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METHODOLOGY ARTICLE

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# From ER $\alpha$ 66 to ER $\alpha$ 36: a generic method for validating a prognosis marker of breast tumor progression

Clémence Chamard-Jovenin<sup>1</sup>, Alain C. Jung<sup>2</sup>, Amand Chesnel<sup>1</sup>, Joseph Abecassis<sup>2</sup>, Stéphane Flament<sup>1</sup>, Sonia Ledrappier<sup>2</sup>, Christine Macabre<sup>2</sup>, Taha Boukhobza<sup>1†</sup> and Hélène Dumond<sup>1\*†</sup>

## Abstract

**Background:** Estrogen receptor alpha36 (ERalpha36), a variant of estrogen receptor alpha (ER) is expressed in about half of breast tumors, independently of the [ER+]/[ER-] status. *In vitro*, ERalpha36 triggers mitogenic non-genomic signaling and migration ability in response to 17beta-estradiol and tamoxifen. *In vivo*, highly ERalpha36 expressing tumors are of poor outcome especially as [ER+] tumors are submitted to tamoxifen treatment which, in turn, enhances ERalpha36 expression.

**Results:** Our study aimed to validate ERalpha36 expression as a reliable prognostic factor for cancer progression from an estrogen dependent proliferative tumor toward an estrogen dispensable metastatic disease. In a retrospective study, we tried to decipher underlying mechanisms of cancer progression by using an original modeling of the relationships between ERalpha36, other estrogen and growth factor receptors and metastatic marker expression. Nonlinear correlation analyses and mutual information computations led to characterize a complex network connecting ERalpha36 to either non-genomic estrogen signaling or to metastatic process.

**Conclusions:** This study identifies ERalpha36 expression level as a relevant classifier which should be taken into account for breast tumors clinical characterization and [ER+] tumor treatment orientation, using a generic approach for the rapid, cheap and relevant evaluation of any candidate gene expression as a predictor of a complex biological process.

**Keywords:** ERalpha36, Breast tumor, Retrospective study, Gene network identification, Metastatic potential, Nonlinear correlation, Distance based tumor classification

## Background

Worldwide, breast cancer remains one of the main causes of cancer-induced morbidity and mortality in women. Breast tumors are usually classified according to clinical parameters (size, grade, lymph node extension) and molecular expression status (ER, PR, HER2, Claudin) [1]. Such a classification allows clinicians ordering the appropriate treatment. For instance, ER-positive/negative ([ER+]/[ER-]) status refers to the expression of the 66kDa nuclear estrogen receptor  $\alpha$  (ER $\alpha$ 66) in tumors, which are consequently cured by endocrine therapeutic

agents such as tamoxifen. Nevertheless, about 30 % therapeutic failure is observed due to unclear resistance mechanisms [2].

Until the recent identification of new membrane bound estrogen receptors, ER $\alpha$ 66 has been considered as the sole functional estrogen receptor in hormone sensitive breast tumor. In 2005, Wang and colleagues [3] cloned a 36-kDa variant of ER-alpha (ER $\alpha$ 36) which lacks both AF-1 and AF-2 transcription activation domains but retains a truncated ligand-binding domain, suggesting that ER $\alpha$ 36 may have a spectrum of ligand selectivity different from ER $\alpha$ 66.

ER $\alpha$ 36 is generated from a promoter located in the first intron of the ESR1 gene, indicating that ER $\alpha$ 36 expression is regulated independently from ER $\alpha$ 66. This is

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consistent with the finding that ER $\alpha$ 36 protein is present in about 40 % of [ER+] and [ER-] breast tumors.

ER $\alpha$ 36 triggers membrane-initiated mitogenic estrogen signaling through non-genomic pathways not only in breast, but also in gastric and laryngeal cancer cells both *in vitro* and *in vivo* [4–7]. In the [ER+] MCF-7 breast tumor cell line, ER $\alpha$ 36 overexpression leads to tamoxifen resistance and enhances metastatic potential [8, 9]. Thus, tamoxifen does not act as a drug for cancer treatment but serves as an ER $\alpha$ 36 agonist, triggering proliferation, migration and invasion. The adverse effect of tamoxifen in ER $\alpha$ 36 highly expressing [ER+] breast tumors may explain why the affected patients display poor outcome and require chemotherapy but not endocrine therapy [10].

