Exploring the intrinsic differences among breast tumor subtypes defined using immunohistochemistry markers based on the decision tree

Yang Li, Xu-Qing Tang, Zhonghu Bai & Xiaofeng Dai

Exploring the intrinsic differences among breast cancer subtypes is of crucial importance for precise diagnosis and therapeutic decision-making in diseases of high heterogeneity. The subtypes defined with several layers of information are related but not consistent, especially using immunohistochemistry markers and gene expression profiling. Here, we explored the intrinsic differences among the subtypes defined by the estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 based on the decision tree. We identified 30 mRNAs and 7 miRNAs differentially expressed along the tree’s branches. The final signature panel contained 30 mRNAs, whose performance was validated using two public datasets based on 3 well-known classifiers. The network and pathway analysis were explored for feature genes, from which key molecules including FOXQ1 and SFRP1 were revealed to be densely connected with other molecules and participate in the validated metabolic pathways. Our study uncovered the differences among the four IHC-defined breast tumor subtypes at the mRNA and miRNA levels, presented a novel signature for breast tumor subtyping, and identified several key molecules potentially driving the heterogeneity of such tumors. The results help us further understand breast tumor heterogeneity, which could be availed in clinics.

Breast cancer (BC) covers a group of heterogeneous diseases with different biologic, clinical, and molecular characteristics. It is important to classify breast cancers into clinically relevant subtypes for therapeutic decision-making and prognosis prediction. Classically, several different subtypes have been defined using immunohistochemistry (IHC) markers together with clinicopathologic indexes. IHC molecules, containing estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), have been traditionally used to classify breast tumors. Notably, PR status is of high correlation with that of ER, leaving ER and HER2 the determinant factors for endocrine and trastuzumab therapy. ER positive and negative tumors have distinctive clinical features and behaviors. Furthermore, HER2 is the member of the epidermal growth factor receptor family, which is well applied for prognosis and used for sub-classifying ER+ or ER− tumors into distinct subgroups, i.e., [ER+ | PR+ | HER2−] (positive ER and PR status, and negative HER2 status), [ER+ | PR+ | HER2+] (positive ER, PR and HER2 status), [ER− | PR− | HER2−] (negative ER and PR status, and positive HER2 status), and [ER− | PR− | HER2+] (negative ER, PR and HER2 status). Other IHC molecules such as the epidermal growth factor receptor (EGFR) have been identified to classify breast cancers and, in particular, among triple negative tumors.

Some work focused on the intrinsic breast cancer subgroups using large-scale gene expression profiling with the aid of gene expression array. Perou et al. identified five subgroups using different gene expression datasets, i.e., Luminal A, Luminal B, HER2, Basal-like tumor, and Normal tumor. The mRNA expression profile of the intrinsic genes was first used by Sørlie et al. in tumor subgroup identification. Parker et al. developed a
classifier, named PAM50, using a 50-gene set to identify the four major intrinsic subtypes. Additionally, microRNAs, a category of small non-coding RNA molecules regulating cell function both at the transcriptional and post-transcriptional levels, complement the prognostic marker discovery using, traditionally, gene expression data\(^{16,17}\). In this domain, a number of miRNAs, such as miR-7, miR-128a, miR-210\(^{17}\), were found differentially expressed among breast cancer subgroups. Dai et al.\(^7\) reported a set of differentially expressed genes (diff-genes), composed of 1015 mRNAs and 69 miRNAs among the four IHC-defined breast tumor subtypes, which was then reduced to a 115 gene set 1 to 3; the redundancies at these two levels. As miRNAs do not improve the classification accuracy, we include only the mRNA set 1 to 3 The mRNAs of gene set 1 to 3;

| Name of set                  | Description                                                                 |
|-----------------------------|-----------------------------------------------------------------------------|
| Gene set1                   | MRNAs and miRNAs differentially expressed between ER+ and ER− tumors;       |
| Gene set2                   | MRNAs and miRNAs differentially expressed between [ER+|PR−|HER2+|] and [ER+|PR−|HER2−|]; |
| Gene set3                   | MRNAs and miRNAs differentially expressed between [ER−|PR−|HER2+|] and [ER−|PR−|HER2−|]; |
| mRNA set 1 to 3             | The mRNAs of gene set 1 to 3;                                               |
| Feature gene                | Unified mRNAs and miRNAs of gene set 1 to 3;                                |
| RSP gene (the rest-subtype  | MRNAs and miRNAs differentially expressed in a pair-wise fashion among the  |
| pairwise genes)             | rest subtype pairs other than those along the constructed decision tree.    |

Table 1. Terminology summary.

Exploration on the molecular differences among breast cancer subtypes is of crucial importance in understanding the heterogeneity of breast tumors. Though the “intrinsic” genes can capture the differences among the defined subtypes, they could not tell the pair-wise-subtype differences, which is essential when applied for clinical use. With this aim, our study reveals the significant differences between pair-wise subgroups defined by the major IHC markers (ER, PR and HER2), integrating mRNA and miRNA expression at the transcriptional level. We explore the functional roles of these signature genes and their relationships regarding information flow using network and pathway analysis. In addition, at the transcriptional level, breast cancer subgroups could be identified hierarchically in a pair-wise fashion based on the decision tree, indicating the hierarchical differentiation pattern of breast tumors.

