A qualitative transcriptional signature to reclassify histological grade of ER-positive breast cancer patients

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Abstract

Purpose Histological grade (HG) is commonly adopted as a prognostic factor for ER-positive breast cancer patients. However, HG evaluation methods, such as the pathological Nottingham grading system, are highly subjective with only 50%-85% inter-observer agreements. Specifically, the subjectivity in the pathological assignment of the intermediate grade (HG2) breast cancers, comprising of about half of breast cancer cases, results in uncertain disease outcomes prediction. Here, we developed a qualitative transcriptional signature, based on within-sample relative expression orderings (REOs) of gene pairs, to define HG1 and HG3 and reclassify pathologically-determined HG2 (denoted as pHG2) breast cancer patients. Results From the gene pairs with significantly stable REOs in pathologically-determined HG1 (denoted as pHG1) samples and reversely stable REOs in pathologically-determined HG3 (denoted as pHG3) samples, concordantly identified from seven datasets, we extracted a signature which could determine the HG state of samples through evaluating whether the within-sample REOs match with the patterns of the pHG1 REOs or pHG3 REOs. A sample was classified into the HG3 group if at least a half of the REOs of the 10 gene pairs signature within this sample voted for HG3; otherwise, HG1. Using four datasets including samples of early stage (I–II) ER-positive breast cancer patients who accepted surgery only, we validated that this signature was able to reclassify pHG2 patients into HG1 and HG3 groups with significantly different survival time. For the original pHG1 and pHG3 patients, the signature could also more accurately and objectively stratify them into distinct prognostic groups. And the up-regulated and down down-regulated genes in HG1 compared with HG3 involved in cell proliferation and extracellular signal transduction pathways respectively. Conclusions The transcriptional qualitative signature can provide an objective assessment of HG states of ER-positive breast cancer patients, especially for reclassifying patients with pHG2, to assist decision making on clinical therapy.

Introduction

Breast cancer has the highest incidence and mortality among females [1]. The microscopic morphological assessment of the degree of tumor cell differentiation, represented as tumor histological grades (HGs), has powerful prognostic prediction capability in breast cancer [2-5] and has been incorporated into the eighth edition of American Joint Commission of Cancer staging system [6]. According to the Nottingham grading system, after assessing tubule formation (tubularity), nuclear pleomorphism (nuclearity) and mitotic count, each patient can be assigned to histologic grade 1 (HG1, well-differentiated, slow-growing tumor), histologic grade 2 (HG2, moderately differentiated, slightly faster growing tumor) or histologic grade 3 (HG3, poorly differentiated, highly proliferative tumor) [5]. The higher grade is associated with lower survival rate [3, 4, 7]: the 5-year survival rates of untreated HG1, HG2 and HG3 patients are 95%, 75% and 50%, respectively [5, 8, 9]. Considering the excellent prognoses, HG1 patients are amenable for a less toxic therapy. On the contrary, HG3 patients require a more toxic therapy. Genomics analysis indicates that HG1 and HG3 breast carcinomas develop independently along different genetic pathways [10, 11], while HG2 patients (comprising ~50% of breast cancer cases) contain a blend of histological features, some of which are common to both HG1 and HG3 tumors, and exhibit a mixed

gene expression profiles of HG1 and HG3 [12, 13]. Thus, HG2 breast carcinomas should not be classified as individual HG, but represent clinical and molecular hybrids between HG1 and HG3 diseases [14, 15]. The heterogeneity of the HG2 breast cancers resulted in uncertain disease outcome prediction and there is no standard treatment protocol for clinical decision making [7, 16].