These findings raise the possibility that, *in vivo*, enhanced ER $\alpha$ 36 expression could drive the growth status switch from estrogen dependent mitogenic signaling to estrogen dispensable migration/invasion ability and consequently stimulates cancer progression. Therefore, we designed a generic method to validate the hypothesis that ER $\alpha$ 36 expression may serve as a reliable therapeutic response prognosis marker for breast cancer patients.

A retrospective study was performed on 118 breast tumor samples in which the expression of genes involved in non-genomic estrogen response as well as metastatic process was analyzed. Potential relationship between these genes was modeled by using nonlinear correlation analyses, mutual information associated to significance analysis [11, 12], which are proven to be more accurate than linear statics techniques even if the latter are simpler to implement [13–17]. These models are represented by so-called “gene co-regulation graphs” which can be drawn for any consistent subclass of the considered 118 samples. Then, we used a metric comparing two gene co-regulation graphs to search the optimal value of ER $\alpha$ 36 expression providing two distinct populations from a gene network point of view. The two obtained graphs were compared and the differences appeared to be of biological significance.

## Results

### [ER+] versus [ER-] gene networks

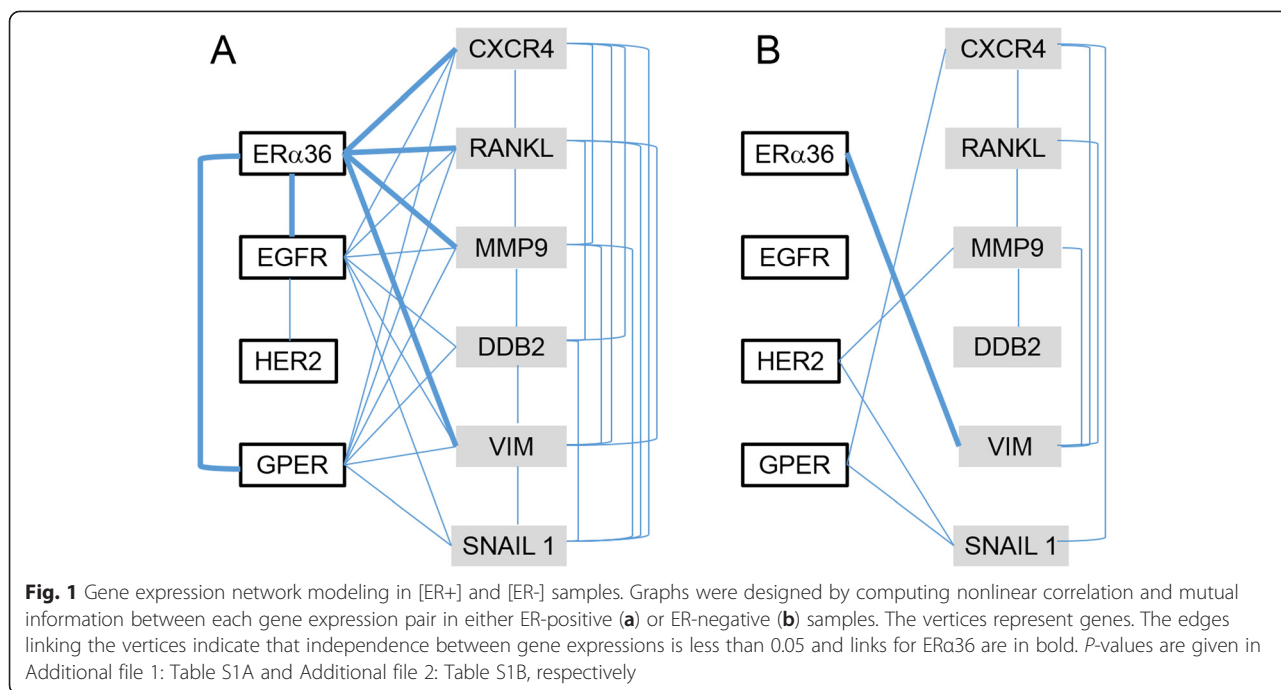
Since breast tumors are usually classified according to their hormone receptor status, tumor samples were first split into two classes according to their respective [ER] status, thus defining a first group of 60 ER $\alpha$ 66 expressing samples ([ER+]), and a second group of 58 samples devoid of ER $\alpha$ 66 expression ([ER-]). [ER+] breast cancer cell lines such as MCF-7 are considered non metastatic and weakly express ER $\alpha$ 36 whereas [ER-] cell lines such as MDA-MB-231 or MDA-MB-235 are highly metastatic and display higher levels of ER $\alpha$ 36 expression. In order