Results
Identification of feature genes. In HEBCS, out of the 183 invasive tumors, 182 are labeled by the ER status and 115 have the marking information on IHC biomarkers. Four subgroups are defined by ER, PR and HER2, i.e., [ER+|PR+|HER2+|], [ER+|PR+|HER2−|], [ER−|PR−|HER2+|] and [ER−|PR−|HER2−|]. In detail, the terminologies of different gene sets are listed in Table 1. The mRNA and miRNA feature genes and RSP genes are listed in Supplementary Tables 1 and 2, respectively. The feature gene set contains 30 mRNAs and 8 miRNAs while the RSP gene set is comprised of 31 mRNAs and 19 miRNAs. Worth noting that, no miRNA was found differentially expressed between [ER+|PR−|HER2+|] and [ER+|PR−|HER2−|]. Both hsa-miR-9 and its low-expression form hsa-miR-9* are over-expressed in ER− tumors. Thus, hsa-miR-9* was removed from the final panel to reduce redundancy. In HEBCS, the classifier using gene set 1 had a prediction accuracy of 0.8736 (naive Bayes classifier) and 0.9066 (naive Bayesian classifier) in subtypes stratified by ER status; it was 0.8804 (naive Bayes classifier) and 0.8804 (naive Bayesian classifier), respectively, using gene set 2 (between subtypes differed by the HER2 status among ER+ tumors), and 0.7692 (naive Bayes classifier) and 0.8804 (naive Bayesian classifier), respectively, using gene set 3 (between two subtypes differed by the HER2 status among ER− tumors). These results are shown in Table 2 and Fig. 1A–C. The performance of mRNA and miRNA signature genes was evaluated using different classifiers (SVM: 0.6667 using mRNA genes; 0.7373 with mRNA; 0.7276 integrating mRNA and miRNA genes, Fig. 1E). Tumor patterns identified using the RSP mRNAs and miRNAs were displayed in Supplementary Figs 1 and 2, respectively. Furthermore, pathway analysis with miRNA targets, RSP genes and feature genes was conducted, respectively (Supplementary Table 3). The overlapping pathways suggest the core signaling controlling breast cancer differentiation, such as signaling pathways regulating pluripotency of stem cells and VEGF signaling pathway. MiRNA targets fall into the same pathways with feature mRNAs, indicating the redundancies at these two levels. As miRNAs do not improve the classification accuracy, we include only the 30 mRNAs in the final signature gene panel.

Validation of feature genes using public datasets. The performance of the feature genes in breast tumor subtyping is validated using GSE22220 and TCGA (Fig. 1D for GSE22220 and Fig. 1F for TCGA). The naive Bayesian classifiers based on the methods of obtaining the prior knowledge regardless of different platforms were compared with the SVM classifier possessing the training process. The classification accuracies were summarized in Table 2. In GSE22220, the tumors were labeled by ER status, and the classifiers were applied using the mRNA set 1. Using the prior knowledge obtained from HEBCS, the nearest-center and naive Bayesian classifiers achieve an accuracy of 0.7963 and 0.8565, respectively, in GSE22220, and an accuracy of 0.6152 and 0.6242, respectively, in TCGA. Obviously, naive Bayesian classifier performs better than the nearest-center classifier in identifying tumor subtypes using the feature genes. The SVM classifier comprising of our feature genes is able to differentiate subtypes with an accuracy of 0.8469 and 0.7696, respectively, in GSE22220 and TCGA, which outperforms the other two methods when the training data is available. The results of different classifiers though differ, suggest that the proposed signature gene panel has a good generality. Several classifiers with obtained
The feature genes were compared with the RSP genes. Among the 30 mRNA feature genes, 6 overlapped with the RSP feature mRNAs; and out of the 7 miRNA feature genes, 5 overlapped with the 19 RSP miRNAs (Table 4). These overlapping mRNA and miRNA feature genes might be the key molecules driving breast tumor heterogeneity.

### Pathway and network analysis using the signature genes.
The expressions of the feature genes were opposite in a subtype pair-wise fashion, as they are selected based on the differentially expressed degree. These feature genes are rarely shared among different pairs (Fig. 2), suggesting succinctness of the feature genes.

Several important genes associated with breast cancer, such as ESR1\textsuperscript{17}, FZD9\textsuperscript{19} and CXCL14\textsuperscript{20}, were unveiled. The targets of miRNA feature genes were explored using miRecords. Furthermore, KEGG and KOBAS pathway analysis reveal that several cancer core pathways were enriched in the signature genes, feature genes, as well as their miRNA targets. For example, ESR1\textsuperscript{17}, responsive to estrogen related signal and so far the most important molecule distinguishing breast tumor subtypes, is present in the feature set; several well-known molecules associated with Jak-STAT signaling pathway\textsuperscript{21} such as CLEC3A, etc, are enriched in the feature genes and miRNA targets (Supplementary Table 3). FZD9 (from the RSP genes) and several targets of the miRNA feature genes (AKT2, KRAS and Nfat1) are enriched in mTOR (p = 0.007) and VEGF(p = 0.007) signalings\textsuperscript{22}. In addition, we checked the diseases relevant to the feature genes, RSP genes and mRNA targets using KEGG disease (Supplementary Table 4), with 56.7% of the enriched diseases being cancers. Gene interaction network was constructed using GeneMANIA according to the physical properties such as co-expression, genetic interaction and pathway. The network of the signature genes contains 49 mRNAs with 20 related genes subjoined and 668 links, among which co-expression attributes 79.5% and co-localization 8% (Fig. 3). In the network constructed using RSP genes (Supplementary Fig. 3), 45 genes in total were connected by 1102 links (co-expression: 81.93%, co-localization: 4.2%, genetic interactions: 4.2% and shared protein domains: 1.79%) with 20 related mRNAs added. Some key genes are densely connected, such as FOXQ1 and SFRP1, which are well-known molecules driving the heterogeneity and progress of breast tumors.

