Signaling Biomarker Pattern Discovery Using Reverse Phase Protein Microarray

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***Abstract*—Understanding the molecular mechanism of aging will allow human beings to develop rationale strategies for therapeutic interventions in aging related diseases. The goal of this study is to investigate the effect of a new protein, Klotho protein, on FGF (fibroblast growth factor) signaling. To identify the signaling molecules, two emerging technologies, high-throughput siRNA (small inference RNA) and reverse phase protein microarray (RPPM) are utilized. To quantitatively analyze the patterns of siRNA knockdowns, we present a Discriminative Feature Pattern Identification System (DFPIS) to identify contributing nuclear hormone receptors. Computational analysis results using HEK293 (human embryonic kidney) cells knocked down from siRNAs and screened by protein microarray were presented.**

I. Introduction

Suppression of aging is a far-reaching dream of human beings. Aging is an extremely complex phenomenon influenced by various genetic and environmental factors. However, recent studies have demonstrated that single gene mutations can extend life span and delay aging processes in various experimental animals [3], [5], [7], [8], [13], [15]. Our pioneering research has been focused on elucidating the function of Klotho gene [9], [10]. Klotho gene was originally identified by our collaborator, Dr. Makoto Kuro-O, which is involved in the suppression of aging in mammals [9] and is expressed in limited tissues, notably in kidney and brain. It encodes a single-pass transmembrane protein and functions as an aging suppressor that extends life span when over-expressed and accelerates the development of multiple aging-like disorders when disrupted in mice.

Klotho is a multi-functional protein that regulates insulin/IGF1 signaling, FGF (fibroblast growth factor) signaling and vitamin D metabolism, all of which are potentially associated with its anti-aging properties. In this paper, we will present the effect of Klotho on FGF signaling. As the newest member of the FGF ligand gene family, FGF23 can inhibit phosphate re-absorption in the kidney and lower serum vitamin D. Many aging-like phenotypes in these mutant mice are rescued by ablation of vitamin D activity [16], [17]. Klotho is necessary for FGF23 to activate FGF signaling in various types of cultured cells. To investigate the mechanism by which Klotho and FGF23 regulate FGF signaling, protein microarray screening will be utilized and a computational method will be presented to discover the regulating molecule patterns of data output from protein microarray.

II. Research Methods

A. siRNA Knockdown for Validation and Global Screening

To identify the global structure of the FGF signaling networks in the regulation by Klotho and FGF23, we have developed a high throughput siRNA (small interfering RNA) library screening system as an experimental platform. Currently, we have a library consisting of three non-overlapping siRNAs (siRNA_A, siRNA_B, and siRNA_C) to 48 human steroid hormone receptors. These siRNAs are chemically modified to overcome some of the limitations of standard siRNAs-specifically, the "off target" effects, or non-specific interactions of siRNAs with other, nontargeted RNAs. These reagents sold by Invitrogen (Stealth RNAi Collection, Invitrogen) are blunt ended, double stranded and modified in a manner that prevents sense strand activity (a modified sense strand backbone that cannot fit inside the RISC structure), eliminating sense-strand-mediated off-target activity. The three non-overlapping antisense strands are designed to the target RNAs using available software and the sequences searched against the genome database to weed out binding to other gene targets.

B. Reverse Phase Protein Microarray (RPPM)

To establish high-throughput screening of siRNA knockdown, we have developed a Reverse-Phase Protein Microarray (RPPM) technology. Different from matured gene microarray technology, protein microarray is a new emerging, quantitative assay technology. In contrast to other protein arrays that immobilize the probe, RPPM immobilizes the whole repertoire of sample proteins. It allows numerous samples to be analyzed in parallel using only minute (nanoliter) amounts of sample for making quantitative measurements to profile changes in activity of different candidate signaling molecules in cell lines knockdown and not-knockdown [6]. The RPPM technology was especially designed for profiling changes in protein activity (e.g. phosphorylation, cleavage activation, etc.) rather than just protein expression levels.