to assess if such a link between ER $\alpha$ 36 expression level and metastatic ability may be observed *in vivo*, nuclear (ER $\alpha$ 66) or membrane-associated estrogen receptors (ER $\alpha$ 36, GPER), their counterparts in non-genomic estrogen signaling (EGFR, HER2) as well as metastatic marker (SNAIL1, CXCR4, RANKL, VIM and MMP9) mRNA expression levels were determined by real-time PCR analyses. Among the growing amount of biomarkers related to the ER status (DDB2), the migration/invasion process (MMP9, VIM, CXCR4, RANKL, SNAIL) or the estrogen-response pathways (GPR30, EGFR), those listed above were picked up because they were previously shown to be related to ER $\alpha$ 36 [18–20]. Then, we identified the gene networks for each class of tumors by using nonlinear correlation analyses and transfer entropy computation (see Additional file 1: Table S1A and Additional file 2: Table S1B). The processed data obtained from [ER+] samples indicated that ER $\alpha$ 36 was a key node of a complex gene network, which involves other steroid and growth factor receptors as well as metastatic markers as a whole (Fig. 1a). On the other hand, ER $\alpha$ 36 was connected to the single metastatic marker VIM in the [ER-] network (Fig. 1b). These huge differences displayed by the two networks implied different functioning modes according to the tumor [ER] status and suggested that there could be a quantifiable link between ER $\alpha$ 36 position into the network and/or its expression level and tumor metastatic progression.

### ER $\alpha$ 36 based classification of breast tumor samples

To check if ER $\alpha$ 36 mRNA expression level could be a relevant classifier of a particular breast tumor phenotype, we drew a gene network for each ER $\alpha$ 36 expression value. Then, we quantified the differences between the networks as a function of ER $\alpha$ 36 relative expression, and designed a metric playing the role of a distance between graphs. The metric is an integer number standing for the structural differences between two graphs. More precisely, we compared the edges in the two graphs: when an edge existed in a graph and not in the other the distance was incremented with 1, if the edge existed in both graphs but did not represent the same linking way, the distance was incremented with 2. The obtained distance is then a metric.