### Discussion
The mRNA and miRNA feature genes, identified using HEBCS, could efficiently differentiate the four IHC-defined tumor subtypes as indicated by the statistics obtained using two other public datasets (Table 2). This suggests that decision tree is an effective approach for identifying feature genes differentiating breast cancer subtypes. We found 6 mRNAs and 5 miRNAs overlapping between the feature genes and RSP genes (Table 3), 3 mRNAs (ESR1, NFIX, SFRP1) overlapping among the signature genes, the Sorlie's signature and PAM50 genes. Most of the feature genes are shared with Dai's diff-genes (Table 5), except for C8orf85, CENPW, CENPV, CXCL14 and has-miR-1238, which are revealed only from the decision tree. These overlapping genes may drive breast cancer differentiation, and the 5 genes exceptionally obtained using the decision tree capture the pair-wise differences along the constructed tree assuming that ER is the predominant differentiation factor followed by HER2. With the obtained feature genes, three classifiers are applied to subtype the tumors in the specific usage (Table 4). By using network and pathway analysis, it reveals that co-expression accounts for the most physical properties (79.5% in feature gene network, 81.93% in the RSP gene network), because the gene expression profiling is the key factor to predict the interactions. Some genes, well-known in breast cancer subtyping and involved in the cancer-relevant pathways, are the hubs of the network, e.g., FOXQ1, SFRP1 and ESR1. From the biological view, the roles of selected genes in the regulation of cancer should be analyzed.

| Dataset  | Gene          | Dimension | Nearest-center classifier | Naïve Bayesian classifier | SVM | Purpose          |
|---------|---------------|-----------|---------------------------|----------------------------|-----|-----------------|
| HEBCS   | mRNA set 1    | 10        | 0.8736                    | 0.9066                     |     | Identification  |
|         | mRNA set 2    | 10        | 0.8804                    | 0.8804                     |     | Identification  |
|         | mRNA set 3    | 10        | 0.7692                    | 0.8846                     |     | Identification  |
|         | mRNA feature genes | 30    | 0.7203                    | 0.7712                     | 0.7373 | Validation  |
|         | miRNA feature genes | 8     | —                         | —                          | 0.6666 | Validation  |
|         | Feature genes  | 38        | —                         | —                          | 0.7276 | Validation  |
| GSE22220 | Gene set 1    | 6         | 0.7963                    | 0.8565                     | 0.8469 | Validation  |
| TCGA    | mRNA feature genes | 24    | 0.6152                    | 0.6242                     | 0.7696 | Validation  |

**Table 2. Comparison of classification accuracy based on the differentially expressed genes using different datasets.** Note: The gene set 1 to 3, along the decision tree can distinguish the subtypes hierarchically, using feature genes are not able to be used for subtyping validation here using neither nearest center classifier nor naïve Bayesian classifier as the hierarchical decision tree was broken with no miRNA found differentially expressed between [ER+|^PR^+|^HER2^+| and |ER^+|^PR^+|^HER2^−|. SVM classifier is applied to the miRNA feature genes for subtyping validation using 5-fold cross-validation. Only the mRNA set1 was used for subtyping validation in GSE22220 as only ER status is available in this dataset.
No differentially expressed miRNA was found between the [ER⁺|PR⁺|HER2⁺] and [ER⁺|PR⁺|HER2−] subgroups, indicating that ER⁺ tumors are less diverse than ER− tumors. The fact that rather few miRNA was found and less accuracy was obtained once miRNA was included, which might be caused by the following two reasons. First, miRNAs function in driving phenotypic differences through regulating mRNA expression, thus information at these two levels is redundant to some extent. Second, miRNA regulation is a complex and indirect process, with many steps potentially introducing noise, e.g., the feature miRNAs may regulate some non-feature mRNAs, complicating the subtyping process. No overlap was observed between mRNA feature genes and the validated targets of miRNAs, elucidating the concision of the feature genes. Hsa-miR-135a and hsa-miR-135b play crucial roles in distinguishing breast tumors by ER status, which has been extensively

**Figure 1. Heatmaps measuring the performance of different gene sets.** Using (A) mRNA set1 to identify ER⁺ and ER− tumors in HEBCS; (B) mRNA set2 to distinguish [ER⁺|PR⁺|HER2⁺] and [ER⁺|PR⁺|HER2−] in HEBCS; (C) mRNA set3 to identify [ER−|PR−|HER2⁺] and [ER−|PR−|HER2−] in HEBCS; (D) gene set 1 to identify ER⁺ and ER− tumors in GSE22220; (E) the feature mRNA sets to classify breast tumors in HEBCS; (F) the mRNA feature genes to identify breast tumor subtypes in TCGA.

