | 44/100    | 0.002   | 85   | Cell movement; cell cycle; cell death; cell growth and proliferation; gene expression       |
| VDR    | Vitamin D receptor                            | 41/100    | 0.016   | 10   | Cell growth and proliferation; cell development; cell cycle; protein synthesis; cellular compromise |
| ZNF350 | Zinc finger protein 350                       | 38/100    | 0.032   | 50   | Cell death; cell morphology; cell development; cell-to-cell signaling and interaction; cell assembly and organization |
| TRIM63 | Tripartite motif-containing 63                | 36/100    | 0.003   | 72   | Cell cycle; cell growth and proliferation; cell death; gene expression; cell development |
| MAF    | V-maf musculo-aponeurotic fibrosarcoma oncogene | 33/100   | 0.001   | 64   | Cell cycle; cell death; cell growth and proliferation; cell development; DNA replication, recombination, and repair |
| MYC    | V-myc myeloma-cytomatosis viral oncogene      | 32/100    | 0.020   | 115  | Cell death; cell cycle; cell growth and proliferation; cell development; cell morphology |

*Frequency of significant enrichments of the top binding motif in 100 resampled sample sets; *P value for differential expression; *number of genes in the genomic neighborhood.

Figure 3. Robust transcription factor binding motifs. The motifs are indicated by the name of the corresponding transcription factor with a running number in the case that has more than one motif per factor. The left side indicates the number of differentially expressed genes that have the binding motif in their promoter region. The right side shows the percentage of resampled signatures where the motif was enriched as a measure of robustness.

Key transcription factors control genes with distinct biological functions

To further investigate the roles of the 9 master regulators in creating the clinical differences between GIST and LMS, their genomic neighborhoods were constructed as described in Methods. The sizes of the resulting gene regulatory networks varied from 10 genes in the VDR neighborhood to 159 genes in the SRF neighborhood. We went on to associate biological processes to the neighborhoods using IPA gene annotations and enrichment analysis to the respective genes. The top 5 processes for each genomic neighborhood are listed in Table 1. Invariably, common
cancer-related processes, such as cell cycle, cell death and cell proliferation, appeared on the top of each list. To further establish the functional differences and similarities in neighborhoods of the 9 master regulators, and to relate those to the set of 2330 differentially expressed genes between GIST and LMS ($q < 0.005$) in the entire cohort, we systematically compared their functional enrichment scores in several categories. An illustration in Figure 5 shows negative log-transformed enrichment $P$ values in 18 broad biological processes that were relevant for all the gene sets.

We observed that the broadest terms, genetic disorder and cancer, were the most enriched for the set of all differentially expressed genes. This was expected because the global signature is a composition of all the cancer-related processes that are different between GIST and LMS, and therefore, the only unifying functional themes must be very broad. Among the more specialized terms, there was a large variation of enrichment scores between the neighborhoods. Some neighborhoods greatly exceeded the score of the global gene signature in many categories. Since the network genes are functionally coherent subsets of all differentially expressed genes, we expected them to gain a much higher score in specialized categories and possibly a lower score in broad categories. Overall, the highest significance scores in most categories were given to the SRF, LEF1, and CCDC6 neighborhoods. The diversity of SRF’s cellular functions was also seen as a significant enrichment in most functional categories (Figure 5).

We provided schematics of the genomic neighborhoods of SRF (Figure 6A) and ZNF350 (Figure 6B). In Figure 6A, we highlighted three partially overlapping biological processes to which the SRF neighborhood is very significantly associated: cancer, muscle development, and cellular movement. To show the link from SRF to three generally cancer-related signaling pathways, we included PTEN, VEGF and ATM signaling, which are also more strictly defined than most processes and, therefore, contain fewer genes in general. For ZNF350 (Figure 6B), we highlighted the genes that are associated with cancer, cell death, and integrin signaling.

Experimental identification of binding sites by ChIP-seq experiments validated 28 differentially expressed predicted targets of SRF, such as a computational marker PRUNE2. In addition to validating

Figure 4. Correlation between master regulator expression and predicted target gene expression. The most strongly expressed predicted target genes of 4 master regulators (SRF, MAF, CCDC6 and NKX2-5) are sorted based on their normalized expression. Red color implies high expression and blue color low expression values. A general enhancing effect is clearly seen for the first 3 regulators whereas NKX2-5 seems to inhibit most of its targets.
Figure 5. Functional enrichment analysis for genomic neighborhoods. Enrichment scores of genes in 9 neighborhoods are compared against the global signature (all differentially expressed genes) in 18 categories. Interestingly, the global signature reaches the highest score in the broadest categories: cancer and genetic disorder. The neighborhoods score the highest in more specialized categories.
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Cancer
- Dev. of muscle
- Cellular movement
- PTEN signaling
- VEGF signaling
- ATM signaling