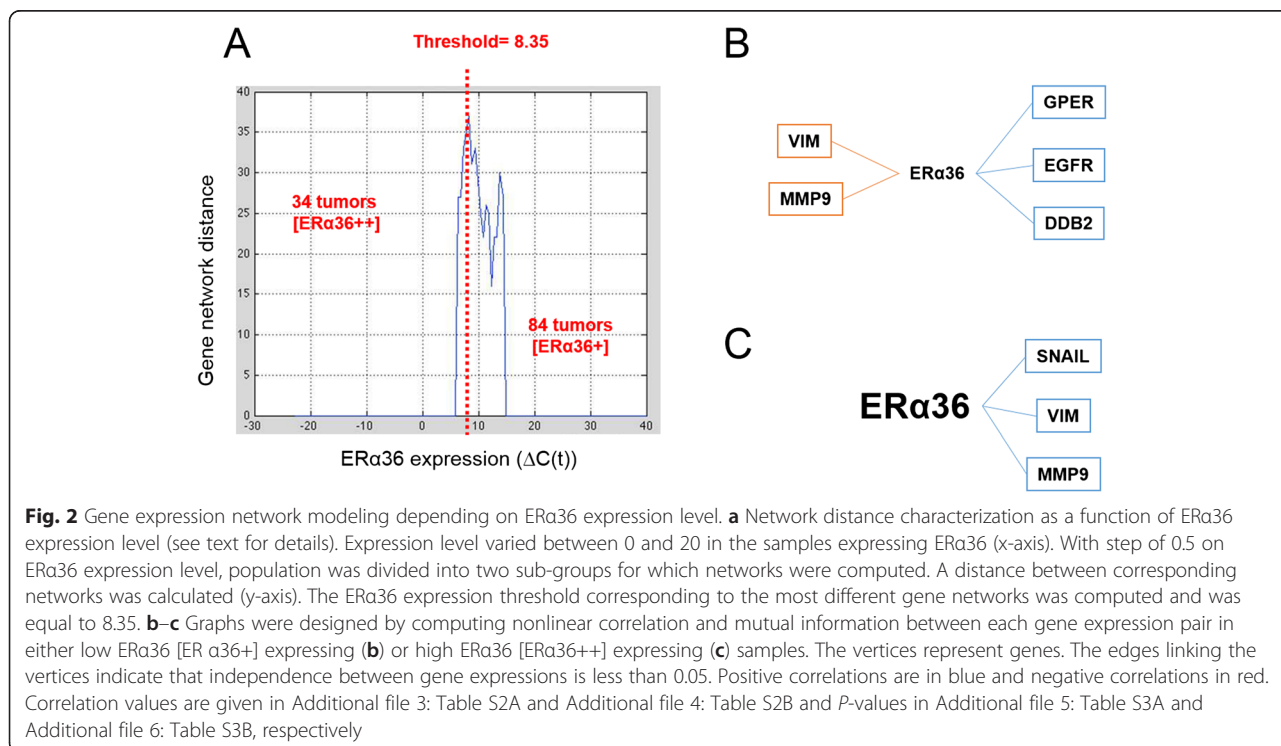
According to this metric, we determined the best threshold for ER $\alpha$ 36 to subdivide the samples into two populations, in order to obtain the most different networks probably defining the most different tumor phenotypes related to ER $\alpha$ 36 expression (Fig. 2a). Among ER $\alpha$ 36 expressing samples, the “best” threshold (which leads to the highest network difference score) was  $\Delta C(t) = 8.35$  and allowed to segregate a high ER $\alpha$ 36 expressing class ([ER $\alpha$ 36++]) of 24 tumors and a low ER $\alpha$ 36 expressing class ([ER $\alpha$ 36+]) of 84 tumors.



**ERα36 and metastatic progression**

In a last step, the previous modeling procedure was applied to either [ERα36+] or [ERα36++] subgroups. When ERα36 expression was low (Fig. 2b), it was clearly related to other receptors (GPER, EGFR) and DDB2 as well as inversely correlated to metastatic markers (MMP9, VIM)

(see Additional file 3: Table S2A and Additional file 4: Table S2B). Conversely, in the context of a high ERα36 expression (Fig. 2c), the network indicated a positive relationship to metastatic markers (SNAIL1, VIM and MMP9) independent from other receptors (see Additional file 5: Table S3A and Additional file 6: Table S3B).



## Discussion

In the present study, we examined ER $\alpha$ 36 expression in breast tumor specimens from 118 patients. We report that the majority of [ER+] tumors also express high levels of ER $\alpha$ 36.