**Feature miRNAs.** No differentially expressed miRNA was found between the [ER⁺|PR⁺|HER2⁺] and [ER⁺|PR⁺|HER2−] subgroups, indicating that ER⁺ tumors are less diverse than ER− tumors. The fact that rather few miRNA was found and less accuracy was obtained once miRNA was included, which might be caused by the following two reasons. First, miRNAs function in driving phenotypic differences through regulating mRNA expression, thus information at these two levels is redundant to some extent. Second, miRNA regulation is a complex and indirect process, with many steps potentially introducing noise, e.g., the feature miRNAs may regulate some non-feature mRNAs, complicating the subtyping process. No overlap was observed between mRNA feature genes and the validated targets of miRNAs, elucidating the concision of the feature genes. Hsa-miR-135a and hsa-miR-135b play crucial roles in distinguishing breast tumors by ER status, which has been extensively
These suggest that metastasis potential is another important index here to differentiate known as basal markers, also has a member, i.e., KRT6A, found in this gene set. It is reported to have potential which together with AGR2 are both associated with breast cancer and ovarian cancer. BCMP11, was originally identified as a membrane protein from breast cancer cell lines, and has been traditionally applied for breast tumor subtyping and prognosis. As expected, it is found within this set and over-expressed in ER− tumors. Similarly, CA12 and AGR3 are also up-regulated in ER+ tumors. It is reported that carbonic anhydrase XII (CA12), encoding a zinc metalloenzyme responsible for acidification of the microenvironment of cancer cells, is regulated by estrogen via ERs in breast cancer cells, and that this regulation involves a distal estrogen-responsive enhancer region in human breast tumors. Moreover, several other keratins (e.g., KRT14, KRT15) are included to construct theFeature mRNA set1. Genes belonging to this set stratify breast cancer by ER status. ER, also named ESR1, mediates the biological effects of estrogens through the estrogen response elements (EREs) of the target genes, and has been traditionally applied for breast tumor subtyping and prognosis. As expected, it is found within this set and over-expressed in ER− tumors. Similarly, CA12 and AGR3 are also up-regulated in ER+ tumors. It is reported that carbonic anhydrase XII (CA12), encoding a zinc metalloenzyme responsible for acidification of the microenvironment of cancer cells, is regulated by estrogen via ERs in breast cancer cells, and that this regulation involves a distal estrogen-responsive enhancer region in human breast tumors. AGR3, also named breast cancer secreted protease inhibitor α-secreted protease inhibitor α-2-macroglobulin (A2M)-like-1, activates mutations in signal transducers of the RAS/mitogen-activated protein kinase (MAPK) pathway. FZD9 encodes WNT receptors and is an important factor affecting WNT signaling. MAPK and WNT pathways both contribute in cell proliferation control, suggesting that cell proliferation is a key property driving the differences between ER+ and ER− tumors. Keratin, known as basal markers, also has a member, i.e., KRT6A, found in this gene set. It is reported to have potential relevance to circulating tumor cells, which might function as an early marker for breast cancer metastasis or monitor therapy efficacy. These suggest that metastasis potential is another important index here to differentiate ER+ and ER− tumors. Moreover, several other keratins (e.g., KRT14, KRT15) are included to construct the gene interaction network. VGLL1 (Vestigial-like 1) is a gene encoding a transcriptional co-activator modulating the Hippo pathway, which is known to be associated with a basal-like phenotype in breast cancer. Participation of FZD9 in carcinogenesis has been reported in various cancers, indicating their potential roles in breast cancer, such as mTOR signaling pathway.

### Table 3. The comparison of nearest-center classifier, naïve Bayesian classifier and SVM classifier.

| Classifier           | Advantage                              | Disadvantage                                           |
|----------------------|----------------------------------------|--------------------------------------------------------|
| Nearest-center classifier | Convenient to be applied to the clinical prediction | Linear classifier with unsatisfactory accuracy.          |
| The naïve Bayesian classifier | Obtain the priori probability distribution; Identify the subgroups in same or other datasets. | The prior knowledge obtained based on relatively abundant labeled patterns. |
| SVM classifier       | Predict with an acceptable accuracy if training data is available. | Challenging to apply the results to the different datasets |

### Table 4. Overlapping genes between the feature genes and RSP genes.

| mRNA    | miRNA      | miRNA    |
|---------|------------|----------|
| P13     | VGLL1      | FZD9     |
| LOC400578 | KRT6A     | SOX8     |
|         | hsa-miR-190b | hsa-miR-184 |
|         | hsa-miR-135a | hsa-miR-1238 |
Feature mRNA set2. Genes belonging to this set differentiate ER+ tumors by HER2 status. In particular, TCN1, SFRP1, NKX3-1 and NFIX are suppressed in the [ER+|PR+|HER2+] subtype and up-regulated in the [ER+|PR+|HER2−] subtype. TCN1 is reported to be a breast cancer-related gene, which affects replication timing with expression significantly differ between normal and malignant cell lines. SFRP1 encodes the secreted frizzled-related protein 1 which is a soluble Wnt antagonist, and its inactivation is known to be associated with unfavorable prognosis among breast cancer patients. NKX3-1, a prostate-specific tumor suppressor gene is the earliest known marker of prostate epithelium during embryogenesis and is subsequently expressed at all stages of prostate differentiation in vivo. Hypermethylated NFIX is identified in the breast cancer model, its differential expression among ER positive tumors as stratified by HER2 status suggests the role of methylation in regulating such a phenotypic difference.