However, the pathological Nottingham grading system, the most employed HG evaluation method, is dependent on adequately prepared hematoxylin-eosin-stained tumor tissue sections to be assessed by an appropriately trained pathologist, which is highly subjective with only 50%–85% inter-observer agreements [17-20]. And the consensus was more lower for HG2 samples [15, 21, 22]. Therefore, many studies have tried to identify transcriptional signatures to reclassify pathologically-determined HG states especially HG2 (pHG2) status of patients in order to improve the therapeutic planning for breast cancer patients [13, 16, 23-25]. However, most of the previously proposed signatures for classifying samples were based on summarized expression measurements of the signature genes, which lack robustness for clinical applications due to widespread batch effects and quality uncertainties of clinical samples [26-29]. The data normalization of samples collected in advance also hinders the feasibility of these signatures in routine clinical practice [16]. In contrast, the qualitative transcriptional signatures based on the within-sample relative expression orderings (REOs) of gene pairs are robust against experimental batch effects [27-29], varied proportions of the tumor epithelial cell in tumor tissues sampled from different tumor locations of the same patient [30], partial RNA degradation during specimen preparation and storage [31] and amplification bias for minimum specimens even with about 15-25 cancer cells [32], which are common factors that can lead to failures of quantitative transcriptional signatures in clinical applications. Besides, the qualitative signatures can be applied at the individual level [33]. Based on the within-sample relative expression orderings (REOs) of gene pairs, we have developed prognostic signatures for many cancer types [28, 34-36] and demonstrated their robustness in both inter-laboratories and across-platforms tests [29, 37].

Approximately 70% breast cancer patient express estrogen receptor (ER) according to American Cancer Society [1], adjuvant endocrine therapy is the routine regimen, and only for ER-positive patients with high HG, combined chemotherapy is suggested. It has been reported that there is a significant transcriptional difference between ER-positive and ER-negative cohorts [38]. ER-positive status is associated with a heterogeneous mixture of histologic grades, whereas ER-negative status is generally associated with HG3 [39].

In this study, we aimed to develop a qualitative transcriptional signature to identify HG states objectively in ER-positive breast cancers. Using gene expression profiles of 932 ER-positive early stage breast cancer patients, we developed a qualitative signature to allocate each patient into the pathologically-determined HG1 (denoted as pHG1) or HG3 (denoted as pHG3) group. Using four independent validation datasets including a total 524 samples of ER-positive breast cancer patients who accepted surgery only, the signature could find out a certain percentage of pHG1 patients as HG3 patients with worse prognoses and some pHG3 patients identified as HG1 patients with better prognoses. Especially, we adopted an objective approach to validate the signature through evaluating whether the pHG2 patients reclassified as HG1 had better prognoses than the pHG2 patients reclassified as HG3.

Results

Development of the REO-based grade signature

For each of the seven train datasets (Table 1), with FDR < 0.1, we firstly identified gene pairs with significantly stable REOs in the pHG1 and pHG3 groups, respectively, and then identified gene pairs with reversal REOs between the two groups. Then, 437 gene pairs were commonly identified from the seven datasets and they consistently showed the same reversal REO patterns between the pHG1 and pHG3 groups in the seven datasets. Next, we performed a forward-stepwise selection procedure to search a set of gene pairs that achieved the highest F-score according to the classification rule as follows: a sample was classified into the HG3 group if at least a half of the REOs of the set of gene pairs within the sample voted for HG3; otherwise, into the HG1 group. Finally, we obtained 10 gene pairs, denoted as 10-GPS (Table 2), to distinguish different histological grades with the highest F-score (0.8884). In the train data, the apparent specificity for HG1 samples was 90.77% and the apparent sensitivity for HG3 samples was 86.99%. The performance of the transcriptional grade signature in each train dataset can be found in Additional file 1: Table S1. Notably, the apparently imperfect performance should be reasonable because HG evaluation based on the pathological Nottingham grading system is highly subjective with only 50%–85% inter-observer agreements [17-20]. We speculated that the 10-GPS could provide a more objective and clinically relevant measure of tumor grade with prognostic significance.

Table 1. Description of the datasets used in this study

Table 2. The REO-based transcriptomic grade signature

We validated the above speculation based on the knowledge that HG3 patients were with lower survival rate than HG1 patients [3, 7]. Here, we collected another four independent datasets (Table 1) including samples with RFS or OS data of early stage ER-positive breast cancer patients who accepted surgery only. When the 10-GPS was applied to these datasets, the averaged apparent sensitivity for all HG3 samples was 83.1% and the average apparent specificity for all HG1 samples was 78.4%. In a merged dataset from the three validation datasets with the RFS information, the 12 pHG3 patients reclassified as HG1 by the signature had significantly higher 10-years RFS rate than that of the 46 HG3 patients confirmed by the signature (p=0.0143; HR=8.17, 95% CI: 1.10-60.6; C-index = 0.61, Fig. 1a). And, we also compared 10-years RFS rates between the 13 pHG1 patients reclassified as HG3 by the 10-GPS and the 93 HG1 patients confirmed by the 10-GPS. Despite no statistical difference was, there was trend to be different between the two groups (p=0.3583; HR=1.65, 95% CI: 0.56-4.86; C-index = 0.54, Fig. 1b). There was also trend of difference between the RFS rate of 12 pHG3 patients reclassified as HG1 by the signature and that of 13 pHG1 patients reclassified as HG3 by the 10-GPS (p=0.1847; HR=3.95, 95% CI: 0.44-35.35; C-index = 0.65, Fig. 1c).

