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Integrative investigation on breast cancer in ER, PR and HER2-defined subgroups using mRNA and miRNA expression profiling

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Exploring the molecular difference among breast cancer subtypes is of crucial importance in understanding its heterogeneity and seeking its effective clinical treatment. For this, several layers of information including immunohistochemical markers and a variety of high-throughput genomics approaches have been intensively used. Here we have explored the intrinsic differences among breast cancer subgroups defined by immunohistochemical expression (IHC) of hormone receptors ER and PR as well as human epidermal growth factor receptor 2 (HER2) using the mRNA and miRNA expression profiles of 115 tumors. A core basal group was further defined by epidermal growth factor receptor and cytokeratin 5/6 IHC expression and compared to triple negative group. A set of differentially expressed genes including 1015 mRNAs and 69 miRNAs was found to distinguish tumor subtypes whose generality was demonstrated using two independent data sets. The network was explored for each subtype and biomass synthesis signaling was found to play an important role in the core basal subgroup. This study contributes to elucidating the intrinsic relations among breast cancer subgroups defined by ER, PR and HER2 expression via integrating mRNA and miRNA expression. The results can avail functional studies of breast cancer with translational potential for clinical use.

Breast cancer is a heterogeneous disease where classically, immunohistochemistry (IHC) markers, together with clinicopathologic variables are used for prognosis prediction and treatment selection^{1,2}. The commonly used IHC markers include estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). ER-positive tumors account for >70% of breast cancer³, respond to anti-estrogen or aromatase inhibitors and have a more indolent clinical course². ER-negative tumors are hormone-independent and characterized by a more aggressive behavior⁴. PR is induced by estrogen and is a favorable prognostic marker¹. HER2 is the most well-known prognostic member of the epidermal growth factor receptor family, whose amplification or over-expression is predictive of poor survival¹. HER2 status can be used to subclassify also [ER-/PR-] tumors into distinct subgroups of [ER-/PR-]HER2+ (breast tumors with negative ER and PR status, and positive HER2 status) and [ER-/PR-]HER2- (breast tumors with negative ER, PR and HER2 status), where [ER-/PR-]HER2-, called triple negative phenotypic tumors (TNP) given their negative status of all the three receptors, forms a clinically challenging group with poor prognosis and difficult to treat⁵. A core basal subgroup (CoreBasal) with distinct clinical and immunophenotypic differences has been more recently distinguished within the triple negative group using the epidermal growth factor receptor (EGFR, also known as HER1) which is associated with a lack of ER and poor prognosis¹ and cytokeratins such as KRT5, KRT6, KRT14, KRT17 which are over-expressed in core basal tumors⁶. Particularly, triple negative tumors with positive EGFR or KRT5/6 staining are referred to as core basal tumors⁷. The 2012 IMPAKT task force investigated the medical usefulness of current methods for breast cancer subgroup classification and recommended the use of ER and HER2 for the identification of clinically relevant breast cancer subgroups⁸.

Breast cancer subgroup classification based on IHC markers is widely used in both clinical and research settings due to its reliability and reproducibility⁹. Several so called intrinsic breast cancer subgroups have been identified by large-scale gene expression profiling and shown to be associated with differential prognosis of the patients¹⁰. In year 2000, Perou et al. proposed that breast tumors could be classified into at least four intrinsic subgroups, i.e.,



Table 1 | Gene number in each diff-gene set. The unified diff-genes includes the unique genes in the diff-gene sets of [ER+/PR+]HER2-, [ER+/PR+]HER2+, [ER-/PR-]HER2+ and TNP subgroups

	[ER+/PR+]HER2-	[ER+/PR+]HER2+	[ER-/PR-]HER2+	TNP	CoreBasal	Union
mRNA	379	65	152	777	535	1015
miRNA	30	3	5	58	64	69

luminal-like, HER2 positive, basal-like and normal-like tumors¹¹. These subgroups are similar but not identical to the classification according to receptor classification using IHC. Particularly, luminal A and luminal B intrinsic subgroups are often considered to represent the [ER+/PR+]HER2- (breast tumors with positive ER and PR status, and negative HER2 status) and [ER+/PR+]HER2+ (breast tumors with positive ER, PR and HER2 status) subgroups, respectively¹², and the intrinsic HER2 subgroup refers to [ER-/PR-]HER2+ tumors¹². Recently, it was reported that only part of luminal B tumors are HER2 positive and additional luminal B tumors can be distinguished from luminal A tumors by the proliferation marker Ki67¹³. MiRNAs, a category of small (approximately 22-nucleotide) non-coding RNAs with regulatory activity, have attracted much attention in tumor subgroup characterization due to their regulatory role in mRNA expression¹⁴. A number of differentially expressed miRNAs among breast cancer subgroups have been identified, and the miRNA patterns distinguish between the basal-like and luminal tumors (luminal A and luminal B)¹⁴.

A number of studies have been conducted to further define tumor subgroups, indicating also finer-grained subgroups within each major group^{15,16}. Curtis et al. recently identified 10 intrinsic breast cancer subgroups by jointly clustering the copy number and gene expression data of 2000 breast tumors¹⁵. The Cancer Genome Atlas (TCGA) network investigated breast cancer subgroups by incorporating information from multiple platforms, i.e., genomic DNA copy number arrays, DNA methylation, exome sequencing, mRNA arrays, miRNA sequencing and reverse-phase protein arrays. They conclude that diverse genetic and epigenetic alterations converge phenotypically into four major expression-only breast tumor subgroups, i.e., luminal A, luminal B, HER2 positive and triple negative¹⁶.

With the aim of uncovering the intrinsic differences and heterogeneity of breast cancer at multiple levels, we integrated mRNA and miRNA expression and network analysis in characterizing breast cancer subgroups defined by expression of the major hormonal and growth factor receptors, i.e., ER, PR and HER2, and additionally identified the differences between the core basal and triple negative subgroups at the gene and network levels.