For the final output of high-throughput RPPM data, to quantitatively analyze the patterns of siRNA knockdown to

nuclear receptors, visual inspections are not always obvious or accurate. We present a discriminative feature patterns identification system called DFPIS. The framework starts with a *feature selection* performed by building a connection between pattern frequency (pattern support value) and discriminative measures. Once redundant features are removed, *feature patterns identification* algorithm is performed. The interaction patterns of the selected genes will be discovered by Strong Jumping Emerging Patterns (SJEPs). Finally, *feature pattern annotation* assigns a set of characteristics to feature pattern and thus obtains relevant information for the interpretation of experimental results. In the following sections, we will introduce the background knowledge and our proposed methodology.

III. Problem formulation

For each numerical attribute from RPPM data output, its value range is discretized into two or more intervals. Each (attribute, continuous-interval) pair is called an item. ($gene_{M26383}$, [59.8, +∞)) is an example of items. Let I be a set of all items. Then a set X of items is called an itemset which is defined as a subset of I. $X(f_i)$ is defined as an itemset of the feature f_i which contains all continuous-interval items of the attribute f_i. For example, the discretization method partitions genes each into two disjoint intervals. $X(M_{26383})$={($gene_{M26383}$, (-∞, 59.8)),($gene_{M26383}$, [59.8, +∞))}. $sp_D(X)$ is the support of an itemset X in a data set D calculated by $count_D(X)/|D|$, where $count_D(X)$ is the number of samples in D containing X. Suppose D contains two different classes: D_1 and D_2. For an item $i \in I$, there is a single itemset $\{i\} \subset I$.

Definition 1. *(Pattern Significance) Given $\xi > 0$ as a minimum support threshold, the significance of an item $\{i\}$, denoted as S($\{i\}$), is defined as*

$$S(\{i\}) = \begin{cases} 0 & \text{if } sp_{D_1}(\{i\}) < \xi \wedge \\ & \quad sp_{D_2}(\{i\}) < \xi, \\ sp_{D_2}(\{i\}) & \text{if } sp_{D_1}(\{i\}) = 0 \wedge \\ & \quad sp_{D_2}(\{i\}) \geq \xi, \\ sp_{D_1}(\{i\}) & \text{if } sp_{D_1}(\{i\}) \geq \xi \wedge \\ & \quad sp_{D_2}(\{i\}) = 0, \\ |sp_{D_1}(\{i\}) - sp_{D_2}(\{i\})| & \text{otherwise.} \end{cases}$$

The larger the significance of an item, the sharper the discriminating power associated with the item. If $S(\{i\}) = sp_{D_1}(\{i\})$ or $S(\{i\}) = sp_{D_2}(\{i\})$, we call an item $\{i\}$ as an SJEP (Strong Jumping Emerging Pattern) which is the shortest JEPs satisfying the support constraint. Let $J = \{j_1, j_2, ..., j_p\}$ be the set of all items appearing in $X(f_i)$.

Definition 2. *(Feature Significance) A significance measure S is a function mapping a feature $f \in F$ to a real value such that $S(f)$ is a degree of interestingness of the feature f. $S(f)$ is defined as $S(f) = \sum_{i=1}^{p} S(J(i))/|J|_{S(J)\neq 0}$.*

Initialization
- Feature set: F = {f_1, f_2, ..., f_r}

Iteration
- Discretize training samples
- Repeat until F=[ ]
  - Compute relative feature significance $S_i(\cdot|f_i)$ of f_i using Def. 1-3
  - Find the feature subset Sub_i with the highest significance score S_i
  - Train SVM with the training samples with features contained in Sub_i
  - Compute the weight vector **w** using Eq. (5)
  - Compute the weight W_i using Eq. (7)
- Compute the average of feature weights obtained from Sub_1, ... Sub_r
- Find the feature subset Sub_{final} with the highest weights
- Backward feature selection with features in Sub_{final}
  - Classify the test samples with selected features
- Analysis of measurements

Fig. 1. A feature selection method

Example 1. S($gene_{M26383}$)=(S(($gene_{M26383}$, (-∞, 59.8)))+S(($gene_{M26383}$, [59.8, +∞))))/2.

We define the significance of the feature f as the combined significance of items in $X(f)$. Given significance measures, we can define the relative significance between two features. Let $J = \{j_1, j_2, ..., j_p\}$ be the set of all items appearing in $X(f_i)$ and $K = \{k_1, k_2, ..., k_q\}$ be the set of all items appearing in $X(f_j)$.