Fig. 1 is about here

In each of the three validation datasets with the RFS information, we also compared the survival between the pHG1 and pHG3 patients diagnosed by the pathological Nottingham grading system and the survival between the HG1 and HG3 patients reclassified by the 10-GPS from the pHG1 and pHG3 patients.

Significant difference of RFS between the pHG1 and pHG3 patients was observed only in GSE4922 dataset. However, the HG1 patients showed significantly better RFS than those of HG3 patients in all the three datasets (Fig. 2). These results demonstrated that the 10-GPS can more accurately and objectively stratify samples into distinct prognostic groups.

Fig. 2 is about here

Application of the signature to reclassification of HG2 samples

Then, we used the 10-GPS to reclassify the pHG2 samples of the above four validation datasets with RFS or OS information (Table 1) into the HG1 and HG3 groups, respectively, and evaluated their prognostic differences.

Firstly, for the 68 pHG2 samples of the GSE7390 dataset, the 10-GPS signature allocated 38 and 30 patients into the HG1 and HG3 groups, respectively. And, the former ones had significantly higher RFS rate than the latter ones (p=5.55E-03; HR=2.53, 95% CI: 1.28-4.97; C-index = 0.64; Fig. 3a). Then, in the 91 pHG2 patients combined from the datasets of GSE6532 and GSE4922 with small sample sizes, the RFS rate of the 65 patients stratified into the HG1 group was significantly higher than that of the 26 patients stratified into the HG3 group (p=9.06E-03; HR=2.64, 95% CI: 1.24-5.62; C-index = 0.61; Fig. 3b). In the EGA dataset, the 71 HG1 patients classified by the 10-GPS also displayed significant higher OS rate than that of the 49 HG3 patients classified by the 10-GPS (p=6.92E-03; HR=2.10, 95% CI: 1.21-3.64; C-index = 0.61; Fig. 3c).

Fig. 3 is about here

Transcriptional characteristics of the low-HG and high-HG samples recognized by the 10-GPS

In the TCGA-BRCA dataset, we used the limma algorithm and found 2,875 differentially expressed genes (DEGs) between the 58 pHG1 and 170 pHG3 samples diagnosed by the pathological Nottingham grading system (FDR<0.05). Applying the 10-GPS to these samples, 66 samples were allocated into the HG1 group and the other 162 samples were allocated into the HG3 group. We identified 3,282 DEGs between the two reclassified groups with the same FDR control. And of these genes, up-regulated genes were significantly associated with proliferation and down-regulated were significantly associated with extracellular signal transduction (Fig. 4, Additional file 1: Table S2). When comparing the two DEG lists (Additional file 2: Fig. S1a), we found that 2,568 (89.32%) of the 2,875 DEGs between the original HG1-HG3 groups were also included in the DEGs identified after sample reclassification and the dysregulation directions of the overlapped genes reached up to 100% (binomial test, p < 1.10E–16). We also identified 1361 DEGs between 95 HG1 (denoted as LHG2) samples and 121 HG3 (denoted as HHG2) samples recognized from the pHG2 samples with the aid of 10-GPS. About 85.53% of the 1,361 DEGs were also included in the 3,282 DEGs (Additional file 2: Fig. S1b). The concordance score of the 1,164 overlapped DEGs was 100%, which was unlikely to happen by chance (binomial test, p < 1.10E–16). The clearer transcriptional differences between the two reclassified groups indicated that the 10-GPS could more accurately and objectively stratify samples into distinct histological grade groups.

