Experimental Model Construction and Validation of the ErbB Signaling Pathway

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***Abstract*—The importance of ErbB receptor signaling in breast cancer is consistent with its functional role in normal development of mammary gland. The study of the ErbB signaling network and its bidirectional cross-talk with hormonal receptors, such as estrogen receptor (ER) encloses information about the molecular mechanisms on breast cancer evolution, progression and endocrine resistance. With this analysis we attempt to examine the differences in activation/inhibition of intracellular signaling molecules within ErbB signaling cascade on ER+ and ER- breast cancer patients. With the proposed framework we model the genetic interactions in the ErbB signaling pathway directly from expression data as Gaussian approximations and compare them with the KEGG canonical ErbB pathway in order to identify significant molecular deformations characterizing the studied population. The results indicate a distinct profile of activation/inhibition between the two ER populations and highlight the primary role of PI3K/Akt pathway in breast cancer progression and targeted treatment strategies.**

I. Introduction

The ErbB receptor family consists of four transmembrane tyrosine kinases: EGFR/ErbB1/HER1, ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4. It activates numerous key intracellular pathways that govern major biological processes including proliferation, cell migration, metabolism and survival. ErbB signaling is stimulated by the epidermal growth factor (EGF) family of peptides, members of which are receptor specific. The ability of ErbB receptors to regulate crucial intracellular pathways is due to their aptitude for interaction with many signal transducers [1]. In addition, their differentiation in expression between breast cancer populations suggests numerous novel ways of interaction. This implies many different states in the ErbB pathway's genetic expression among the subjects, whose decoding would give insight in their oncogenic potential, but also highlight their utility as therapeutic targets.

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A variety of computational methods have been considered for modeling genetic regulations in pathways, such as linear models [2] and Gaussian networks [3] that aim to provide suitable mathematical models for describing stochastic associations and dependence structures in complex high-dimensional data. Although graphical models are promising for regulation analysis, their main drawback is their limited performance when the experimental data is insufficient. In addition, the simplicity of modeling complex dependencies between molecules introduces high uncertainty. Thus, there is an imperative need to develop robust methods with validated performance to real genomic applications.

In this study we focus on a signal transduction network, the ErbB receptor signaling, which is dysregulated in many cancers including breast cancer. This signaling network reveals the biological sequence of molecular events in normal cellular conditions. Instead, we use the experimental gene expression data from ER+ and ER- subjects to model the activations/inhibitions of the implicated ErbB signaling molecules and compare the resulting network with the actual ErbB pathway as expressed on normal subjects. The purpose of this comparison is twofold; first to study the ability of the experimental model to express sound biological relations as in the ErbB pathway and second, to examine differences in molecular signaling and inconsistencies between the two populations, which might be relevant to the progression of the cancer pathology. In addition, we consider the response of the involved molecules upon activation by EGF, in an attempt to examine the ability of the proposed framework to predict the response to such molecular deficiencies. Our proposed framework represents nonlinear relations between genes and relies on Gaussian modeling through Kernel density estimation (KDE). We apply this framework on data from breast cancer and focus on the ErbB pathway. The results indicate the important role of the intracellular pathway PI3K/Akt of ErbB signaling, responsible for cancer evolution and progression.

II. Methodology

This study attempts to identify which molecules are activated/inhibited in a signaling pathway, based on thresholds T. A gene is considered inhibited if the probability of expression $e \in [0, T]$ is lower than 0.5 (section II.B). In contrast, activation probabilities are considered higher than 0.5 for $e \geq T$. In this way, we can estimate the genetic expression profile for all genes in the pathway based

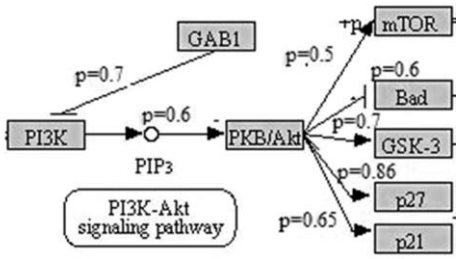

Fig. 1. An example of the proposed methodology for the PI3K/Akt sub-pathway. Given that *GAB1* is inhibited, we predict the impact of its inhibition on the other molecules. For each gene we compute *T* considering as inhibition probability $p>0.5$ in the range $e \in [0, T]$ and as activation $p>0.5$ for $e>T$.

on the available expression data. In the same context, we can predict the impact of involved genes in the entire pathway. We pose inference queries conditioned on the observation of a specific gene that play an important role. An example is illustrated in Fig.1, where we compute the probabilities of activation/inhibition for the involved genes in the PI3K/Akt pathway given that *GAB1* was found inhibited.