In a previous clinical study, ER $\alpha$ 36 expression was shown to correlate with poor outcome in patients with [ER+] tumors treated by tamoxifen and the same tendency was observed in patients with [ER-] tumors [10]. Therefore, a high level of ER $\alpha$ 36 expression seemed to be an unfavorable factor of survival in breast cancer patients, independently of ER status. Besides, recent *in vitro* data indicate that ER $\alpha$ 36 expression (i) controls metastatic potential in [ER-] HCC38 cells and (ii) confers estrogen-hypersensitivity to [ER+] MCF-7 cells [9, 18]. In order to confirm that ER $\alpha$ 36 can trigger the progression of breast cancer in the primary tumor as well as during metastasis and to characterize the underlying mechanisms of high ER $\alpha$ 36-dependent phenotypes, we developed modeling tools. Expression analyses and network modeling of estrogen and growth factor receptor encoding genes, well known markers involved in tumor cell migration or invasion, and selected ER $\alpha$ 36 target genes [18] suggest that ER $\alpha$ 36 could be a key node of estrogen responsive pro-metastatic gene network in [ER+] tumors. These results are in line with recent *in vitro* analyses in MCF-7 cells, which show that the activation of ER $\alpha$ 36 expression triggers adaptive changes characterized by enhanced survival and migration during acquired tamoxifen resistance process [8, 21]. Similar data were obtained from endometrial cancer cells wherein ER $\alpha$ 36 was shown to promote tamoxifen agonist action *via* the MAPK/ERK and PI3K/Akt pathways [22–24]. Taken together, our results and others clearly suggest that [ER+] tumors highly expressing ER $\alpha$ 36 should not be cured by tamoxifen because the treatment could drive metastatic progression.

The developed approach to validate ER $\alpha$ 36 as relevant prognostic marker is quite generic and can be applied to other genes as well as to a subset of genes  $G_0$ . Indeed, the only modification, in this case, is to consider that we search for the maxima of multivariable function. Then, a classification can be done according to the expression of each gene to obtain  $2^n$  classes, where  $n$  is the cardinality of the considered subset  $G_0$ . Moreover, the robustness of the proposed method is attested by the fact that we proceed as described in [25], by using a shuffling method which generates more than 20 000 data for each of the dependency computation done between each pair of the studied genes.

Among the genes tested in this study, ER $\alpha$ 36 was identified as the best classifier candidate based on its ability to discriminate between two separate networks: one connecting ER $\alpha$ 36 to membrane receptors and the

second relating ER $\alpha$ 36 expression to those of metastatic markers. Therefore, comprehensive analysis and modeling of gene expression combined to colocalization analysis of ER $\alpha$ 36 and ER $\alpha$ 66 in breast tumors will contribute to characterize the cascade and timing of events that trigger ER $\alpha$ 36 expression during [ER+] metastatic tumor progression.

## Conclusions

In conclusion, this study (i) identifies ER $\alpha$ 36 as a relevant classifier whose expression level should be taken into account for breast tumors clinical characterization and [ER+] tumor treatment orientation, (ii) confirms *ex vivo* previous *in vitro* data connecting high ER $\alpha$ 36 expression to enhanced expression of migration/invasion markers and (iii) generates a novel approach for the rapid, cheap and relevant evaluation of any candidate gene expression as a predictor of a complex biological process.

## Methods

### Patients

Tumor specimen from 118 women with primary breast cancer expressing the canonical long form of ER $\alpha$  (ER $\alpha$ 66) [ER+] or not [ER-] were collected between 1980 and 1998, stored in the Paul Strauss Cancer Center biobank and used with the patients' verbal informed consent with the approval of the hospital ethic committee. Since the tumor pieces used in this study were regarded as post-operative waste materials, verbal consent was recorded by the surgeon during the preoperative examination. The Hospital Ethic Committee for Clinical Research localized into the Paul Strauss Center for Anticancer Research, 3 rue Porte de l'Hôpital, 67000 Strasbourg, France, approved the procedure. 60 [ER+] as well as 58 [ER-] tumor samples were included in the retrospective study. Immediately after resection, one half of each tumor was cryogenized into liquid nitrogen whereas the other part was fixed in 4 % formalin and further used for immunohistological analyses. [ER] status was assayed by standard ligand binding assay. In short, snap frozen tumor samples were pulverized and cytosols were extracted by ultracentrifugation. Human serum albumin was used as a standard control for protein normalization. Cytosol (10  $\mu$ L) was incubated with 5 nmol/L [ $H^3$ ] estradiol. After incubation, 100  $\mu$ L supernatant were transferred to an isoelectric focusing gel, in order to separate bound, unbound and unspecifically bound hormone. Samples with >10 fmol/mg bound ER were considered to be [ER+].