Also found in this set are MAL2, ORMDL3, SYT13, CST6, PGAP3 and CLEC3A, which are lowly expressed in the [ER+|PR+|HER2−] subtype but highly expressed in the [ER+|PR+]HER2+ subgroup. MAL2, Mal, T-cell differentiation protein 2, has been identified as a molecule predictive of metastases whose increased expression has been validated in ovarian, colorectal and pancreatic cancer. CST6 is a breast tumor suppressor expressed in normal breast epithelium, but epigenetically silenced as a consequence of promoter hypermethylation in metastatic breast cancer cell lines, which suggests the mechanism of CST6 loss during breast tumorigenesis and/or progression to metastasis. PGAP3 was reported to be specifically expressed in HER2+ tumor cells but not in...
stroma or HER2 non-amplified breast tumor samples. Its differential expression in [ER+|PR+|HER2−] and [ER+|PR+|HER2+] with over-expression in HER2+ luminal tumors is concordant with the previous reports. Synaptotagmin 13 (SYT13) is identified as a putative liver tumor suppressor gene, complementing a molecular defect in GN6TF liver tumor cells and giving rise to tumor suppression through induction of rat WT1. CLEC3A is a heparin-binding, cell adhesion modulator, whose cleavage in tumor microenvironments may affect tumor cell invasion and metastasis by modulating tumor cell adhesion and the plasminogen/plasminogen-activator interaction.

Figure 3. The gene network constructed using GeneMANIA. The network, constructed by the feature genes and 20 related genes, contains 668 links. Different attribution links are labeled by different colors and the mRNA feature genes are indicated with stripes in the gene network.

Table 5. The shared genes compared with Dai’s diff-genes.
system. By pathway analysis, CLEC3A participates in signaling pathways regulating pluripotency of stem cells. Genes involved in JAK-STAT signaling are enriched in the gene set 2, with p value = 0.038. Several basic pathways were also involved, such as signaling pathways regulating pluripotency of stem cells and viral carcinogenesis. Variants of ORMDL3 were expressed in human breast cancer cell lines, but the functional relevance of ORMDL3 in breast cancers has not been reported. In our study, it is differentially expressed in ER+ tumors, which provides evidence for their relation.

**Feature mRNA set 3.** Genes within this set differentiate tumors within ER− tumors as stratified by HER2 status. RH110, C8orf85, FOXQ1, CENPW and CENPV are suppressed in the [ER−PR−HER2+] subgroup, but elevated in the [ER−PR−HER2−] subgroup. RDH10 was reported to play critical oncogenic roles in tumor progression for patients of non-small-cell lung cancer, and was involved in tumors with lymph node invasion. FOXQ1 expression is regulated by TGFB-31 and involved in the EMT process. Here, its high expression in [ER−PR−HER2−] tumors as compared with [ER−PR−HER2+] accords with our conception that metastasis is an easily gained property of triple negative cancers and suggests its importance in differentiating tumors of these two subtypes. CENPV is required for centromere organization, chromosome alignment and cytokinesis, and CENPW plays crucial roles in the formation of a functional kinetochore involved in cell division during mitosis. Over-expression of these two genes in [ER−PR−HER2−] tumors suggests the crucial role of irregular cell cycle signaling in triple negative tumors that distinguish it from [ER−PR−HER2+] tumors.

**Conclusion**

We studied the intrinsic molecular differences of breast cancer subtypes labeled by the three major IHC markers (ER, PR and HER2) in a pair-wise fashion following a decision tree. By presenting a set of feature genes, we capture the differences on molecular profiling among breast cancer subtypes pair-wisely, rather than re-define them into finely grained subgroups. This is fundamentally different from, where genes differentiating breast tumor subtypes are identified in an ensemble fashion. According to the decision tree constructed using ER, PR and HER2, the feature genes along each branch (gene sets 1 to 3) as well as those differentiating cross-branch pairs (subtype-specific genes) are presented. Gene sets 1 to 3 altogether compose the feature genes. Besides availing in precise diagnosis, genes revealed here could also be utilized to achieve efficient therapeutic treatment of triple negative tumors via modulating the expression of the pivotal genes controlling breast tumor subtype switches. That is, while daunorubicin is a commercial drug for luminal A breast cancers, we could apply it for triple negative tumor treatment after applying therapies transiting triple negative tumors into the Luminal A subtype. This could be achieved via modulating the expression of the genes in the corresponding pair. Therefore, these pair-wisely revealed genes have profound clinical implications.