A. Kernel Density Estimation

In previous studies [4], we focused on approaches for estimating the structure of a gene-gene network. Based on the KDE approach, we now estimate the network structure using only expression data. After this design step, we examine the modeling of the inter-genetic dependencies using a non-linear analysis.

Kernel density estimation [5] is a non-parametric framework that estimates the probability density function (pdf) of a random variable. Assume that a generic network is developed based on a limited genomic i.i.d dataset $X=(x_1,..x_n)$,where x_i denotes the sample i of gene X. The KDE allows the estimation of X as follows:

$$\hat{f}_h = \frac{1}{nh}\sum_{i=1}^{n} K\left(\frac{x-x_i}{h}\right) \quad (1)$$

where $K(.)$ is a symmetric positive definite Gaussian function $K(u)=\frac{1}{2\pi}e^{\frac{-1}{2}u^2}$, n is dataset's size of the gene X and $h=1.47\sigma n^{1/6}$ is the optimal Gaussian bandwidth parameter, with σ standard sample deviation.

B. Conditional Probability Distribution

Gaussian graphical models (GGMs) are forms of graphical models for representing complex associations among Gaussian random variables. A gene corresponds to a random variable, while gene interactions are shown by edges. Thus, interactions with parental nodes are modeled by the conditional probability distribution (CPD) of each gene. We use KDE as a non-parametric framework in order to capture the dependencies from parental nodes that underlie on experimental data.

Suppose we have p sets of microarrays and n genes where $X_i=(x_{i1},\ldots,x_{ip})^T$ is a p dimensional expression vector obtained for ith gene. Let P_{ai} be the parents of gene X_i then direct dependencies are encoded according to a conditional probability. In order to model these dependencies, we find the joint distributions with Standard Gaussian Kernel (SGK) as follows:

$$f(X_i \mid P_{a_i}) = \frac{\sum_{j=1}^{p} K_{h_1}(x - x_{ij})\, K_{h_2}(p_{a_i} - p_{a_{ij}})}{\sum_{j=1}^{p} K_{h_2}(p_{a_i} - p_{a_{ij}})} \quad (2)$$

where K(.) is a Gaussian kernel function described as (1), p is dataset's size and $h_1=c_1 n^{-1/6}$, $h_2=c_2 n^{-1/6}$ for $c_1,c_2>0$ are the smoothing parameters selected as optimal approximations of Gaussians basis functions [5], [6].

Equation (2) implies that the conditional density estimate is an asymptotic approximation of Gaussian [5], [6] $N(\theta_1,\sigma_1^2)$ with $R(K)=\int K(u)^2 du$ and parameters as follows:

$$\theta_1=\frac{\sigma_\kappa^2}{2\sqrt{c_1c_2}}(c_1^2 f^{(2)}(X_i|P_{a_i})+c_2^2 f_{(2)}(X_i|P_{a_i})+2c_2^2 f_{(1)}(X_i|P_{a_i}) f^{(1)}(X_i|P_{a_i}) \quad (3)$$

$$\sigma_1^2 = \frac{R(K)^2 f(X_i \mid P_{a_i})}{c_1 c_2 f(P_{a_i})} \quad (4)$$

Hence, (3) and (4) encode a Gaussian model that captures non-linear dependencies of network parameters. If a gene has no parents its mean and variance are taken from KDE.

C. Genes' Thresholds and Interactions

In order to establish a mathematical base of gene's interactions (activation/ inhibition), we have to (i) establish a robust threshold for gene expressions and (ii) define simple rules which can map the gene associations into interactions.

The threshold on gene expression follows a two-interval discretization of expression patterns which is based on an information-theoretic setting as follows [7]. For each gene we sort the expression values in descending order in the form $V = n_1, n_2, \ldots n_i$ where $n_1 < n_2 < \ldots < n_i$. For all consecutive pairs in V, the midpoints are computed. For each midpoint μ_κ, two subsets of V are formed, i.e. $H_k=\{n_i \in V \,\&\, n_i > \mu_\kappa\}$ for high or up regulated class and $L_k=\{n_i \in V \,\&\, n_i < \mu_\kappa\}$ for low or down regulated class.