### RT-QPCR analysis

ER $\alpha$ 66, ER $\alpha$ 36, GPER, EGFR and HER2, as well as SNAIL1, CXCR4, RANKL, DDB2, VIM and MMP9



expression levels were determined by real-time PCR analyses. Large ribosomal protein (RPLPO) encoding gene was used as a control to obtain normalized values. Primers are listed in [see Additional file 7: Table S4]. Assays were performed at least in triplicate, and the mean values were used to calculate expression levels, using the  $\Delta C(t)$  method referring to RPLPO housekeeping gene expression. Briefly, total RNA was extracted using RNeasy Plus Universal tissue Mini (Qiagen, Courtabœuf, France) and reverse transcribed (GoScript Reverse Transcription System, Promega, Charbonnières-les-Bains, France). Real-time PCR analyses were then performed by using iTaq Universal SYBR Green Supermix (Bio-Rad, France) in Opticon2 thermocycler (Bio-Rad) as described elsewhere [26].

### Statistical analysis and modeling

Mathematical modeling of biological processes has recently emerged and developed as an essential tool to help cancer biologists and clinician pathologists improving personalized diagnosis, therapy and prognosis. Mainly, the first step in many gene regulation network-modeling task is the identification of the co-regulated or co-expressed genes. To this purpose, most of the works are based on a linear correlation computation and statistical hypothesis tests. Nevertheless, these tools do not detect nonlinear relationship between gene expressions, which is generally the case [13, 14]. That is why we propose to use nonlinear correlation and conditional mutual information techniques on the gene expressions in order to detect more accurately and exhaustively the co-regulated genes. More precisely, to confirm that there exists a relationship between two gene expressions, we cross two hypothesis tests. The first one is based on a nonlinear correlation computation based on the Spearman's rank correlation coefficient. We associate to this number a hypothesis test on the dependence of the considered gene expressions. When the  $p$ -value of this test is less or equal to a fixed threshold (0.05 or 0.01 for our study), we conclude on the possible link between these genes that must be confirmed by a second computation based on the mutual information value associated to a significance analysis.

We consider statistical significance testing for the mutual information measurement  $M(X, Y)$ , where  $X$  and  $Y$  represent the random variables associated to the considered two gene expressions. The null hypothesis  $H_0$  of this test is that  $X$  and  $Y$  are independent. The Mutual Information is a measure of the variables' mutual dependence. Here we use it to measure this dependence for every pair of genes. In this context, we consider two random variables  $X$  and  $Y$  associated to the expression of two genes among the target genes.

The expression of  $M(X, Y)$  is given by:

$M(X, Y) = H(X) + H(Y) - H(X, Y)$ , where  $H(X)$  and  $H(Y)$  are the marginal entropies and  $H(X, Y)$  is the joint entropy (or the Shannon entropy) of  $X$  and  $Y$ .

Here, the computation of marginal entropy is given by, for the samples  $(x_i)_{i=1, \dots, n}$