Definition 3. *(Relative Feature Significance) Given the significance measure S, the relative significance between two features f_i and f_j is defined as*

$$\begin{aligned} S(f_j|f_i) &= \Big[\sum_{i=1}^{p}\sum_{j=1}^{q} S(K(j)|J(i))\Big] \Big/ (|K| + |J|), \\ &= \Big[\sum_{i=1}^{p}\sum_{j=1}^{q} S(K(j)) - R(J(i), K(j))\Big] \Big/ \\ &\qquad (|K| + |J|) \end{aligned}$$

where $S(J(i), S(K(j)) > 0$ and $R(J(i), K(j))$ denotes the redundancy between two patterns $J(i)$ and $K(j)$.

IV. A Discriminative Feature Pattern Identification System (DFPIS)

Feature patterns (combination of features) identification techniques could be used to capture more underlying semantics than single feature. However, it is very hard to find meaningful patterns in large datasets like microarray data because of the huge search space. Furthermore, infrequent patterns are often irrelevant or do not improve the accuracy of the classification. To tackle these problems, we designed a discriminative feature patterns identification system named DFPIS.

Our framework starts with a feature selection performed by building a connection between pattern frequency (pattern support value) and discriminative measures. This method defines

a feature subset relevant to each feature which includes the d lowest correlated features of a given feature, based on a relative feature significance measure. With the low correlated feature subset, we run the linear SVMs algorithm where $\frac{2}{3}$ samples are utilized for training and the remaining $\frac{1}{3}$ for testing. Then, we compute the weight for each feature based on the idea proposed in [14].

$$Z_k = \begin{cases} \dfrac{|w_k|S(f_k)}{\sum_{j=1}^{d+1}|w_j|S(f_j)} \times \beta \times \delta, & \text{for } \gamma \leq \beta, \\ \left(1 - \dfrac{|w_k|S(f_k)}{\sum_{j=1}^{d+1}|w_j|S(f_j)}\right) \times (\gamma - \beta) \times \delta, & \text{for } \gamma > \beta, \end{cases} \quad (1)$$

where

$$\delta = \begin{cases} 1, & \text{for } \gamma \leq \beta \\ -1, & \text{for } \gamma > \beta \end{cases} \quad (2)$$

and β is the accuracy using testing samples, γ is a predefined threshold and $|w_k|$ is the absolute SVM weight. Each $|w_k|S(f_k)$ is normalized by dividing the summed $|w_k|S(f_k)$ value of all the features in the subset. $S(f_k)$ is the feature significance using Def 2. To prevent the feature weight from being multiplied by zero, a very small value is summed to $|w_k|$ and $S(f_k)$. In our approach, $S(f_k)$ is additionally multiplied on the equation from Oh et al. [14] because this feature significance is an important measure to be able to show if the feature is globally discriminant, not locally in the feature subset. Finally, backward selection (elimination) started with a certain number of features selected by the weights of features. The process stops when decreasing the size of current best subset leads to a lower prediction rate. This algorithm is summarized in Fig. 1.

Once redundant features are removed, feature patterns identification algorithm is performed. To efficiently mine SJEPs (Strong Jumping Emerging Patterns), we employed SJEPs mining algorithm based on the contrast pattern tree (CP-tree) [4]. The CP-tree is constructed by using the new ordering of each transaction based on the feature weight from Eq. 1, while the original CP-tree reorders transactions based on the feature support value. The order of CP-tree is very important to extract SJEPs. However, there are some critical issues when we use only the feature support value for reordering. First, there are many cases that the support values of features are equivalent. Second, feature support value only is not enough to rank features. Therefore, reordering based on the feature weight has the strong advantage to efficiently extract SJEPs. Because every training instance is sorted by its weight when inserting into the CP-tree, items with high weight, which are more likely to appear in an SJEP, are closer to the root. Using the predefined order, we can produce the complete set of paths (item sets) systematically through depth-first searches of the CP-tree. We start from the root to search the CP-tree depth-first for SJEPs. The item set, which is initially empty, will grow one item at a time. After completing the search of the CP-tree, we select only those minimal patterns by filtering out those that are supersets of others. The remaining minimal ones are SJEPs since they satisfy the minimum support threshold.