Then, the information-gain in [8] is utilized and computed as $IG(\mu_\kappa)=E(V) - E(V/\mu_\kappa)$, where E($V$) stands for the entropy of the system and $E(V/\mu_\kappa)$ for the entropy of the system when the set V is split into the disjoint sets H_k and L_k. The latter term is obtained from the absolute entropy subtraction of class H_k from L_k [9]. The midpoint that exhibits the maximum information-gain is selected as the threshold that best splits the expressions in V and best expresses the regulation of the studied gene.

Thresholds on expression levels also give us the ability to characterize the identified gene interactions. In the gene-interaction network, genes are represented as nodes and interactions as edges. An edge is considered to be activation

TABLE I. EFFECT OF EGF ACTIVATION ON A SUB NETWORK PORTION OF THE ErbB SIGNALING PATHWAY

	RTKs	ErbB intracellular pathways	PI3K/Akt pathway
A. ER+ and ER- phenotypes			
(i) Activation	EGFR	-(PLCγ) PLCG2 → (CAMK) CAMK2A |-(PLCγ) PLCG2 → (PKC) PRKCA; PRKCB |-(Cbl) CBLC; CBLB -(PAK) PAK1; PAK2 → (JNKK) MAP2K7 → (JNK) MAPK9 → (Jun) JUN -(Sos) SOS2 → (Ras) NRAS → (Raf) ARAF; BRAF; RAF1 → (MEK) MAP2K1; MAP2K2 → (ERK) MAPK3	-(PI3K) PIK3CA; PIK3R1; PIK3R3 -(PKB/Akt) AKT2 -(p70S6K) RPS6KB1 |-(Bad) BAD |-(p27) CDKN1B
(ii) Activation upon ↑ by EGF (0.5<P≤1.0)	EGFR	-(PLCγ) PLCG2 → (CAMK) CAMK2A |-(PLCγ) PLCG2 → (PKC) PRKCA; PRKCB; **PRKCG** -(Cbl) CBLC; CBLB |-**(Src) SRC** |-**(Abl) ABL2** |-(PAK) PAK1; PAK2 → (JNKK) MAP2K7→ (JNK) MAPK9 → (Jun) JUN |-**(Shc) SHC1** → (Sos) SOS2 → (Ras) NRAS; **HRAS** → (Raf) ARAF; BRAF; RAF1→ (MEK) MAP2K1; MAP2K2 → (ERK) MAPK3 |-**(STAT5) STAT5A; STAT5B**	-(PI3K) **PIK3CB**; PIK3CA; **PIK3CD**; PIK3R1; PIK3R3 |-(PKB/Akt) AKT2, **AKT3** |-(mTOR) **MTOR** -(p70S6K) RPS6KB1 |-(Bad) BAD -(GSK-3) **GSK3B** |-(p27) CDKN1B
(i) Inhibition	ERBB2 ERBB3	-(PLCγ) PLCG1 |-(Cbl) CBL |-(Crk) CRK; CRKL → (Abl) ABL1 -(JNK) MAPK8; MAPK10 → (Elk) ELK1 |-(Grb2) GRB2 → (Ras) KRAS → (ERK) MAPK1	-(PI3K) PIK3CG |-(PKB/Akt) AKT1| -(p70S6K) RPS6KB2
B. ER+ positive phenotype			
(i) Activation		-(PKC) PRKCG |-(Src) SRC |-(Abl) ABL2 |-(Shc) SHC3; SHC1; SHC2 → (Ras) HRAS -(STAT5) STAT5A; STAT5B	-(PI3K) PIK3CD; PIK3CB |-(PKB/Akt) AKT3 -(mTOR) MTOR |-(GSK-3) GSK3B
(ii) Activation upon ↑ by EGF	**EGFR**	-(Shc) SHC3	
(i) Inhibition	ERBB4	-(CAMK) CAMK2B; CAMK2G |-(FAK) PTK2 |-(Nck) NCK1; NCK2 |-(PAK) PAK3; PAK4; PAK6; PAK7 → (JNKK) MAP2K4 |-(Sos) SOS1 → (Myc) MYC |-(GAB1) GAB1	-(PI3K) PIK3R5; PIK3R2 |-(eIF-4EBP) EIF4EBP1 -(p21) CDKN1A
(ii) Inhibition upon ↑ by EGF (0.5<P≤1.0)	**ERBB2** **ERBB3** ERBB4	**-(PLCγ) PLCG1** → (CAMK) CAMK2B; CAMK2G |-**(Cbl) CBL** |-(FAK) PTK2 **-(Crk) CRK; CRKL → (Abl) ABL1** |-(Nck) NCK1; NCK2 → (PAK) PAK3; PAK4; PAK6; PAK7 → (JNKK) MAP2K4 → **(JNK) MAPK8; MAPK10 → (Elk) ELK1** |-(Grb2) GRB2 → (Sos) SOS1 → (Ras) KRAS → **(ERK) MAPK1** → (Myc) MYC |-(Grb2) → (GAB1) GAB1 → (PI3K)	-(PI3K) **PIK3CG**; PIK3R5; PIK3R2 -(PKB/Akt) **AKT1**|**-(p70S6K) RPS6KB2** -(eIF-4EBP) EIF4EBP1 -(p21) CDKN1A
C. ER- negative phenotype			
(i) Activation	ERBB4	-(CAMK) CAMK2B; CAMK2G |-(FAK) PTK2 |-(Nck) NCK1; NCK2 → (PAK) PAK3 PAK4; PAK6; PAK7 → (JNKK) MAP2K4 |-(Sos) SOS1 → (Myc) MYC |-(GAB1) GAB1	-(PI3K) PIK3R5; PIK3R2 |-(eIF-4EBP) EIF4EBP1 -(p21) CDKN1A
(ii) Activation upon ↑ by EGF (0.5<P≤1.0)	**ERBB3** ERBB4	-(CAMK) CAMK2B |-**(Cbl) CBL** |-(FAK) PTK2 |-**(Crk) CRK; CRKL → (Abl) ABL1** |-(Nck) NCK1; NCK2 →(PAK) PAK3 PAK4; PAK6; PAK7→ (JNKK) MAP2K4 → (JNK) MAPK8; MAPK10→ **(Elk) ELK1** |-**(Grb2)** **GRB2** →(Sos) SOS1 → **(Ras) KRAS → (ERK) MAPK1** → (Myc) MYC |-(Grb2) → (GAB1) GAB1→ (PI3K)	-(PI3K) **PIK3CG**; PIK3R5; PIK3R2 -(PKB/Akt) **AKT1** |-(p70S6K) **RPS6KB2** -(eIF-4EBP) EIF4EBP1 |-(p21) CDKN1A
(i) Inhibition		-(PKC) PRKCG |-(Src) SRC |-(Abl) ABL2 |-(Shc) SHC3; SHC1; SHC2 → (Ras) HRAS -(STAT5) STAT5A; STAT5B	-(PI3K) PIK3CD; PIK3CB |-(PKB/Akt) AKT3 -(mTOR) MTOR |-(GSK-3) GSK3B
(ii) Inhibition upon ↑ by EGF	**EGFR**	-(Shc) SHC3	