Network and pathway analysis revealed the physical interaction and relationships among the selected feature genes and importance of genes in the gene network and metabolic pathways. Though computational analysis facilitates our understanding towards the functions of these genes, solid experimental validations and functional studies are indispensable to further consolidate our findings before clinical use.

Conclusively, our study bridges the gap between immunohistochemistry markers and gene expression profiling in breast tumor subtyping at the mRNA and miRNA levels, which helps us better understand breast cancer heterogeneity in a pair-wise fashion. More importantly, these genes deepen our understandings towards breast cancer differentiation, and imply an indirect efficient therapeutic strategy for subtypes without targeted therapy.

**Material and Method**

**Materials.** The three gene expression datasets used in ref. 7 for feature gene identification among breast tumors have been employed in this study. HEBCS was used to identify the differentially expressed gene sets among the IHC-defined subtypes, and GSE22220 together with TCGA was applied to validate the selected gene biomarkers. HEBCS is comprised of mRNA (GSE24450) and miRNA (GSE43040) expression data and retrieved from the GEO database (Gene Expression Omnibus), with the experiments carried out at SCIBLU Genomics Centre, Lund University, Sweden. This dataset harbors 24660 mRNAs (Illuma HumanHT-12_V3 Expression BeadChips) and 1104 miRNAs (Illumina HumanMI V2 BeadChips) for 183 primary breast tumor samples from the department of Oncology of the Helsinki University Central Hospital (HUCH) and department of Surgery. Among them, 115 tumors were labeled unambiguously by the status of ER, PR and HER2, which were grouped
into four subtypes i.e., \([\text{ER}^+|\text{PR}^+]\text{HER}2^-, [\text{ER}^+|\text{PR}^+]\text{HER}2^+, [\text{ER}^-|\text{PR}^-]\text{HER}2^+\) and \([\text{ER}^-|\text{PR}^-]\text{HER}2^-,\)

based on these markers.

GSE22220 is composed of mRNA (GSE22219) and miRNA (GSE22216) expression profiling from GEO54. GSE22219 contains 24332 Entrez Gene entities for 216 tumor samples that were processed and hybridized to Illumina Human Ref-8 V1 expression Bead Chips. GSE22216 contains 734 probes (Illumina HumanMI V1 BeadChips) for 207 samples. Only ER status is available in GSE22220, based on which these samples were grouped into \(\text{ER}^+\) and \(\text{ER}^-\) tumors.

TCGA dataset (level 3) was retrieved from the TCGA portal at http://tcga.cancer.gov/dataportal, which contains 17814 mRNAs for 451 samples and 1046 miRNAs (IlluminaGA_mRNASeq) for 315 patients. The mRNA dataset was produced from the Agilent 244 K Custom Gene Expression G4502A-07-3 platform, and the miRNA data was generated using IlluminaGA_mRNASeq7. These primary solid tumor samples were classified into the four IHC-characterized subtypes as defined in the HEBCS data.

**Methods**

**Data normalization.** Normalization of gene expression data from different platforms was conducted. The regulatory direction of the listed genes in each set was denoted by \(\alpha \in \{-1, 0, 1\}\), with \(-1, 0, 1\) each representing down-, normal- and up-expression, respectively. The average gene expression \(x\) is denoted by \(\bar{x}\), with the standard deviation being marked by \(\delta\). The gene expression data was discredited into the status of genes \(\bar{x}\), by the following rules:

\[
\text{The gene is} \quad \begin{cases} 
\text{down - expressed} & x < \bar{x} - \delta \\
\text{normal} & x \in [\bar{x} - \delta, \bar{x} + \delta], \\
\text{up - expressed} & x > \bar{x} + \delta.
\end{cases}
\] (1)

**Decision tree construction and feature gene identification.** Breast tumors can be grouped by ER status into \(\text{ER}^+\)-positive and \(\text{ER}^-\)-negative tumors. Tumors of these two branches could be each further divided into two subtypes by \(\text{HER}2\) status, resulting in four subgroups, i.e., \([\text{ER}^+|\text{PR}^+]\text{HER}2^+, [\text{ER}^+|\text{PR}^+]\text{HER}2^-, [\text{ER}^-|\text{PR}^-]\text{HER}2^+\) and \([\text{ER}^-|\text{PR}^-]\text{HER}2^-\). Note that \(\text{PR}\) status is in consistent with that of \(\text{ER}\) in most cases. This subtype identification procedure can be described by a decision tree (Fig. 4), which splits this complex partitioning process into a union of several simple decisions38.

HEBCS data was used to detect the differentially expressed genes which was pre-processed following instructions in ref. 7. Differentially expressed genes were identified in a pair-wise fashion (i.e., \(\text{ER}^+\) vs. \(\text{ER}^-, [\text{ER}^+|\text{PR}^+]\text{HER}2^+\) vs. \([\text{ER}^+|\text{PR}^+]\text{HER}2^-, [\text{ER}^-|\text{PR}^-]\text{HER}2^+\) and \([\text{ER}^-|\text{PR}^-]\text{HER}2^-\)), assuming that \(\text{ER}\) drives the major difference as indicated by many studies33,37,38. Differentially expressed genes among breast cancer subgroups were selected according to the following rules:

I) Significant difference on gene expression was observed between pair-wise subtypes under comparison.