Fig. 4 is about here

Discussion

In this study, we developed a histological grade signature consisting of 10 gene pairs (10-GPS) to reclassify the ER-positive breast cancer patients to distinct prognostic groups. This transcriptional qualitative signature, which is based on REOs in an individual sample, was highly robust against experimental batch effects, varied proportions of the tumor epithelial cell in tumor tissues [30], RNA degradation [31], and amplification bias for minimum specimens [32]. All of these merits make it possible to apply the 10-GPS into clinical practices. The 10-GPS could not only objectively and accurately allocate HG1 and HG3 patients but also reclassify HG2 patients into two groups with significantly different survival rates. For clinical application, the patients allocated into the HG3 group should receive adjuvant chemotherapy followed by endocrine therapy; and the patients allocated into the HG1 group were recommended the endocrine therapy only.

Fortunately, based on the working assumption that the majority labels of the pHG1 and pHG3 samples were right, thus we employed a supervised learning method to develop the signature. Imperfect F-score of 0.8884 just suggested that the 10-GPS did not over-fit the train dataset. There's no surprise that the apparent sensitivity for all HG3 samples was 83.10% and the apparent specificity for all HG1 samples was 78.40% in the validation datasets. In this study, we adopted a more objective approach to validate the signature through evaluating whether the reclassified HG1 patients could have better prognosis than that of HG3 patients. In four independent validation datasets, the reclassified HG1 and HG3 groups recognized by the 10-GPS from the original HG2 patients or from the original HG1 and HG3 patients had significantly different survival.

We expected that the 10-GPS can replace or serve as auxiliary reference of the pathological Nottingham grading system to stratify ER-positive patients into two distinct groups in clinical practices. When applying the 10-GPS to all 132 samples of the GSE7390 dataset, the grade signature classified 66 patients into the HG1 group and 66 patients into the HG3 group. The RFS rate of the former group was significantly higher than that of the latter group (p=7.74E-04; HR=2.49, 95% CI: 1.44-4.33; C-index = 0.64; Additional file 3: Fig. S2 a). Significantly different survival time between HG1 and HG3 groups reclassified by the 10-GPS were also observed in another three independent validation datasets (Additional file 3: Fig. 2b-d).

Some signatures had been developed to re-classify the HG status of BC samples, for example, during the development of GGI, samples with ≥100ng and a RIN≥7 were considered as qualified, and the quantitative threshold has been adopted during the re-classification process [40], which results lacking of reproducibility for datasets generated by different labs or platforms and limitation of individual application. Meanwhile, the qualitative signature 10-GPS is with wider application to trace samples [32], samples with lower RIN [31] and samples with low tumor-purity [30].

A limitation of this study is that we were unable to directly evaluate the signature in RNA-sequencing, such as those archived in the TCGA database, where no patients accepted surgery only. Here, we only indirectly validate the 10-GPS in RNA-seq data of the TCGA-BRCA dataset through analysis of DEGs between the HG1 and HG3 groups. In the future, we will evaluate the performance of the signature developed in this study for expression data produced by RNA-sequencing or PCR platforms.

Conclusions

Pathological histological grade evaluation methods are with high subjectivity, especially for the evaluation of HG2 breast cancer specimens. The transcriptional qualitative signature is objective for the evaluation and is robust for application of trace samples, samples with lower RIN and samples with low tumor-purity, and can assist making on clinical therapy especially for patients with pHG2.

Methods

Data collection and Pre-processing

We collected gene expression profiles of 932 ER-positive breast cancer samples with pHG1 or pHG3 diagnosed by the pathological Nottingham grading system. To evaluate whether the reclassified two groups have significantly different survival time, we also collected independent expression data of 524 early stage (I–II) ER-positive breast cancer patients who accepted surgery only. All the breast cancer datasets used in this study were summarized in Table 1. The overall pathological histologic grades of TCGA samples were obtained from the study of Zheng Ping et al [41].

For Affymetrix array data, raw intensity files (.cel), downloaded from the Gene Expression Omnibus database, were processed with the Robust Multichip Average algorithm (RMA) algorithm for background adjustment without quantile normalization [42]. For Illumina beadchip data, the normalized expression data under accession number EGAD00010000210 and EGAD00010000211 [43] were downloaded from the European Genome-Phenome Archive (http://www.ebi.ac.uk/ega/). When processing the data of the two platforms, each probe set ID was mapped to Gene ID according the corresponding annotation files, and then probe sets that mapped to multiple Gene IDs or did not map to any Gene ID were removed. The expression measurements of all probe sets corresponding to the same Gene ID were averaged to obtain a single measurement (on the log2 scale). For RNA-Seq data, the level 3 Fragments per Kilobase of transcript per Million mapped reads (FPKM) [44] values were downloaded from the The Cancer Genome Atlas (TCGA) database. After removing genes with a count of 0 in more than 75% of samples, other zero values were filled with the smallest count in this expression data. The Ensembl gene IDs corresponding to the unique Entrez gene IDs were used. From the seven training datasets, we extracted expression profiles of 11,587 genes commonly measured by the three platforms (Affymetrix array, Illumina beadchip and Illumina HiSeq 2000) for subsequent analysis.