The first column shows the predicted activated/inhibited ErbB receptors; the second column presents the predicted activated and inhibited molecules of intracellular pathways of ErbB signaling network; the third column shows the activated and inhibited PI3K/Akt downstream effectors in A) ER+ and ER- networks, B) in the ER+ network, and C) in the ER- network. Activated molecules upon activation by EGF are in bold; there are not inhibited molecules upon activation by EGF in both ER+ and ER- populations. Activation is denoted by the upwards arrow (↑).

only if both the source and the target nodes (genes) are over-expressed. An edge is considered to be inhibition if the source node is over-expressed and the target node is under-expressed or if the source is under-expressed and the target is over-expressed.

III. RESULTS

The basic purpose of the experimental study is to select a central molecule in the pathway, whose expression is known to affect the involved genes in the same pathway. Our goal is to predict which genes appear to be expressed (activated) or under-expressed (inhibited) following the activation of the central molecule and verify the predicted profiles according to previous studies.

A. Dataset

Most of breast cancer (BC) cases are influenced by both estrogen receptor (ER) and growth factor receptor signaling [9]. In an effort to reveal the underlying mechanisms we used three independent gene-expression studies targeting the ER phenotypic status of the respective patients, i.e., ER+ (ER positive) vs. ER- (ER negative). The details of the gene-expression data from the three studies are as follows: GSE2990 with 183 patients; GSE3494 with 247 patients; and GSE7390 involving 198 patients [10]. We studied two pathways, the ErbB signaling and the mTOR, considering 121 genes, which also include closely related genes or genes encoding protein family members and protein isoforms.