II) The average standard deviations in the same subgroup are relatively small.

III) The correlation coefficients of the selected genes are small in absolute value. This is to ensure that the list contains the most succinct number of genes.

The pair-wise identification process is composed of three steps. First, select the distinguishable genes between pair-wise subgroups. That is, genes with base-2 logarithmic fold change larger than 1 as compared with the average expression of each group were chosen. Second, choose differentially expressed genes in a group pair-wise fashion. In this step, the distinguishable genes were filtrated using moderated t-test. That is, the expression level of genes between two subgroups with \(p\)-value < 0.05 were considered differentially expressed39. Third, measurement of differentially expressed degree was presented. The degree of differential expression was measured by introducing the intra-class difference \(M_{\text{intra}}\) and inter-class difference \(M_{\text{inter}}\) which are formulated as

![Decision tree for pair-wise identification of the feature genes of breast cancer subtypes](image-url)
The differentially expressed genes are sorted using the F index. In this study, we considered the difference between two groups, \( i = \{1, 2\} \). If more than 10 genes were differentially expressed, only the top 10 were selected as the feature gene set. Following this process, genes distinguishing ER+ and ER− tumors are called ‘gene set1’, and those discriminating [ER+ | PR+ | HER2+] vs. [ER+ | PR+ | HER2−] and [ER− | PR− | HER2+] vs. [ER− | PR−] HER2−‘ pairs are identified as ‘gene set2’ and ‘gene set3’, respectively. The union of gene sets 1 to 3 is named the ‘feature genes’. Additionally, the differentially expressed genes between the rest-subtype-pairwise genes (RSP genes) were also explored, which contains [ER+ | PR+ | HER2+] vs. [ER− | PR− | HER2+], [ER+ | PR+ | HER2+ vs. [ER− | PR− | HER2−], [ER− | PR− | HER2+] vs. [ER+ | PR+ | HER2−] and [ER− | PR+ | HER2− vs. [ER− | PR−] HER2−‘ pairs.

**Feature gene validation.** A decision tree is constructed to identify the subtypes hierarchically, which can retrieve the prior knowledge from the discovery dataset and apply it to a new dataset based on the normalized gene expression regardless of the experimental platform. The priori probability distribution of feature genes in each subgroup is obtained from the discovery dataset and then applied for subtype identification in a new dataset by the naïve Bayesian classifier using normalized gene expression. Nearest center principle, on the other hand, is a traditional technique for subtype identification and applied here as a comparison. Additionally, as a comparison, SVM classifier is applied to identify the subtypes with kernel functions, equipped with training sets within same platform.

**The naïve Bayesian classifier.** The naïve Bayesian classifier\(^{61,62}\) was applied to calculate the probability that one tumor sample belongs to a certain subgroup. Assuming the signature gene expression is conditionally independent, the conditional probability \( P(x | c_j) \) is expressed as \( P(x | c_j) = \prod_{i=1}^{n} p(x_{ij} | c_j) \), where \( C \) is the tumor subtypes and \( c_j \in C \). We use HEBCS as the discovery data set to train the naïve Bayesian classifier. Given a new pattern with the gene status, the classifier produces a posterior probability distribution over the possible subgroups, i.e.,

\[
P(c_j | x) = \frac{p(x | c_j) p(c_j)}{p(x)}.
\]

With the goal of assigning tumor samples to the subgroups with the highest accuracy, the objective function is written as

\[
C_{map} = \arg \max_j P(c_j | x).
\]

**Nearest center principle.** The tumor samples \( T_x \) were assigned to the closest group as measured by Euclidean distance, and the nearest-center classifier was designed\(^{63}\) as
Network and pathway analysis using feature genes. To investigate the intrinsic heterogeneity of breast cancer, metabolic pathway and network analysis were applied to the obtained feature genes.

MiRecords44 is a resource for predicting miRNA targets, which integrates experimentally validated miRNA targets having systematic experimental support and predicted miRNA targets using 11 established prediction algorithms (DIANA-microT, MirTarget2 and TargetScan/TARGETScanS, etc). It was used to find the targets of the feature miRNA genes. The gene network was constructed using GeneMANIA45 (physical attributions: co-expression, co-localization, genetic interactions, pathway, physical interactions, predicted and shared protein domains; automatically selected weighting method was used) to further elucidate the functional roles of the feature genes and the characteristics of each subtype. In addition, we used DAVID66 (in functional annotation clustering, similarity term overlap: 3; threshold: 0.5; enrichment thresholds: 1.0 and Benjamini is used. In functional annotation chart, threshold count: 2; ease: 0.1; display way: Benjamini) and KOBAS20 (statistical method: hypergeometric test/fisher’s exact test; FDR correction method: Benjamini and Hochberg; Small term cutoff: 5) to interpret the enrichment of gene ontology, metabolic pathways and relevant diseases of these unified feature miRNAs and miRNA targets. The whole process for feature gene identification, validation, and breast tumor heterogeneity exploration, is illustrated in Fig. 5.