Development of the transcriptional signature for histological grade

First, we separately identified the significantly stable REOs in pHG1 and pHG3 groups of each training dataset. For a given gene pair (*Gi*, *Gj*), let *s* denote the number of samples in which gene *i* has a higher (or lower) expression level than gene *j* in a total of *n* samples, the significance of the REO pattern is determined by a binomial test [45] as follows,

[The formula could not be inserted here due to technical limitations. It can be found in the supplemental files.](1)

where $p0$ is the probability of observing a certain REO pattern ($Gi > Gj$ or $Gi < Gj$) in a sample by chance ($p0 = 0.5$). The Benjamini-Hochberg multiple testing correction was used to estimate the false discovery rate (FDR) [46]. Then, we identified the gene pairs with stable REOs in each group but reversal REO patterns between the pHG1 and pHG3 groups in each train dataset.
After selecting gene pairs with concordant reversal REOs among the seven training datasets, a forward-stepwise selection algorithm was performed to search for optimal subset of these gene pairs that resulted in the highest F-score. The F-score, harmonic mean of sensitivity and specificity, was calculated as follows,

[The formula could not be inserted here due to technical limitations. It can be found in the supplemental files.](2)
where sensitivity was defined as the proportion of correctly identified HG3 samples among all pHG3 samples, and specificity was defined as the proportion of correctly identified HG1 samples among all pHG1 samples.

Survival analysis

Recurrence-free survival (RFS) and overall survival (OS) served as the prognosis endpoint. Kaplan-Meier survival plots and log-rank tests [47] were used to evaluate the differences in RFS and OS of distinct groups. The Cox proportional-hazards model was also performed to calculate the hazard ratios (HRs) and their 95% confidence intervals (CIs) [48]. To evaluate the predictive performance of a signature we also adopted the concordance index (C-index), which is a measure of overall concordance between predicted risk scores and observed survival [49, 50].

Differential expression and functional enrichment analysis

The expression values of all tumor samples of TCGA-BRCA dataset were corrected with an empirical Bayesian algorithm to remove batch effects. The batch effect adjusted data of the TCGA-BRCA dataset were downloaded from the TCGA MBatch website (http://bioinformatics.mdanderson.org/tcgambatch/) for differential expression analysis. Limma was conducted to identify differentially expressed genes between two groups of samples. The GO-function algorithm [51] was used to determine the significance of biological pathways enriched with a set of interested genes by hypergeometric distribution test.

Abbreviations

HG: histological grade

pHG: pathologically-determined histological grade

C-index: concordance index

CIs: confidence intervals

HRs: hazard ratios

FDR: false discovery rate

REOs: relative expression orderings

RMA, Robust Multi-Array Average

Declarations

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Availability of data and materials

Previously data analyzed in this study should be requested from the authors of the original publications. Please see methods cohort description (Table 1), for references to these publications.

Authors' contributions

ZG, HC and JL conceived the project. JL, WBJ and HLZ performed computational experiments. JL, and WBJ designed data analyses. JL, HLZ, and JY interpreted data. JL, HC and ZG wrote the manuscript. All authors contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Description of the datasets used in this study

Datasets	Platform	HG1	HG2	HG3	#Genes
Training datasets					
GSE19615	Affymetrix array	23	—	25	20486
GSE21653	Affymetrix array	37	—	47	20486
GSE1456	Affymetrix array	26	—	40	12432
GSE3494	Affymetrix array	62	—	33	12432
EGA_210	Illumina beadchip	30	—	221	25186
EGA_211	Illumina beadchip	35	—	125	25186
TCGA	Illumina HiSeq 2000	58	—	170	20720
Validation datasets					
GSE7390	Affymetrix Array	29	68	35	12432
GSE6532	Affymetrix Array	29	31	12	12432
GSE4922	Affymetrix Array	48	60	11	12432
EGA	Illumina beadchip	35	120	46	25186