Results

Differentially expressed genes in each subgroup. Among the 183 samples in the Helsinki Breast Cancer Study (HEBCS) data set, 115

invasive tumors could be determined by our available IHC marker information (Supplementary Tables 1 and 2). Four subgroups, i.e., [ER+/PR+]HER2-, [ER+/PR+]HER2+, [ER-/PR-]HER2+ and triple negative phenotype (TNP) tumors, were defined, with core basal (CoreBasal) tumors distinguished from the TNP subgroup as a separate group. As seen from table 1, we identified the mRNA and miRNA differentially expressed genes (diff-genes) for each of the five IHC defined subgroups (diff-gene sets are listed in Supplementary Tables 3 and 4), and the unified diff-gene set is the union of all the differentially expressed genes containing altogether 1015 mRNAs (the unified mRNA diff-genes) and 69 miRNAs (the unified miRNA diff-genes). The majority of the genes in the unified diff-gene set come from TNP (777 mRNAs, 58 miRNAs) and CoreBasal (535 mRNAs, 64 miRNAs) tumors, which were analysed using the least number of patients (18 TNP including 12 CoreBasal tumors), suggesting that the different number of genes among subgroups within the unified diff-gene set is not driven by the sample size but the intrinsic nature of the subgroups. The Venn diagrams of the diff-genes of the subgroups are presented in Figures 1A (mRNAs) and 1B (miRNAs), respectively. The TNP and [ER+/PR+]HER2- subgroups show the biggest overlap both for mRNAs and miRNAs. Specifically, 78% of [ER+/PR+]HER2- mRNA diff-genes overlap with TNP mRNA diff-genes and 87% of [ER+/PR+]HER2- miRNA diff-genes overlap with TNP miRNA diff-genes. Only one gene, KRT5, is common among the four sets of mRNA diff-genes, and is down-regulated in [ER+/PR+] tumors and up-regulated in [ER-/PR-] tumors. On the other hand, 84% of [ER-/PR-]HER2+ mRNA diff-genes are subgroup specific with only 16% overlap with the other subgroups, and [ER+/PR+]HER2+ miRNA diff-genes are exclusively subgroup specific.

Generality of the differentially expressed genes. The subgroups could be well differentiated with our diff-genes in the public data sets, especially for mRNA (Figure 2). MiRNA diff-genes performed less accurately as compared with mRNA diff-genes. As miRNA and mRNA data share the same sample set, this difference is probably due to the complexity of the conversion from miRNA expression to the phenotypic differences among different breast tumor subgroups, which is mediated and influenced by many factors such as post-transcriptional regulations and generally large number of varieties of miRNA gene targets. Nevertheless, we could not rule out the

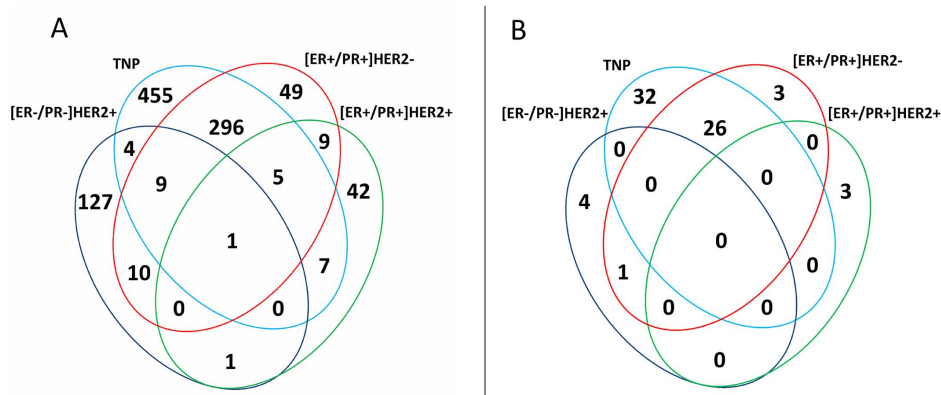


Figure 1 | Venn diagram of subgroup specific diff-genes, indicating numbers of genes in the different or overlapping groups. (A) mRNA, (B) miRNA.