The final step is to provide feature pattern annotation. Feature pattern annotation is important to assign a set of characteristics to feature pattern and thus obtain relevant information for the interpretation of experimental results. Our goal is to generate annotations in order to provide complete and homogeneous feature pattern characterization such as feature significance, relative feature significance, feature prediction ability (classification accuracy) feature pattern significance, and so on, to researchers. Fig. 2 shows the format of our feature pattern annotation.

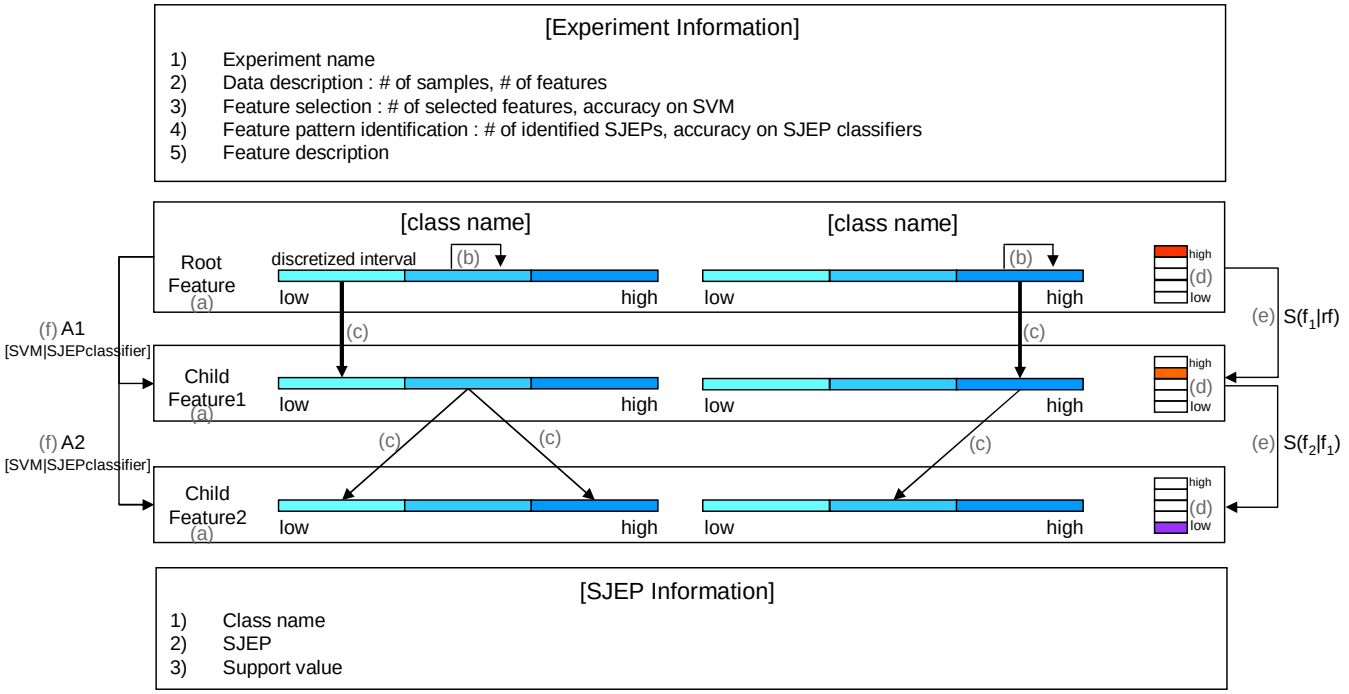
V. Experiments

To test the performance of the proposed DFPIS algorithm, we used quantum dot protein microarray data with linear SVM (soft margin C=1). LOOCV (leave-one-out cross validation) was carried out because of the small number of samples. For comparison, we used four feature selection algorithms, i.e., our proposed feature selection, SVM-RFE, Chi-squared, and information gain feature selection.