$$H(X) = - \sum_i^n P(x_i) \log_2(P(x_i))$$

and the joint entropy is computed by

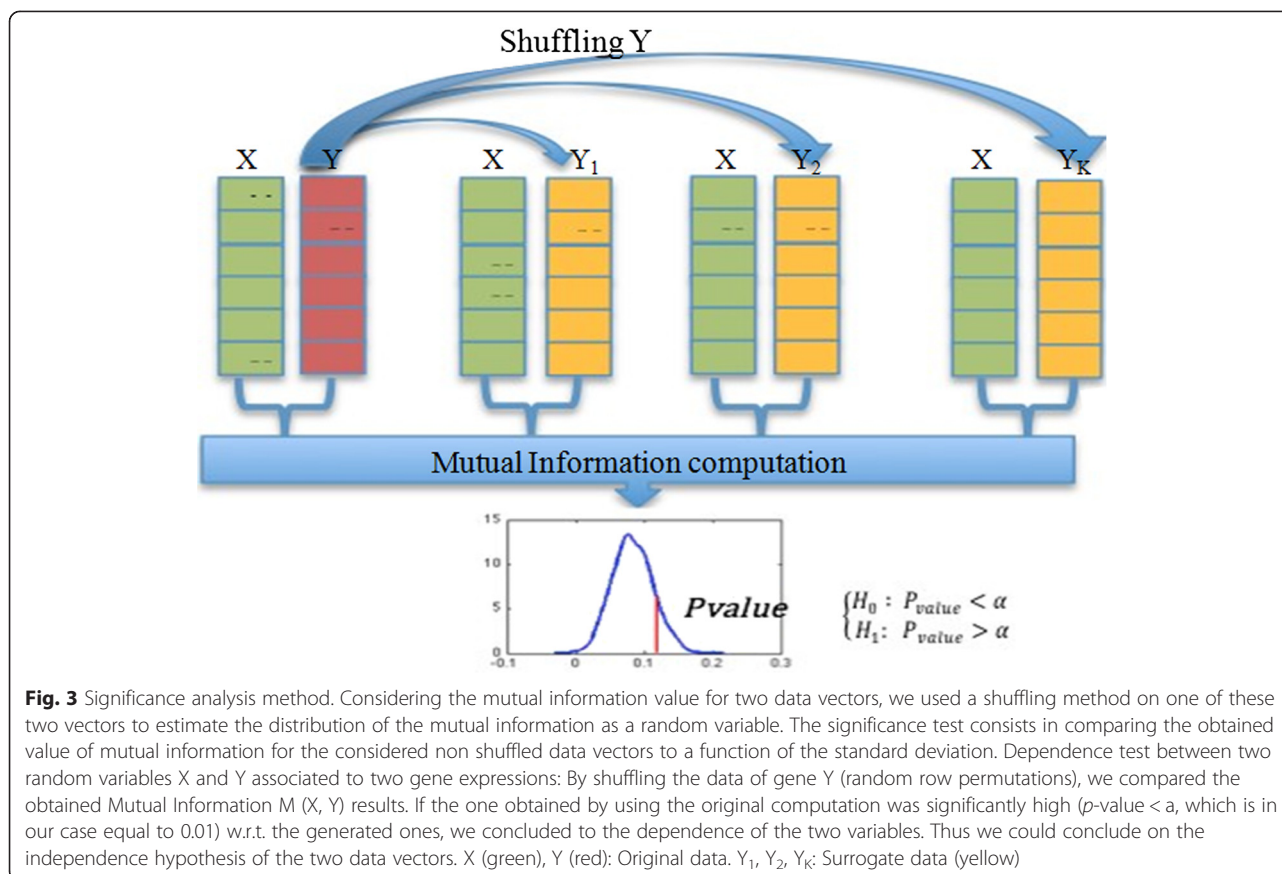
$$H(X, Y) = - \sum_i^n P(x_i, y_j) \log_2(P(x_i, y_j))$$

Intuitively, mutual information measures how much knowing one of these variables reduces the uncertainty about the other. For example, if  $X$  and  $Y$  are independent, then knowing  $X$  does not give any information about  $Y$  and *vice versa*. So their mutual information is zero. At the other extreme, if  $X$  is a deterministic function of  $Y$  and  $Y$  is a deterministic function of  $X$ , then all information conveyed by  $X$  is shared with  $Y$ : knowing  $X$  determines the value of  $Y$  and *vice versa*. As a result, in this case the mutual information is the same as the uncertainty contained in  $Y$  (or  $X$ ) alone, *i.e.* the entropy of  $Y$  (or  $X$ ).