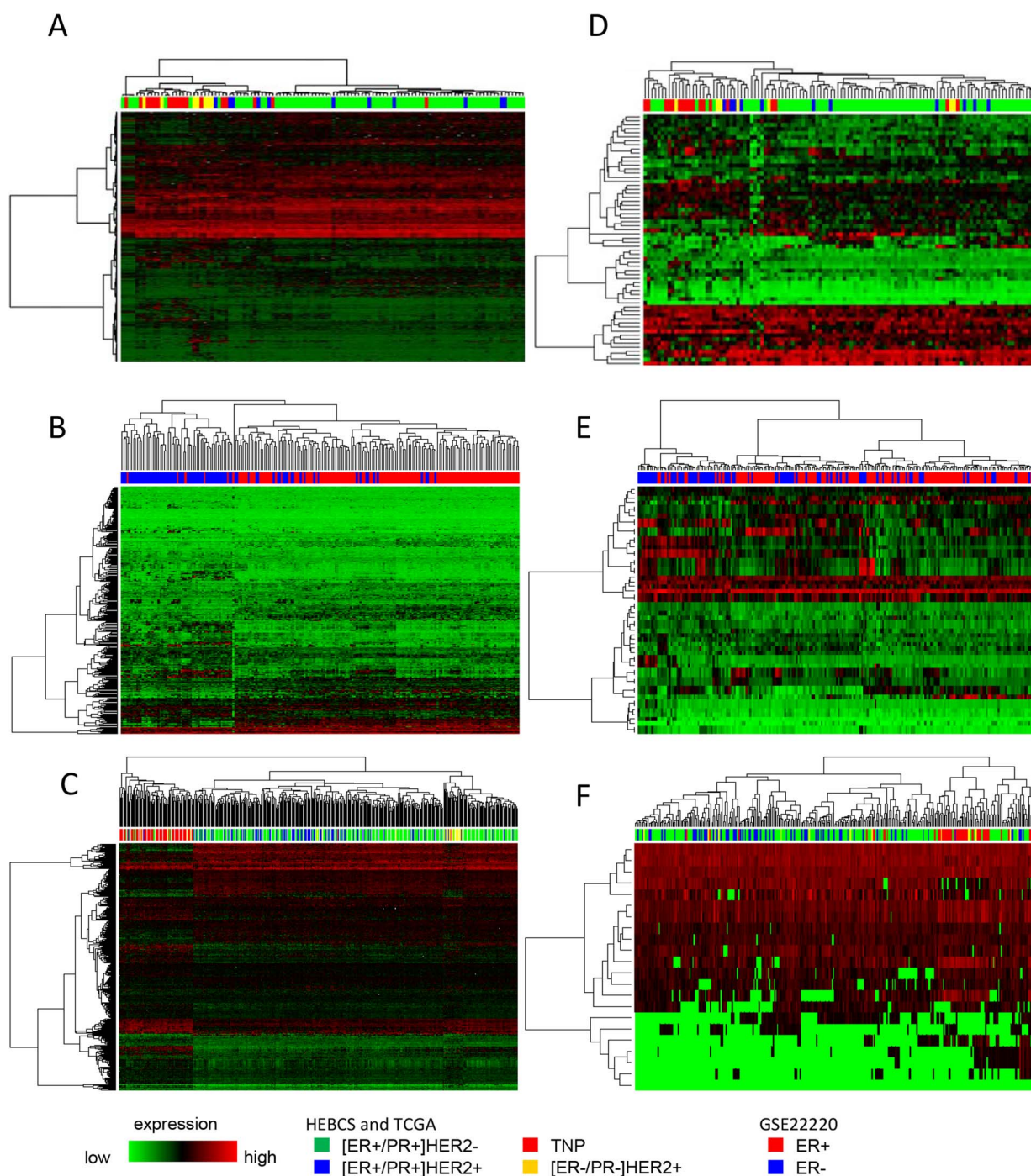


Figure 2 | Tumors from HEBCS and TCGA clustered by four breast cancer subgroups and GSE22220 by two subgroups on the basis of mRNA and miRNA unified diff-genes. (A) Applying the mRNA unified diff-genes to the HEBCS data set. (B) Applying the mRNA unified diff-genes to the GSE22220 data set. (C) Applying the mRNA unified diff-genes to the TCGA data set. (D) Applying the miRNA unified diff-genes to the HEBCS data set. (E) Applying the miRNA unified diff-genes to the GSE22220 data set. (F) Applying the miRNA unified diff-genes to the TCGA data set. The mRNA and miRNA unified diff-genes are listed in supplementary Tables 3 and 4, respectively.

possible effects of a small sample size. Numerically, the computational classifier comprised of our unified diff-genes was able to correctly predict the subgroup in 75% of the tumors in the public data sets (table 2), implying that our diff-genes, although identified from relatively small datasets, have a good generality.

Intrinsic relationship among subtypes revealed by differentially expressed genes. Most of the genes present in multiple tumor subgroups are oppositely regulated between [ER+/PR+] and [ER-/PR-] tumors (Figure 3). We compared mRNA diff-genes

Table 2 | Cross-validation of the diff-gene sets. 'SVM' and 'KNN' represent support vector machine and k-nearest neighbor classifiers, respectively, where $k = 10$ in KNN

Diff-gene	HEBCS		TCGA	
	SVM	KNN	SVM	KNN
mRNA	0.757	0.748	0.735	0.723
miRNA	0.765	0.757	0.549	0.594



Genes	[ER+/PR+]HER2-	[ER+/PR+]HER2+	[ER-/PR-]HER2+	TNP	CoreBasal
KRT5	Green	Green	White	Red	White
FOXC1	Green	Green	White	Red	Red
GABRP	Green	Green	White	Red	Red
ROPN1	Green	Green	White	Red	Red
CDC45L	Green	Red	White	Red	White
LAPTM4B	Green	Red	White	Red	White
CD163L1	Green	Red	White	White	Red
ASCL2	Green	Red	White	White	White
GRB7	Green	Red	White	White	White
MED24	Green	Red	White	White	White
ORMDL3	Green	Red	White	White	White
PGAP3	Green	Red	White	White	White
PSMD3	Green	Red	White	White	White
SLC2A1	Green	Red	White	White	White
TMEM101	Red	Green	White	White	White
ESR1	Red	White	Green	Green	Green
SLPI	Green	White	Red	Red	Red
ST14	Green	White	Red	Red	Red
VGLL1	Green	White	Red	Red	Red
CLIC3	Green	White	Red	Red	White
FUT3	Green	White	Red	Red	White
HDGFRP3	Red	White	Green	Green	White
SLC4A11	Green	White	Red	Red	White
TMPRSS2	Green	White	Red	Red	White
AKR1B15	Green	White	Red	White	White
C6orf127	Green	White	Red	Red	White
GLYATL2	Green	White	Red	Red	White
KRT81	Green	White	Red	Red	White
KYNU	Green	White	Red	Red	White
LBP	Green	White	Red	Red	White
PNMT	Green	White	Red	Red	White
QPRT	Green	White	Red	Red	White
S100A7A	Green	White	Red	Red	White
STAC2	White	Green	Red	White	Green
CAP2	White	Red	White	Red	Green
SFRP1	White	Green	White	Red	Red
ASS1	White	Green	White	Red	White
BBOX1	White	Green	White	Red	White
C17orf58	White	Red	White	Green	White
CRYAB	White	Green	White	Red	White
DEFB1	White	Green	White	Red	White

Figure 3 | Genes shared in the mRNA diff-gene sets among breast cancer subgroups. Genes shared in the mRNA diff-gene sets among [ER+/PR+]HER2-, [ER+/PR+]HER2+, [ER-/PR-]HER2+ and TNP subgroups. The regulation direction of the listed genes are also checked in the CoreBasal subgroup. The over- and under- expression are colored in red and green, respectively, based on the log₂-transformed fold change.

between the two [ER+/PR+] subgroups defined by HER2. Four genes are down-regulated in all ER positive tumors and eleven genes are oppositely regulated between [ER+/PR+]HER2- and [ER+/PR+]HER2+ tumors. The [ER-/PR-]HER2+ subgroup presented 20 genes oppositely regulated compared with ER positive tumors. Among the 20 genes, 19 are differentially expressed between [ER-/PR-]HER2+ and [ER+/PR+]HER2-, and two are differentially expressed between [ER-/PR-]HER2+ and [ER+/PR+]HER2+.