The data sets of HEK293 (Human Embryonic Kidney) cells were generated using the reverse-phase protein microarray platform as described in our previous work [6], [18]. Briefly, cell lysates were arrayed on the slides using a printing robot. In all cases, samples were printed in triplicate. Each slide was probed with anti-antibodies for six antibodies, pERK, SOD2, CuSOD1, mGSS, mHPS60-chaperonin, and Klotho. For background controls, identical slides were incubated without the primary antibody. Finally, a pegylated, streptavidin-conjugated Quantum Dot 655-Sav (Quantum Dot Corp.) was used as a fluorescent detector. The intensities of all antibodies were normalized relative to those of Actin to correct the protein loads between the spots and the mean values of triplicate samples were used. The library screened in this data was the Nuclear Hormone Receptor (NHR) library (48 genes) from Invitrogen. Tab. I shows our data sets used in this experiment. This NHR library had 48 receptors with three siRNAs for each one, namely A, B and C (Invitrogen's naming convention). For each receptor, A,B and C represent three different siRNAs for the same gene differing in the location of the target. Every time a library was spotted, all three sets were spotted. The classes of these datasets were determined by whether FGF (Fibroblast growth factor-23) knockdowns (-FGF) or not (+FGF).

TABLE I
Data description

Dataset	# of classes	# of samples	# of features	Description
Data1	2	10 (5/5)	48	5 antibodies on siRNA A set
Data2	2	10 (5/5)	48	5 antibodies on siRNA B set
Data3	2	10 (5/5)	48	5 antibodies on siRNA C set
Data4	2	30 (15/15)	48	5 antibodies on each siRNA A, B, and C set



(a) Feature name
(b) Arrow represents the relationship of SJEP without interaction with child intervals, only on root feature
(c) Arrow represents the relationship of SJEP with interaction with child intervals
(b),(c) Thickness of arrow represents the support value of the relationship
(d) Feature weight
(e) Relative feature significance
(f) Classification accuracy with selected features on both SVM or/and SJEPclassifier
For A1, accuracy with root feature and child feature1
For A2, accuracy with root feature, child feature1 and child feature2

Fig. 2. Feature Pattern Annotation

A. Computational analysis: feature selection

We accomplished the accuracy of 100% using LOOCV evaluation as shown in Tab. II with a few features out of 48 chosen from our DFPIS-FS (feature selection). For this small sample dataset, even a single siRNA (siRNA_B) was confident enough for the decision making (Data2).

TABLE II
BEST ACCURACY

Data	# of samples	# of features	accuracy	sensitivity	specitivity
SiRNA_A	10	3	100	100	100
SiRNA_B	10	1	100	100	100
SiRNA_C	10	12	100	100	100
SiRNA_All	30	7	100	100	100

To evaluate the performance of our algorithm, we carried out comparison experiments with several commonly used feature ranking algorithms, i.e. ChiSquare, Information Gain and SVM-RFE. Fig. 3(a) shows the experiment results with the corresponding accuracy on Data4 for different algorithms. Note that top ranked five features of DFPIS-FS outperformed. We also found the similar patterns on other data sets. Fig. 3 (b) shows the corresponding weights of features on all data sets. Note that feature 37 (VDR) had a high weight all the time. Also, we can observe the difference of the top 10 ranked feature set on different data sets in Tab. III. However, as observed from the frequency of occurrence, we can see three features, 32 (RXRA), 37 (VDR) and 40 (NR4A3) with higher frequency compared to others. This may reflect that these biomarkers may be used for the decision making regardless of kinds of siRNA or antibodies.

TABLE III
TOP 10 RANKED FEATURE LIST

Rank	Data 1	Data 2	Data 3	Data 4
1	40	25	18	37
2	39	15	3	47
3	22	20	46	40
4	38	24	47	3
5	43	40	37	27
6	35	2	42	2
7	41	37	11	46
8	37	44	6	43
9	36	11	32	32
10	32	1	44	38

B. Computational analysis: feature pattern identification

To explain the results of DFPIS-FPI (feature pattern identification), we explain one of our results, the feature pattern annotation using top 5 ranked features of Data2 in Fig. 4. DFPIS-FPI generated 10 SJEPs using those five features selected from DFPIS-FS where minimum support threshold