Cancer
- Cell death
- Integrin signaling
Figure 6. Graphical representation of the genomic neighborhoods of SRF and ZNF350. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge. All edges are either between the master regulator and its predicted targets or supported by at least one reference from the literature, a textbook, or canonical information stored in the Ingenuity Pathways Knowledge Base. Nodes are displayed using various shapes that represent the functional class of the gene product. In panel A for SRF, colored boxes encircle genes that are annotated to biological processes (cancer, muscle development, and cell movement) or signaling pathway (PTEN signaling, VEGF signaling, and ATM signaling). All genes shown here are differentially expressed between GIST and LMS \((q < 0.001)\). Regulatory connections are shown by dotted arrows pointing the direction of regulation. MKL1 is the only upstream molecule known to regulate SRF, which, in turn, regulates the expression of several marker genes, including the computational marker PRUNE2 and MYOCD, which regulates immunohistochemical marker smooth muscle actin. In panel B for ZNF350, colored boxes encircle genes that are annotated to cancer, cell death, or integrin signaling. Notably, ZNF350 is predicted to bind to the promoter of muscle-specific immunohistochemical marker of LMS, desmin (DES).

some of the predictions, several canonical SRF targets, such as actin genes and FHL2, also showed pronounced peaks in ChIP-seq data. SRF autoregulation by binding to its own promoter and binding to the co-activator MKL1 were clearly observed. However, there were no SRF binding events detected in the promoter of another co-activator, myocardin (Figure 7).

Discussion

In this study, we developed a methodology for studying the global gene expression differences between two groups of samples in a biologically relevant context. Our method is a resampling-based computational approach that couples gene expression data with promoter scanning, database information, and gene set enrichment analysis to find the most robust and biologically relevant transcription factors, the master regulators. A difference between the master regulators and common marker genes is that markers are usually designed to be optimal for classification purposes, whereas the master regulators are likely more biologically relevant. Nevertheless, the master regulators may also be used as a more robust genetic signature, although tumor classification was not included in the scope of this study. The paramount importance of the master regulators is that they can control most of the difference in gene regulation patterns that are observed in the disease phenotypes through their extensive regulatory connections. Despite their biological relevance, the regulators themselves do not always appear on top of the list of the most differentially expressed genes, which makes it hard to identify them with conventional methods.

In addition to identifying the key transcription factors, Figure 7. ChIP-seq validation of SRF neighborhood. The vertical lines represent 30 kb sequence around transcription start sites (arrows point to the direction of transcription) of 9 predicted or known target genes of SRF. Read density profiles from a ChIP-seq experiment are shown for each gene. There is a pronounced region of high read density (“peak”) within the promoter showing autoregulation of SRF, regulation of actin genes (ACTA2, ACTB) and FHL2. SRF has not bound to the promoter of myocardin (MYOCD), one of the established targets of SRF. Predicted target PRUNE2 also shows a peak at their promoter region.
our approach results in marked improvement in the interpretability of the global genetic signature. Only recently have enrichment analyses become the most common analysis for interpreting the biological theme of gene lists. In contrast to individually studying the top genes, enrichment analysis finds statistically significant over- or under-representations of gene categories. Although this is a marked improvement to single gene analysis, applying it directly to the gene list has drawbacks of its own. Mainly, the differentially expressed genes are usually a product of numerous aberrant pathways. Therefore, the signature genes rarely have any real unified biological themes. The presented work extends the standard enrichment analysis workflow towards systems biology by refining the signature and integrating additional information. Linking transcription factors to their differentially expressed up- and down-stream genes results in gene regulatory networks that are functionally coherent subsets of the global genetic signature.

Genomic neighborhoods have several advantages over the use of a global genetic signature. First, enrichment analysis now gives us information not only about the functional difference between the samples but also on the function of the neighborhood. Second, it extends the knowledge on the functional role of the master regulator, even if information on the gene itself is scarce or unavailable. Third, the network topology contains direct information on the biochemical mechanism, which might lead to a differential clinical property in the phenotype. On the other hand, drawbacks include the difficulty of predicting transcription factor binding in mammals, lack of knowledge of gene functionality and regulation, and the inability to measure post-transcriptional regulation. Additionally, the selected significance threshold has an effect on the final network and even the master regulator itself. While computing enrichments alleviates this problem, the method could benefit from inclusion of probabilistic features making strict thresholds unnecessary. Even accounting for these obstacles, we believe that other investigators will benefit from using our methodology in their research.