One gene, AACS, is up-regulated both in [ER-/PR-]HER2+ and [ER+/PR+]HER2- tumors. We found 10 genes altered in the same direction between [ER-/PR-]HER2+ and TNP tumors. The [ER-/PR-]HER2+ subgroup has 10 genes coherently regulated with the TNP subgroup, and 10 genes over-expressed only in [ER-/PR-]HER2+ but not the TNP tumors. The regulatory direction of these genes was also checked in CoreBasal tumors, where 9 genes were disrupted in the same direction in TNP and CoreBasal tumors, including 7 elevated genes and 2 suppressed ones (ESR1, CAP2).



Figure 4 | MicroRNAs shared in the miRNA diff-gene sets among breast cancer subgroups. MiRNAs shared in the miRNA diff-gene sets among [ER+/PR+]HER2-, [ER+/PR+]HER2+, [ER-/PR-]HER2+ and TNP subgroups. The regulation direction of the listed miRNAs are also checked in the CoreBasal subgroup. The over- and under- expression are colored in red and green, respectively, based on the log₂-transformed fold change.

The gene CD163L1 was found up-regulated only in CoreBasal tumors but no differential expression was observed among TNP tumors.

Except for hsa-miR-570 that is oppositely regulated between [ER+/PR+]HER2- and [ER-/PR-]HER2+ tumors, all miRNAs differentially expressed in multiple tumor subgroups are reversely deregulated in the [ER+/PR+]HER2- and TNP subgroups (Figure 4). More precisely, there are 7 miRNAs induced in [ER+/PR+]HER2- tumors and repressed in the TNP subgroup and 19 miRNAs deregulated reversely. According to the miRWalk prediction, two miRNA pairs (miR-135a and miR-135b, miR-519b-5p and miR-519c-5p) in Figure 4 share the same targets (Supplementary Tables 5 and 6) and four miRNAs bind to ER related genes (i.e., hsa-miR-135a, hsa-miR-135b and hsa-miR-365 target ESRR1, hsa-miR-7 targets ESRRG and ESR2).

There are 353 and 121 genes uniquely present in TNP and CoreBasal mRNA diff-genes, respectively (Supplementary Table 7), and 9 and 16 miRNAs specifically shown in these two subgroups correspondingly (Supplementary Table 8).

The subgroup clustering pattern generated according to the identified diff-genes are the same when applied to the HEBCS and TCGA data sets, illustrating the comparability of the data sets (Supplementary Figure 1). The grouping of [ER-/PR-]HER2+ samples differs when miRNA and mRNA diff-genes are used, suggesting that the mRNA expression pattern of [ER-/PR-]HER2+ is more similar to TNP tumors as compared with its miRNA expression profile which is more closely grouped with the ER positive tumors.

Statistically, the mRNA data is highly correlated between HEBCS and TCGA data sets for all the four subgroups, while the miRNA data is less well correlated between different data sets except for [ER-/PR-]HER2+ tumors (table 3). However, when ER positive ([ER+/

PR+]HER2- and [ER+/PR+]HER2+) and ER negative ([ER-/PR-]HER2+ and TNP) tumors are considered, the correlation across different data sets are significantly improved for both miRNAs and mRNAs. TNP tumors have the most varied mRNA expression pattern compared with the other subgroups.

Network and pathway analysis using differentially expressed genes among subtypes. The most significant networks of each subtype are shown in Figure 5 (the diff-genes of each subtype including genes shared among subtypes) and Supplementary Figure 2 (the diff-genes of each subtype that are mutually exclusive among subtypes), with information for the top five networks listed in Supplementary Tables 9 and 10, accordingly.

The most significant network covers 46% ([ER-/PR-]HER2+) to 91% (TNP) of the diff-genes for each subgroup. Each of the networks is assigned with functional annotations associated with genes in a specific network. Genes in the top networks of the [ER+/PR+]HER2- and TNP subgroups associate with cancer, system disorder and reproductive system disease (Figures 5A and 5D, Supplementary Table 9), and genes in the top network of the HER2+ subgroups are related to cellular development (Figures 5B and 5C, Supplementary Table 9).

The most significant network generated using the subgroup specific diff-genes includes 26% ([ER+/PR+]HER2+) to 80% (TNP) diff-genes per subgroup. The genes in [ER+/PR+]HER2- and [ER+/PR+]HER2+ associate with cell death and survival and, in addition, genes in [ER+/PR+]HER2+ are also involved in cell cycle, DNA replication, recombination and repair (Supplementary Figures 2A and 2B, Supplementary Table 10). The genes in the most significant networks for HER2+ subgroups show also associations with dermatological diseases and conditions, with [ER-/PR-]HER2+



Table 3 | Correlation between HEBCS and TCGA data

Subtypes	Cor ^{a1} (mRNA)	P ^{b1} (mRNA)	LM ^{c1} (mRNA)	P ^{d1} (mRNA)	Cor ^{a2} (miRNA)	P ^{b2} (miRNA)	LM ^{c2} (miRNA)	P ^{d2} (miRNA)
[ER+/PR+]HER2-	0.378	<0.001	0.34	<0.001	0.367	0.112	0.094	0.181
[ER+/PR+]HER2+	0.358	<0.001	0.323	<0.001	0.266	0.256	0.069	0.325
[ER-/PR-]HER2+	0.171	0.012	0.123	0.05	0.469	0.038	0.11	0.085
TNP	0.133	0.03	0.137	0.035	0.391	0.089	0.056	0.37
ER+	0.37	<0.001	0.332	<0.001	0.315	0.048	0.081	0.093
ER-	0.15	0.002	0.133	0.003	0.439	0.005	0.084	0.053

^{a1}Spearman correlation score between HEBCS and TCGA data sets for each mRNA diff-gene set.

^{b1}p-value of ^{a1}.

^{c1}Coefficient of the linear model between HEBCS and TCGA data sets for each mRNA diff-gene set.

^{d1}p-value of ^{c1}.

^{a2}Spearman correlation score between HEBCS and TCGA data sets for each miRNA diff-gene set.

^{b2}p-value of ^{a2}.

^{c2}Coefficient of the linear model between HEBCS and TCGA data sets for each miRNA diff-gene set.

^{d2}p-value of ^{c2}.

specifically related to tissue and cell morphology (Supplementary Figure 2C). The diff-genes specific for the TNP subgroup are associated with hematological system development and function, tissue morphology and cancer (Supplementary Figure 2D).