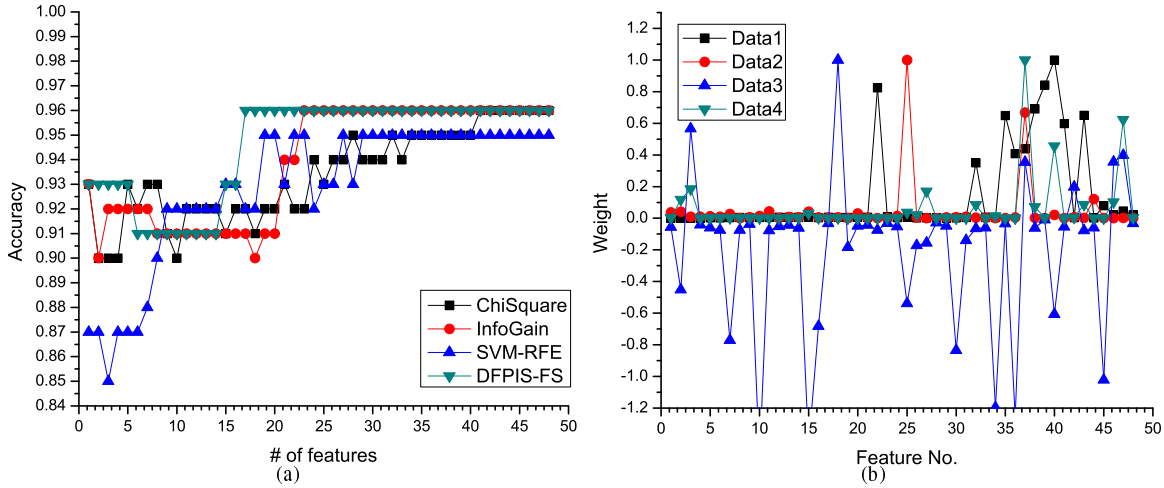


Fig. 3. Comparisons Study: (a) Accuracy on Data4 by ChiSquare, InfoGain, SVM-RFE, and DFPIS-FS when different top ranking SiRNAs are selected. For DFPIS-FS, feature weights were used instead of ranks by backward feature selection. (b) Feature weights of DFPIS-FS.

is 0.25. When looking at the results of DFPIS-FPA (feature pattern annotation), we can see obviously different patterns between two classes. When FGF knockdowns, expression level of all features was decreased. For feature 25, interval itself was a SJEP for each class and these two SJEPs were confident enough for the decision making. As seen in Fig 4., relative feature significance values which are 0.74, 0.39, 0.39 and 0.53 show the significance of the corresponding feature when other feature is already given. For example, relative feature significance of feature 15 when feature 25 is already determined for decision making is 0.75. Other values are relatively lower than this because of redundancy of patterns. Overall, DFPIS-FPA generated annotations to provide homogeneous characteristics for the interpretation of experimental results.

C. Biological observations

We investigated whether expression level of identified features are discriminative as FGF knockdowns. It is interesting to notice that there are two observations such as the difference of expression level of feature 22 (RORC) and feature 32 (RXRA) in Data1 and the difference of expression level of CuSOD1 antibody on all data sets. Unusually, the expression level of feature 22 (RORC) went up as FGF knockdowns in Data1. Feature 32 (RXRA) is over expressed when this feature cooperates with CuSOD1 antibody. Overall, when identified features respond with CuSOD1 antibody, there were relatively large difference of expression level.

VI. Conclusion

This paper presented exploratory work on identifying signaling molecules for aging mechanism. The effect of a new protein, Klotho, on FGF signaling was investigated using siRNA knockdown and reverse phase protein microarray screening. The proposed Discriminative Feature Pattern Identification System (DFPIS) allows us to recognize the contributing genes in the FGF pathway and take into consideration of gene interactions. For feature selection, proteins contributing most to knockdown are identified. The interaction patterns of those selected genes are discovered by employing SJEP pattern mining based on a contrast pattern-tree. The last step of feature pattern annotation provides complete pattern characterization such as single gene significance, relative pair-wise gene significance, and pattern significance.