First we estimate the distribution of the mutual information under  $H_0$ . The main problem using the mutual information measurement is that we do not have a "reference" to say that from a certain value (0.8 for example) the two variables are dependent. In order to decide whether or not the two variables are dependent, we have to make a hypothesis test using the experimental data compared to randomly generated data. These surrogate series of data are obtained by permuting the elements of one of the studied gene expression. Thus, we compare the obtained Mutual Information results: if the one obtained by using the original computation is significantly high w.r.t. the generated ones, we conclude to the dependence of the two variables (here: gene expressions).

Importantly, these surrogates are computed from the same number of observations, and the same distributions for  $X$  and  $Y$  (Fig. 3). We can then determine a one-sided  $p$ -value of the likelihood of our observation of the mutual information *i.e.* the probability of observing a greater mutual information value than that actually measured assuming  $H_0$ . This can be done either by directly counting the proportion of surrogates or assuming a normal distribution of the mutual information and computing the  $p$ -value under a  $z$ -test.

For a given  $p$ -value, which is often 0.05 or 0.01, indicating that the observed results would be highly unlikely under the null hypothesis  $H_0$ , we reject the latter



hypothesis concluding then that a significant relationship between the two gene expressions does exist.

From these networks, we evaluate the pertinence for a unique gene to be assimilated to a breast tumor classifier in three steps. First, after choosing the gene and a classification threshold to separate the samples into two categories, we identify two networks connecting the gene to separate markers by using nonlinear correlation and mutual information techniques. Then, we define and compute the distance between the two networks, which takes into account both the structural differences between the networks (existence or not of relations between the markers, sense of the linking when it exists) and the compartmental differences (behavioral differences in the relationship between genes). Therefore, the distance between both networks represents the classification performance of the classifier gene and allows us finding the more pertinent classifiers.

### Additional files

**Additional file 1: Table S1A.**  $P$ -values given for each gene pair in the [ER+] tumor gene network.

**Additional file 2: Table S1B.**  $P$ -values given for each gene pair in the [ER-] tumor gene network.

**Additional file 3: Table S2A.**  $P$ -values given for each gene pair in the [ER $\alpha$ 36+] tumor gene network.

**Additional file 4: Table S2B.** Correlation values given for each gene pair in the [ER $\alpha$ 36+] tumor gene network.

**Additional file 5: Table S3A.**  $P$ -values given for each gene pair in the [ER $\alpha$ 36++] tumor gene network.

**Additional file 6: Table S3B.** Correlation values given for each gene pair in the [ER $\alpha$ 36++] tumor gene network.

**Additional file 7: Table S4.** Primer list.

### Abbreviations

CXCR4: Chemokine (C-X-C motif) receptor 4; DDB2: Damage-specific DNA binding protein 2; EGFR: Epidermal growth factor receptor; ER: Estrogen receptor alpha; ER $\alpha$ 36: Estrogen receptor alpha 36; [ER $\alpha$ 36++]: High estrogen receptor alpha 36 expression; [ER $\alpha$ 36+]: Low estrogen receptor alpha 36 expression; ER $\alpha$ 66: Estrogen receptor alpha 66; [ER+]: Estrogen receptor alpha 66 positive status; [ER-]: Estrogen receptor alpha 66 negative status; ERK: Extracellular signal-regulated kinases; ESR1: Estrogen receptor 1; GPER: G protein-coupled estrogen receptor 1; HER2: Human epidermal growth factor receptor 2; MAPK: Mitogen-activated protein kinases; MMP9: Matrix metalloproteinase 9; PI3K: Phosphatidylinositol-4, 5-bisphosphate 3-kinase; PR: Progesterone receptor; RANKL: Receptor activator of nuclear factor kappa-B ligand; RPLPO: Large ribosomal protein; SNAIL1: Snail family zinc finger 1; VIM: Vimentin.

### Competing interests

The authors declare that they have no competing interests.

**Authors' contributions**

CCJ and AC performed data acquisition, analysis and interpretation. CM, SL collected the tumor samples. SF performed a critical reading of the manuscript. ACJ and JA performed clinical collection and analysis of tumor samples. HD and TB designed and conducted the study. All authors read and approved the final manuscript.

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