The mRNA diff-genes of the CoreBasal subgroup are largely involved in pathways controlling biomass synthesis, such as glycolysis and steroid biosynthesis (Supplementary Table 11). [ER+/PR+]HER2+ and TNP tumors are enriched with genes communicating with NF-κB (Supplementary Figure 3).

Discussion

We compared our mRNA diff-genes with the gene signature published in¹⁷, which is the first gene signature reported using mRNA profiling and has been implemented for tumor subgroup prediction. There were 81 genes in common (as listed in Supplementary Table 12), among which some are the IHC markers used for tumor subgroup discrimination including ESR1 and KRT5, some are known prognostic markers such as GATA3 whose expression level is strongly associated with the differentiation level of breast cancer¹⁸, and some are shared among any of the four tumor subgroups in our mRNA diff-genes (as listed in Figure 3). The shared genes are KRT5, FOXC1, GABRP, LAPTM4B, GRB7, MED24, SLPI and VGLL1, most of which are generally differentially expressed between ER positive and ER negative tumors, demonstrating the fundamental differences between the two tumor classes and the critical role of ER signaling in driving the phenotypic differences of breast cancer.

Several miRNAs are also consistent with the previous studies. Blenkiron *et al.* carried out a study in 2007, where the miRNA features were determined for subgroups classified using mRNA data¹⁴. Due to the different subgroup categorization methods used, finding the direct correspondence between the two studies is difficult. However, hsa-miR-135b is up-regulated in TNP tumors and down-regulated in [ER+/PR+]HER2- tumors in this study and is elevated in basal-like tumors and suppressed in luminal tumors in [14].

ESR1, the discriminative factor between ER positive and ER negative tumors that mediates the biological effects of estrogens through direct binding to the estrogen response elements (EREs) of the target genes or via protein-protein interactions with other DNA-binding transcription factors in the nucleus¹⁹, is oppositely regulated in the presented diff-gene sets (Figure 3). There are four miRNAs targeting ESR1-related proteins from our miRNA diff-genes, i.e., hsa-miR-135a, hsa-miR-135b, hsa-miR-365 and hsa-miR-7. Specifically, the first three miRNAs target ESRRA and hsa-miR-7 is predicted to bind both ESRRG and ESR2 by miRWalk²⁰. ESR1 and ESRRG belong to the ERR subfamily, which is highly similar to the ESR subfamily regarding its amino acid composition and function²¹. It has been reported that ESRRA, ESR2 and ESR1 share 70% amino acid identity²¹ and ERRs share common target genes and pathways with ESRs²². Thus, it is reasonable to assume that these four miRNAs are crucial in distinguishing ER positive and ER negative breast cancer given their

regulatory roles in ESR1-related proteins. Previously, a predictive signature containing 6 miRNAs, i.e., hsa-miR-135b, hsa-miR-190, hsa-miR-217, hsa-miR-218, hsa-miR-299, hsa-miR-342, was identified to be associated with estrogen signaling using artificial neural networks²³. Also, high hsa-miR-135a expression is associated with good prognosis in ER positive tumors²⁴ and hsa-miR-7 is one of the four miRNAs (hsa-miR-7, hsa-miR-128a, hsa-miR-210 and hsa-miR-516-3p) that are linked with ER positive and lymph node negative breast cancer²⁵. Thus, this study confirmed three miRNAs from the previous reports and identified a new miRNA (hsa-miR-365) that plays critical roles in differentiating tumors by ER status. In addition, hsa-miR-365 has been reported to be cooperatively regulated by SP1 and NF-κB²⁶, where NF-κB is a known mechanism or target in ER- or basal like breast cancer²⁷, further confirming its differentiating roles. The miRNAs diff-genes targeting non-ESR1 related mRNA diff-genes and differentially expressed by the ER status are also found. For instance, hsa-miR-224 is suppressed in the [ER+/PR+]HER2- subgroup and elevated in TNP tumors (including the CoreBasal subgroup) according to our results. By checking its targets using miRWalk²⁰ and the identified diff-genes, the DNA repair gene, MBD4²⁸, was found to be up-regulated in [ER+/PR+]HER2- mRNA diff-genes, and the tumor suppressor whose loss is characteristic of basal-like tumors, INPP4B²⁹, was found down-regulated in TNP mRNA diff-genes. Also reversely regulated between [ER+/PR+]HER2- and TNP (including CoreBasal) tumors are hsa-miR-135a and hsa-miR-135b, which share the same targets, indicating that these miRNAs, although closely related and targeting the same genes, function in different tumor subgroups and have different or even opposite regulatory roles in tumor progression. However, this reasoning highly depends on the accuracy of the computational prediction, and needs to be tested *in vivo*.

HER2 is a critical gene in ligand-activated signalling pathways that regulate cell proliferation and cell death³⁰. Several genes are oppositely regulated by HER2 status (Figure 3), and have been indicated for potential roles in controlling cell growth. For example, CDC45L is known to be associated with proliferation³¹, and ASCL2 is involved in Wnt signalling and is a putative regulator of cell growth³². Several genes up-regulated in [ER-/PR-]HER2+ tumors are closely located with the HER2 gene at chromosome region 17q12 whose amplification is a common mechanism for HER2 activation³³, e.g. PNMT is mapped onto the same chromosome with 17439 bp away from and co-expressed with HER2³⁴. Genes whose deregulation is associated with HER2 status and cell growth are also found from the network analysis. For instance, AGTR1 is a central node in the [ER+/PR+]HER2- subgroup (Supplementary Figure 2A) and specifically over-expressed in such tumors as well as involved in cell growth³⁵. No obvious difference is observed between genes differentially expressed in [ER-/PR-]HER2+ and TNP tumors, indicating the more critical roles of ER compared with HER2 in tumor subgroup classification.