For future work, we plan to use additional kinase library (636 human kinases) for additional cell lines. With more sample collection, the whole framework will be further testified and improved. We will also establish a western blotting procedure to validate results from the siRNA studies.

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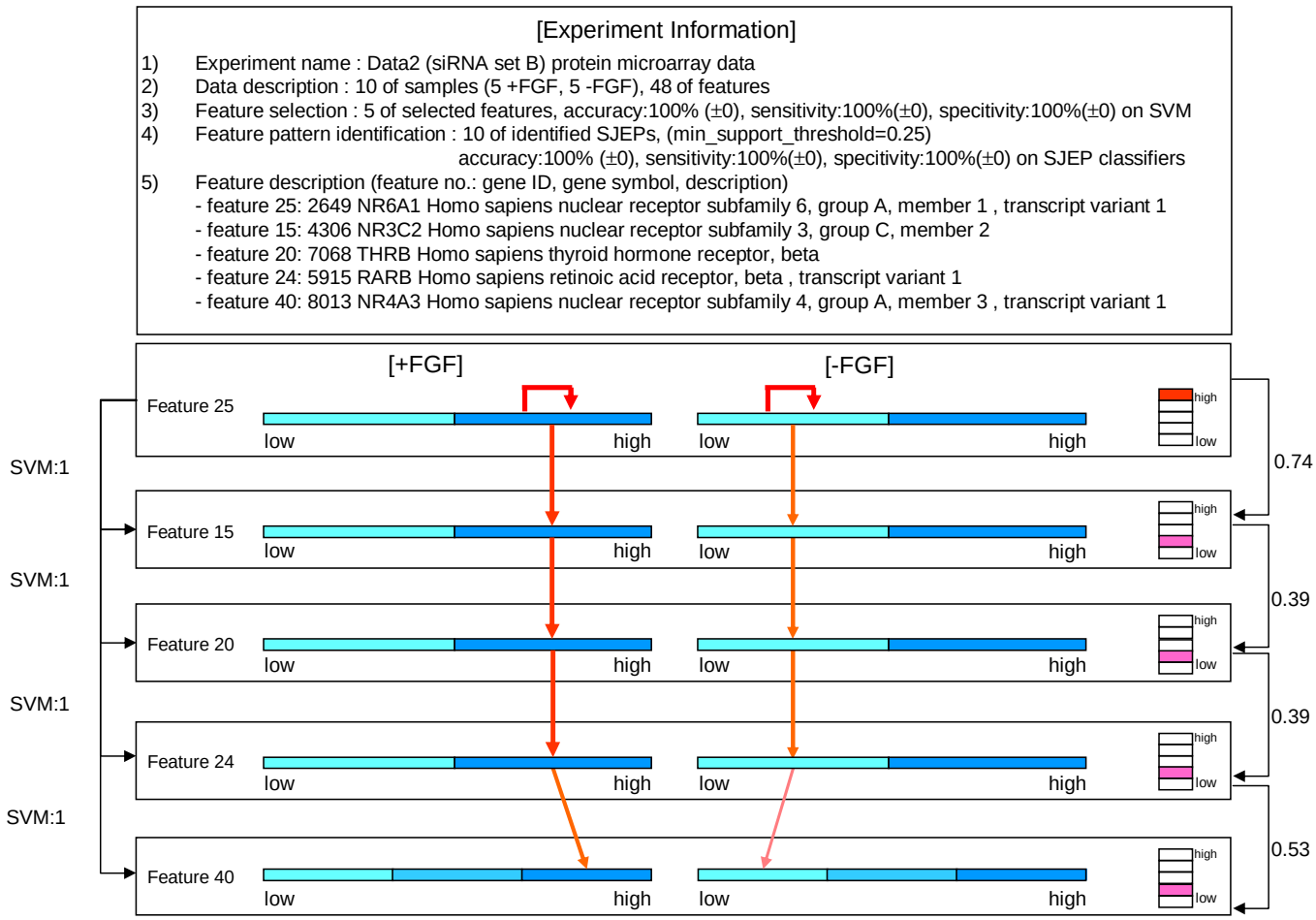

Fig. 4. Feature pattern annotation of Data2 with five features

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