We demonstrated our approach by using it to investigate the biological differences in two soft tissue sarcomas, GIST and LMS. We uncovered 9 transcription factors that were robust and differentially expressed between the tumors. The most prominent regulator SRF is a widely expressed transcription factor that participates in many global and tissue-specific processes like muscle cell differentiation and growth factor-induced cell proliferation, but it has also been linked to human diseases like heart disease and cancer [58]. Previous studies have also shown that overexpression of SRF co-factor myocardin (MYOCD) leads to increased migration ability in LMS [59]. This is well in accordance with our observation that the SRF neighborhood is strongly associated with cellular movement. The second transcription factor, LEF1, is a mediator of Wnt signaling through which it plays a role in tissue specification and apoptosis [57,58]. This is reflected by high enrichment scores in the cell cycle, cell death, and tissue development categories in our study. The remarkably high enrichment of the LEF1 neighborhood in the cellular movement category is also a noteworthy finding. There is less evidence on the functional role of ubiquitously expressed CCDC6, but it has been linked to papillary thyroid carcinomas through fusion to RET proto-oncogene [60]. Also worth noting is the strong association of the MYC neighborhood with cell death, growth, and proliferation, as could be expected as per its well-known biological effects as an oncogene [60]. One concern is that many genes in human genome do not have any functional annotations. This fact affects particularly smaller gene sets which may contain only a few annotated genes and also likely induces a bias towards finding only the known functions for neighborhoods around extensively studied transcription factors such as MYC. The lack of knowledge on regulatory interactions is also one probable cause for the consistently lower significance scores of neighborhoods of NKX2-5, VDR, MAF, TRIM63, ZNF350.

Although SRF and ZNF350 have not been associated with processes like cellular movement, cell death, and integrin signaling before, they have the potential to regulate the processes and signaling pathways through their extensive regulatory connections. Therefore, we associated SRF and ZNF350 with these cellular functions through our analysis and proposed the relevant regulatory relationships in detail. Similarly, we proposed several new associations for all 9 master regulators through functional enrichment analysis of their genomic neighborhoods. The 9 master regulators may directly or indirectly explain the differential regulation of many computational and immunohistochemical markers used to differentiate the clinical groups. For example, an immunohistochemical marker for LMS, desmin, is predicted to be directly regulated by ZNF350. Another example of direct regulation is SRF’s predicted target PRUNE2, which has been used as a computational marker in an earlier 2-gene classifier differentiating between GIST and LMS [60]. In addition, SRF is known to regulate its own muscle-specific co-activator MYOCD, which, together with SRF, forms a complex that is a master regulator of smooth muscle gene expression pattern [61]. One of the genes in this pattern is smooth muscle actin, which is another common immunohistochemical marker used in diagnosing LMS. Cell type-specific regulation for SRF has been shown before [60] and probably accounts for many of the targets without visible peaks.
Uncovering SRF as an extremely robust master regulator differentiating between GIST and LMS is the most important finding of this work. Differential activation of SRF co-activators has been suggested as one way of directing SRF regulation to subsets of target genes in a tissue-specific manner. The only known co-factors that were differentially expressed in our data are NKKX2-5, MYOCDC, and MKL1. While MKL1 is ubiquitously expressed, MYOCDC is required for the expression of smooth muscle specific genes\cite{64}. Association to muscle development, on the other hand, may be explained by the cell lineage-specific expression rather than by the difference in tumor biology as leiomyosarcomas are tumors of the smooth muscle. Thus, they likely have a more smooth muscle cell-specific expression pattern than GISTs, which are thought to arise from interstitial cells of Cajal\cite{64}. Having matching control samples would likely remove cell type-specific findings, but it is often very difficult to obtain such a sample for various practical reasons, such as the limited availability of healthy tissue. Even if some of the transcription factors were unrelated to these cancers, uncovering a fundamental regulatory program like smooth muscle-specific regulation supports our assertion that we can find meaningful gene regulatory networks and key factors behind them.

We showcased here our novel data analysis framework in a topical biological setting and provided solid hypotheses for further experimental work in this field. Particularly, the functional associations of the 9 master regulators warrant experimental validation in the context of GIST and LMS. An in depth investigation into these transcription factors may increase our knowledge of the functional differences between the tumors. Methodologically, genomic neighborhoods could be built similarly for studying different biological settings, such as tumor versus normal tissue, early stage versus late stage tumor, or primary tumor versus metastasis. By identifying key transcription factors, application into the aforementioned scenarios could help us understand the major mechanisms of tumorigenesis, disease progression, and metastasis.

Conclusions

In conclusion, we developed a framework that applies a systems approach to analyze the functional differences in gene expression patterns. The approach integrates several levels of data such as gene expression measurements, transcription factor binding site predictions, regulatory network topology, and information on gene function. Using a set of expression measurements from GIST and LMS, we demonstrated the applicability of our approach. Analyzing the differences in the expression patterns yielded many interesting starting points for future research that can potentially lead to better diagnostic methods and deeper understanding into biological differences of these tumors. Specifically, we uncovered 9 differentially expressed transcription factors around which we generated gene regulatory networks of differentially expressed genes using promoter analysis and literature-based database information. These master regulators and their genomic neighborhoods may be the epicenter of different clinical properties of GIST and LMS.

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Further studies are needed to clarify the role of these pathways in the context of other types of cancers. The identification of specific therapeutic targets in these pathways could offer new opportunities for cancer treatment. The results of this study provide important insights into the molecular mechanisms underlying cancer progression and suggest potential strategies for the development of novel therapeutic approaches.
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