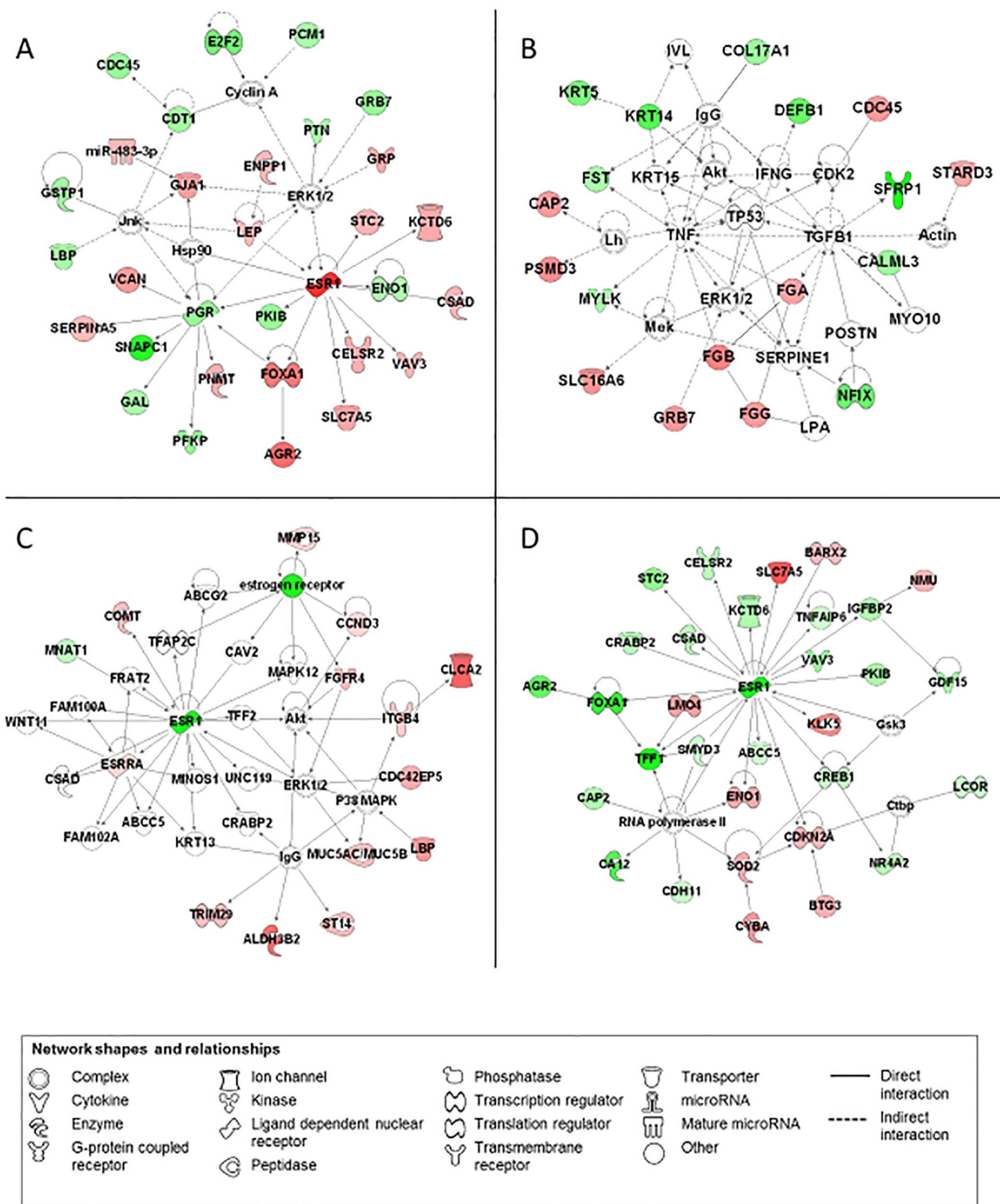


Figure 5 | Schematic representations of the most significant networks generated by IPA. (A) [ER+/PR+]HER2-, (B) Triple negative, (C) [ER+/PR+]HER2+ and (D) [ER-/PR-]HER2+. Molecules shown in red (increased expression) and green (decreased expression) identified in our set of genes. Molecules with no coloring represent genes not present in our data set but relevant to biological pathways and incorporated to generate networks (description and fold change associated with differentially expressed genes are described in Supplementary Tables 3 and 4).



Overall, proliferation is an important character in differentiating breast cancer subgroups, with poor prognosis associated with high tumor cell proliferation³⁶. Several proliferation markers were found significant in the diff-gene sets. For example, topoisomerase II α (TOP2A), a proliferation marker whose over-expression is reported to be associated with shorter survival and HER2 amplification (or over-expression) in breast cancer³⁷, is a diff-gene of [ER+/PR+]HER2+ tumors in this study. Since TOP2A is the target enzyme for top II α inhibitors such as anthracycline, our results are consistent with the efficacy of anthracycline treatment in [ER+/PR+]HER2+ breast cancer. Indeed, cells co-amplifying TOP2A and HER2 are reported to be highly sensitive to anthracyclines³⁸. Similarly, other proliferation markers such as thymidine kinase (TK)³⁹ and mini-chromosome maintenance protein 2 (MCM2)⁴⁰ were found to be characteristic of [ER+/PR+]HER2- and TNP tumors, respectively. Several genes associated with cellular proliferation were found oppositely regulated between ER positive and ER negative tumors in this study. For example, KRT5, which has a role in maintaining cell proliferation potential in the basal layer of stratified epithelia and modulating PI3K/Akt-mediated cell proliferation⁴¹, is down-regulated in ER positive and up-regulated in ER negative tumors. In addition, there are 8 diff-genes associated with cell division and 8 related to cyclin dependent kinases or their inhibitors, most of which are over-expressed in TNP tumors and down-regulated in [ER+/PR+]HER2- tumors (Supplementary Table 13), consistent with the positive association of proliferation with TNP tumor aggressiveness.

The [ER+/PR+]HER2- and [ER+/PR+]HER2+ subgroups are always grouped together regardless of the data set (HEBCS, TCGA) used or the type of the genes (mRNA or miRNA) applied (Supplementary Figure 1), indicating their close relationships at both the mRNA and miRNA levels. However, the grouping of ER negative tumors does depend on the type of the genes, i.e., looser grouping between [ER-/PR-]HER2+ and TNP tumors is observed at the miRNA level than mRNA level. These results are consistent with the fact that ER positive and ER negative tumors are two major classes in breast cancer⁹, which could be well distinguished from each other at both the miRNA and mRNA levels. However, fine-grained classification within each major group may not be as clear as such especially at the miRNA level within the ER positive tumors. Similarly, [ER-/PR-]HER2+ tumors are shown to be more closely related to the TNP subgroup regarding their mRNA expression compared with the miRNA profiling. Overall, it is not surprising that the consistency scores within the major groups of ER positive or ER negative tumors are higher than those within each finer-grained subgroup, since more associations and less clear boundaries exist among subgroups than the two major branches.