B. Implications of Activation/Inhibition

We focus on the expression profile prediction for the 121 studied molecules. For each gene we compute the appropriate threshold T and consider expressions higher than T as activation. More specifically, the information of genetic expression is enclosed in a Gaussian CPD, according to our methodology. Hence, we establish the genetic expression profile for all genes in the pathway based on the available expression data. In addition, we can predict the impact of specific genes in the pathway, by posing inference queries conditioned on the observation of the specific gene. For instance, the probability of gene *ERBB2* to be inhibited when EGF is activated is summarized as the conditional probability of the former given the expression level of the latter gene.

For the 121 studied genes we isolated the EGF, for which we predict its influence in the ErbB signaling pathway for the ER+ and ER- networks. It is well-known that EGF binds specifically to EGFR and its expression in breast carcinomas was associated with tumor size [11]. Our goal is to identify differences between the ER+ and ER- networks.

Table I presents the predicted expression levels based on (i) the expression data and (ii) the effects upon activation by EGF, in ER+ and ER- networks. Key points of interest are:

- There is a distinct gene expression profile for activation and/or inhibition in both ER+ and ER-.
- The observation of activated genes in ER-, which at the same time are inhibited in ER+ and *vice versa*, highlights

molecules with inverse properties in the ER populations.

- Upon activation by EGF, different ErbB receptor family members, signal transmitters and downstream effectors of ErbB intracellular cascades are activated in both ER+ and ER- (EGFR, SRC, STAT5A, STAT5B) or activated in ER- and inhibited in ER+ (ERBB3, CBL, ABL1, ELK1).

In general, we observe distinct gene expression profiles across the ErbB signaling network in ER populations, which is consistent with recent findings [10], [12]. As expected, the major signaling pathways activated by ErbB receptors are mediated by PI3 kinase, Ras-Raf (MAPK), JNK, PLCγ and facilitate a multiplicity of cellular functions [13]. Nevertheless, as illustrated in Table I the intracellular signaling cascades of ErbB network are preferentially implemented via specific, closely related human genes (RAS genes), or encoded protein family members (Crk) and isoforms (Akt), or different effector molecules (STAT, Myc, Elk) in each ER subtype. Numerous studies have demonstrated that the ErbB signaling network, including the intracellular pathways such as PI3K/Akt pathway are activated in BC. Moreover, the influence of PI3Ks proteins in oncogenesis has been validated by several studies indicating that aberrations in this pathway are potential causes of cell transformation and, more significant, that PI3K pathway inhibition causes tumor regression. By restricting our discussion to the PI3K/Akt pathway, we observed distinct activation and inhibition profiles of ER+/ER- subtypes. Given that PI3K/Akt drives proliferation, as well as tumor cell survival, it is likely expected that tumor cells attempt to maintain constitutive activation of this pathway [13], [14]. This is also observed in our study with the appearance of distinct activation profiles based on gene expression data and upon activation by EGF (Table I). As reported recently in [14], each molecular aberration may have a different clinical impact depending on the breast cancer molecular background, the presence of other aberrations, and/or the treatment received.

Furthermore, the proposed framework provides a useful tool for the discovery of activated or inhibited molecules that may reveal novel mechanisms of PI3K pathway-activation in BC subtypes. A bidirectional cross talk, where the PI3K pathway affects the levels and activity of ER, and the endogenous membrane ER can stimulate growth factor receptors and PI3K/Akt pathway [9], [14], indicating the significance of the above observations. Recent studies provide also evidence that the frequency and type of PI3K pathway aberrations vary among the different breast cancer subtypes, such ER+/ER- status [14], [12]; thus confirming our results.

Another important aspect of our study is the ability to focus to specific down and upstream effectors of the PI3K signaling pathway, as they comprise potential targets for drug development in BC, by posing specific queries. This can come with important therapeutic implications, because the differences of genetic expression on ER patients could be exploited for the production of individualized medications based on the expression profile of the ER patient, as the newly developed targeted therapies (AKT inhibitors) [14].

IV. Conclusion

With this study we examined the sequence of molecular events of the ErbB signaling cascades on ER+ and ER- subjects and identified their differences in activation/inhibition. Upon activation by EGF, apart from the common activated or inhibited genes, we observed a distinct expression pattern of affected molecules, activated on ER+, inhibited on ER- and *vice versa*. This observation could be exploited for therapeutic use for the studied disease on the ER+ and ER- patients. Our study highlights the impact of specific molecules (activation/inhibition) through the PI3K pathway and more widely of ErbB pathway, and to identify key molecules of these aberrant pathways in the different subtypes of breast cancer. At a subsequent level, the proposed framework may be very useful for targeted therapy at different points of the PI3K pathway and the entire ErbB signaling network.

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