References
1. Simpson, F. T., Reis-Filho, J. S., Gale, T. & Lakhani, S. R. Molecular evolution of breast cancer. J pathol 205, 248–254 (2005).
2. Dunnwald, L. K., Rossing, M. A. & Li, C. I. Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. Breast Cancer Res 9, R6 (2007).
3. Dai, X. et al. Breast cancer intrinsic subtype classification, clinical use and future trends. A j cancer res 5, 3292 (2015).
4. Blows, F. M. et al. Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS med 7, e1000279, doi: 10.1371/journal.pmed.1000279 (2010).
5. Pusztai, L. et al. Effect of Molecular Disease Subsets on Disease-Free Survival in Randomized Adjuvant Chemotherapy Trials for Estrogen Receptor–Positive Breast Cancer. j Clin Oncol 26, 4679–4683 (2008).
6. Vallejos, C. S. et al. Breast cancer classification according to immunohistochemistry markers: subtypes and association with clinicopathologic variables in a peruvian hospital database. Clin breast cancer 10, 294–300 (2010).
7. Dai, X., Chen, A. & Bai, Z. Integrative investigation on breast cancer in ER, PR and HER2–defined subgroups using miRNA and miRNA expression profiling. Sci rep 4 (2014).
8. Walker, R. A. Immunohistochemical markers as predictive tools for breast cancers. J clin pathol 61, 689–696 (2008).
9. Charafae-Jaffreft, E. et al. Gene expression profiling of breast cell lines identifies potential new basal markers. Oncogene 25, 2273–2284 (2006).
10. Rakha, E. A. et al. Triple-negative breast cancer: distinguishing between basal and nonbasal subtypes. Clin Cancer Res 15, 2302–2310 (2009).
11. van’t Veer, L. J., Paik, S. & Hayes, D. F. Gene expression profiling of breast cancer: a new tumor marker. J clin oncol 23, 1631–1635 (2005).
12. Sorlie, T. et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. PNAS 98, 10869–10874 (2001).
13. Perou, C. M. et al. Molecular portraits of human breast tumours. Nature 406, 747–752 (2000).
14. Sorlie, T. et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. PNAS 100, 8418–8423 (2003).
15. Parker, J. S. et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J clin oncology 27, 1160–1167, doi: 10.1200/ JCO.2008.18.1570 (2009).
16. Blenkiron, C. et al. MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol 8, R214 (2007).
17. Buffa, F. M. et al. microRNA-associated progression pathways and potential therapeutic targets identified by integrated miRNA and microRNA expression profiling in breast cancer. Cancer res 71, 5635–5645 (2011).
18. Dai, X., Li, Y., Bai, Z. & Tang, X.-Q. Molecular portraits revealing the heterogeneity of breast tumor subtypes defined using immunohistochemistry markers. Sci rep 5 (2015).
19. Winn, R. A. et al. Antitumorigenic effect of Wnt 7a and Fzd 9 in non-small cell lung cancer cells is mediated through ERK5-dependent activation of peroxisome proliferator-activated receptor γ. J Biol Chem 281, 26943–26950 (2006).
20. Gu, X.-L. et al. Expression of CXCL14 and its anticancer role in breast cancer. Breast cancer res treat 135, 725–735 (2012).
21. Buettner, R., Mora, L. B. & Jove, R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin cancer res 8, 945–954 (2002).
22. McMahon, G. VEGF receptor signaling in tumor angiogenesis. The Oncologist 5, 3–10 (2000).
23. Hildebrandt, M. et al. Hsa-miR-9 methylation status is associated with cancer development and metastatic recurrence in patients with clear cell renal cell carcinoma. Oncogene 29, 5724–5728 (2010).
24. Kastl, L., Brown, I. & Schofield, A. miRNA-34a is associated with docetaxel resistance in human breast cancer cells. Breast cancer res treat 131, 445–454 (2012).
25. Oka, H. et al. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. Cancer res 53, 1696–1701 (1993).
26. HWANG, J. T. et al. Resveratrol induces apoptosis in chemoresistant cancer cells via modulation of AMPK signaling pathway. Ann N Y Acad Sci 1095, 441–448 (2007).
27. Zhou, M. et al. A novel onco-miR-365 induces cutaneous squamous cell carcinoma. Carcinogenesis 34, 1653–1659 (2013).
28. Bellacosa, A. et al. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int J cancer 64, 280–285 (1995).
29. Paranjape, T. et al. A 3′-untranslated region KRAS variant and triple-negative breast cancer: a case-control and genetic analysis. The lancet oncol 12, 377–386 (2011).
30. Marotta, L. et al. The JAK2/STAT3 signaling pathway is required for growth of CD44+ CD24–stem cell–like breast cancer cells in human tumors. J clin Invest 121, 2723–2735 (2011).
31. Barnett, D. H. et al. Estrogen receptor regulation of carbonic anhydrase XII through a distal enhancer in breast cancer. Cancer res 68, 3505–3515 (2008).
The authors declare no competing financial interests. Competing financial interests: accompanies this paper at http://www.nature.com/srep

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.
How to cite this article: Li, Y. et al. Exploring the intrinsic differences among breast tumor subtypes defined using immunohistochemistry markers based on the decision tree. Sci. Rep. 6, 35773; doi: 10.1038/srep35773 (2016).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2016