Due to the more aggressive features and lack of therapeutic targets, triple negative and especially core basal tumors have become a key topic that has gained particular clinical and research interest⁷. We identified the core basal tumors within the TNP subgroup using EGFR and KRT5/6 to study the properties differentiating the core basal subgroup from TNP tumors with more aggressiveness. There are 111 mRNAs and 16 miRNAs whose expression is aberrant only in CoreBasal tumors (Supplementary Tables 7 and 8). Among these mRNAs, EIF4EBP1, LRR6, RBM34, CKS1B, ADAM15, ZBP1, GGPS1, RCOR3, LOC149134 are amplified in 11%, 10%, 6%, 5%, 5%, 5%, 5%, 5% of the 825 breast invasive carcinomas in TCGA¹⁶, respectively, as queried using cBio Cancer Genomics Portal (cbioportal.org). Network analysis shows that CoreBasal specific mRNAs and miRNAs diff-genes are mostly related to infectious diseases, inflammation and cell signaling as compared to TNP tumors (Supplementary Table 10, Supplementary Figure 3), indicating the importance of tumor-promoting inflammation in facilitating neoplasias to acquire core cancer hallmarks⁴² in this subgroup.

Signalings controlling the biomass synthesis process such as glycolysis or gluconeogenesis and steroid biosynthesis are associated

with the 111 mRNAs (Supplementary Table 7) differentially expressed in CoreBasal tumors (Supplementary Table 11), suggesting the crucial importance of another emerging cancer hallmark, i.e., reprogramming of energy metabolism, in this subgroup. NF- κ B pathway was revealed as an important signaling in two clinically challenging subgroups²⁷, i.e., [ER+/PR+]HER2+ and TNP, as represented by its central roles played in these networks (Supplementary Figures 2B and 2D). NF- κ B aberration has been mostly reported in basal like breast cancer²⁷. NF- κ B is a critical transcription factor in regulating inflammation, whose crosstalk with glucocorticoid receptor has been proposed as the potential target for breast cancer treatment²⁷.

Conclusion

We study the intrinsic differences of five breast cancer subgroups defined using the major receptor IHC markers, by analysing the differentially expressed genes among them and the pathways and networks within each subtype. Network analysis revealed high level relationships and interactions among the mRNA and miRNA diff-genes within the tumor subgroups. Further intrinsic relationships among the four tumor subgroups were revealed at the mRNA and miRNA levels. A novel miRNA differentiating breast tumors with different ER status, hsa-miR-365, was identified and worth testing *in vivo*. Energy metabolism reprogramming was found to play important roles in forming the aggressive nature of the CoreBasal subgroup.

Due to the limited sample size in our study, we did not aim to define fine-grained subgroups or detect subtle differences between breast tumors. Instead, we studied the differences among subtypes revealed by our dataset and explored their generality using two independent publically available datasets. On the other hand, we focused on the major characteristics of the four subgroups defined by the receptor immunohistochemistry markers as well as the differences between TNP and CoreBasal tumors here, where other markers such as those for proliferation could also be explored given data. Altogether, our results complement other relevant investigations, and avail functional studies of breast cancer with further translational potential for clinical use.

Methods

Tumor samples. The mRNA and miRNA HEBCS data were used for determining differentially expressed genes. There are 183 primary breast tumor samples, among which 151 were collected as a part of the unselected series at the department of Oncology of the Helsinki University Central Hospital (HUCH) in 1997, 1998 and 2000⁴³ and at the department of Surgery from 2001 to 2004⁴⁴. The remaining 32 patients belong to an ongoing collection of additional familial breast cancer series from the department of Clinical Genetics at HUCH. Out of the 183 samples, 115 tumors have unambiguous IHC marker information, which have been used for subgroup determination and further analysis.

Immunohistochemical marker status determination. The information on ER and PR status was collected from the pathology reports (positive when >10% of cells were stained)⁴⁵. HER2 status was determined by gene amplification with chromogenic *in situ* hybridization (CISH) and immunohistochemical staining. In particular, samples with ≤ 5 replications from CISH or less than 10% of the cells stained were considered negative, those with ≥ 6 replications or over 90% cells stained were defined as positive, with CISH data preferred⁴⁶. The EGFR and CK5/6 expression were analyzed using immunohistochemistry with more than 10% of the cells stained taken as positive and otherwise negative^{9,45}.

Gene expression profiling. For mRNA data, total RNA was extracted from the 183 primary breast tumors, and the samples were processed and hybridized to Illumina HumanHT-12_V3 Expression BeadChips, containing 24660 Entrez Gene entities, according to the manufacturer recommendations (<http://www.illumina.com>). Similarly, for the miRNA profiling, the processed samples (183 patients) were hybridized onto the Illumina HumanMI_V2 BeadChips, containing 1104 known and predicted miRNAs. Both mRNA (GSE24450 at the Gene Expression Omnibus (GEO) database⁴⁷) and miRNA (GSE43040) expression profilings were carried out at SCIBLU Genomics Centre, Lund University, Sweden.

Public data sets. To explore the generality of the differences identified among breast cancer subtypes, public data sets were used to distinguish subtypes by the identified differentially expressed genes. They are the breast cancer data set from TCGA ([SCIENTIFIC REPORTS | 4 : 6566 | DOI: 10.1038/srep06566](http://</p>
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cancergenome.nih.gov) retrieved on 21th November 2011, and the GSE22220 data set²⁴ from GEO database⁴⁷. We took the primary solid tumor data from the TCGA data set (available from TCGA portal at <http://tcga.cancer.gov/dataportal>), which included mRNA data on 451 samples and 17814 genes and miRNA data on 315 patients and 1046 genes. Samples with unambiguous IHC marker subgroup classification were used in the analysis, including 433 and 311 samples from the mRNA and miRNA data sets, respectively. The mRNA data has been produced using Agilent 244 K Custom Gene Expression G4502A-07-3 platform, and the miRNA data has been generated by miRNA sequencing technique using IlluminaGA_miRNASeq. The level 3 data was used for both mRNA and miRNA data sets, i.e., the mRNA data was lowest normalized followed by log₂-transformation of the ratio between two channels, and miRNA was normalized using appropriate method. To make the distribution of miRNA data comparable with the mRNA data, the logarithm 2 transformation was taken before usage.

The GSE22220 data set is composed of the mRNA data (GSE22219) where the samples from 216 patients were hybridized onto Illumina HumanRef-8_V1 expression BeadChips containing 24332 probes, and miRNA data (GSE22216) which was generated using Illumina HumanMI_V1 BeadChips containing 734 probes for 207 patients. Both GSE22219 and GSE22216 are quantile normalized.

Identification of subgroups and their differentially expressed genes. *Defining tumor subgroups using immunohistochemical markers.* Three IHC markers, i.e., ER, PR and HER2, were used to define the breast tumors into four subgroups, i.e., [ER+/PR+]/HER2-, [ER+/PR+]/HER2+, [ER-/PR-]/HER2+ and TNP tumors. Within the TNP subgroup, we also identified the CoreBasal tumors using information on EGFR and KRT5/6. In particular, [ER+/PR+]/HER2- tumors are defined as ER positive or PR positive tumors with negative HER2 status, [ER+/PR+]/HER2+ tumors are ER positive or PR positive tumors with positive HER2 status, [ER-/PR-]/HER2+ tumors are those that have negative ER and PR statuses but are HER2 positive, tumors with negative ER, PR and HER2 status are defined as TNP subgroup, among which those that are EGFR or KRT5/6 positive are defined as Core Basal tumors. Collectively, [ER+/PR+]/HER2- and [ER+/PR+]/HER2+ tumors are similar to the luminal, and [ER-/PR-]/HER2+ and TNP are close to the non-luminal tumors as categorized using the expression profiles.

Gene expression data preprocessing. HEBCS data was used to identify the differentially expressed genes for each breast tumor subgroup. Microarray raw data was imported into R⁴⁸ and processed by the methods included in the BioConductor facilities. Briefly, after quality control, the data was normalized using the quantile method⁴⁹ and the gene expression matrix was obtained by averaging the probes mapping to the same Entrez Gene IDs. For the miRNA data, the gene expression matrix was obtained by averaging the probes mapped to the same miRNA ID.

Identification of the differentially expressed genes. Differentially expressed miRNAs and mRNAs between the breast cancer subgroups, as defined by IHC markers, were retrieved using linear models followed by moderated t-test. Genes with p-value < 0.05 after Benjamini and Hochberg post hoc correction were considered differentially expressed. The miRNAs differentially expressed in [ER+/PR+]/HER2+ subgroup were selected to have nominal p-value < 0.05. Using the average expression of the other subtypes as the reference cohort, the genes (including both mRNA and miRNA) with base-2 logarithmic fold change larger than 0.58 or smaller than -0.58 were defined as differentially expressed genes (named 'diff-genes') for each tumor subgroup.

The union of the differentially expressed genes for each subgroup was considered the unified diff-genes.

Generality exploration using public datasets. The subtypes were grouped using the unified diff-genes by two classes of machine learning methods, i.e., unsupervised clustering and supervised classification, using two public data sets, i.e., TCGA (<http://cancergenome.nih.gov>) and GSE22220²⁴ from GEO database⁴⁷. Both methods assign objects into proper groups, which differ in that classification requires prior information on the ground-truth categories and involves a training process for the classifier while clustering does not.

The expression patterns of the unified diff-genes were used to cluster the samples in the two public data sets (TCGA and GSE22220), respectively. The TCGA samples are composed of four groups, i.e., [ER+/PR+]/HER2-, [ER+/PR+]/HER2+, [ER-/PR-]/HER2+ and TNP subgroups, where the sample categories were determined based on the four IHC markers. The GSE22220 samples can only be classified into ER+ and ER- tumors based on ER status given its limited IHC marker information. Hierarchical clustering method was used here, where the distance matrix and agglomeration method used were optimized to produce the optimal heatmaps. In particular, the ward agglomeration method was used to generate the HEBCS mRNA and GSE22220 miRNA clusterings, and the complete agglomeration method was used to generate HEBCS miRNA, GSE22220 mRNA, TCGA mRNA, and miRNA clusterings. The Euclidean distance matrix was used in all clusterings, except for the TCGA miRNA clustering where the Manhattan distance matrix was used.

To assess the generality of our diff-gene sets numerically, the predictive power of the miRNA and mRNA unified diff-genes was evaluated by two classification methods, i.e., support vector machine (SVM) and k-nearest neighbor (KNN). Both SVM and KNN are supervised machine learning methods widely applied in classification. SVM constructs a set of hyperplanes in a high-dimensional space, and the classification is achieved by the hyperplane that has the largest distance to the nearest

training data point of any class. KNN classifies an object by taking a vote of its 'k' nearest neighbors, and the object is assigned to the class that is voted by the majority of the 'k' neighbor. In this study, k = 10. Both methods are coupled with leave-one-out cross validation. We use HEBCS and TCGA data sets in the classification given that they have all the four subgroup grouping information available.

Network and pathway analysis. In order to understand the biological interactions among the subtypes as defined, we performed network analyses of the diff-gene sets using the Ingenuity Pathway Analysis (IPA) tool, (Ingenuity Systems, www.ingenuity.com). We used two sets of input for IPA, i.e., the combined mRNA and miRNA diff-genes for each subgroup and, in a separate analysis, the subgroup specific diff-genes (including both mRNAs and miRNAs). The number of molecules shown in the network was set to a default limit of 35, i.e., only the most important genes with the maximum connectivity were included. The resulting networks were scored through Fisher's exact test, with the most significant (highest IPA scoring) networks selected.

In addition, we used DAVID⁵⁰ to predict the enrichment of biological themes by the mRNA diff-genes, which performs an enrichment analysis for a given gene list and compares them to the KEGG pathways.

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Author contributions

X.D. designed, implemented the study, and prepared the draft. A.C. helped in formatting. A.C. and Z.B. are involved in finalizing the manuscript. All authors have read and approved the final manuscript.

Additional information

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