Table 2. The REO-based transcriptomic grade signature

Gene A		Gene B	
Gene ID	Gene symbol	Gene ID	Gene symbol
80127	BBOF1	9319	TRIP13
22885	ABLIM3	24137	KIF4A
1848	DUSP6	11065	UBE2C
9486	CHST10	9319	TRIP13
11122	PTPRT	9833	MELK
6271	S100A1	9319	TRIP13
23403	FBXO46	8140	SLC7A5
23303	KIF13B	27346	TMEM97
1101	CHAD	11004	KIF2C
51310	SLC22A17	9212	AURKB

Note Gene A has a higher expression level than Gene B in HG1 groups

Additional File Legends

Additional file 1: Table S1. The performance of the transcriptional grade signature in each train dataset, shown with apparent specificity, sensitivity and F-score. Table S2. The enriched biological pathways by DEGs between the LHG and HHG group, performed by accumulated hypergeometric analysis.

Additional file 2: Fig. S1. **Analysis of differentially expressed genes by Venn**. (a) Differential mRNAs expression in HG1 vs HG3 patients and pHG1 vs pHG3 patients. (b) Differential mRNAs expression in HG1 vs HG3 patients and LHG2 vs HHG2 patients.

Additional file 3: Fig. S2. **Kaplan–Meier estimates of survival**. (a) Relapse-free survival curves for HG1 and HG3 patients reclassified from all breast cancer patients in dataset GSE7390. (b) Relapse-free survival curves for HG1 and HG3 patients reclassified from all breast cancer patients in dataset GSE6532

. (c) Relapse-free survival curves for HG1 and HG3 patients reclassified from all breast cancer patients in dataset GSE4922. (d) Overall survival curves for HG1 and HG3 patients reclassified from all breast cancer patients in dataset EGA.

Figures

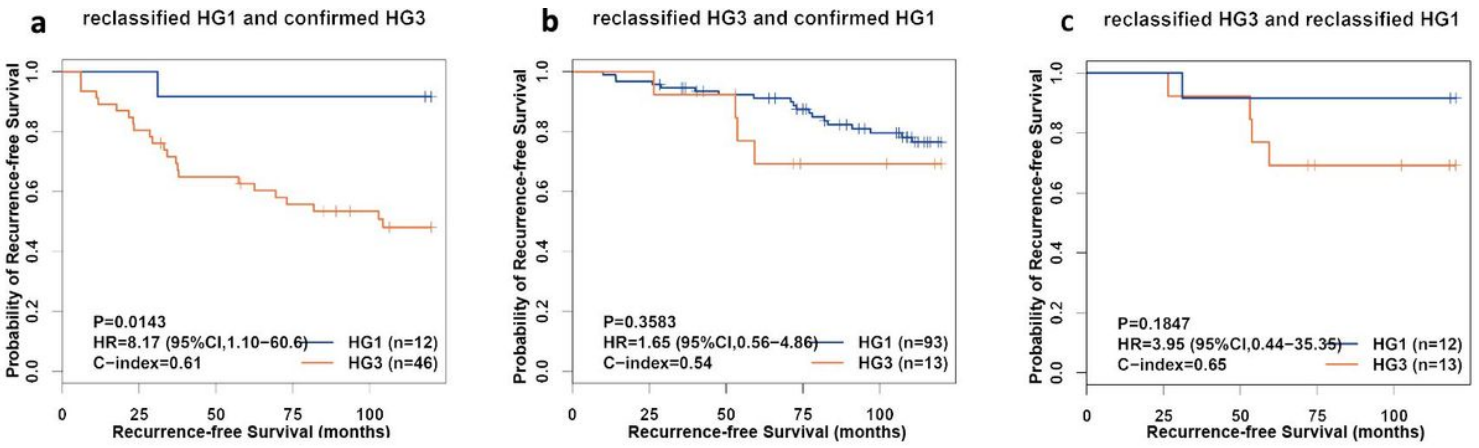

Figure 1

Kaplan–Meier estimates of relapse-free survival in dataset merged from GSE7390, GSE6532 and GSE4922. (a) Relapse-free survival curves for reclassified HG3 and confirmed HG1 breast cancer patients. (b) Relapse-free survival curves for reclassified HG1 and confirmed HG3 breast cancer patients. (c) Relapse-free survival curves for reclassified HG3 and reclassified HG1 breast cancer patients.

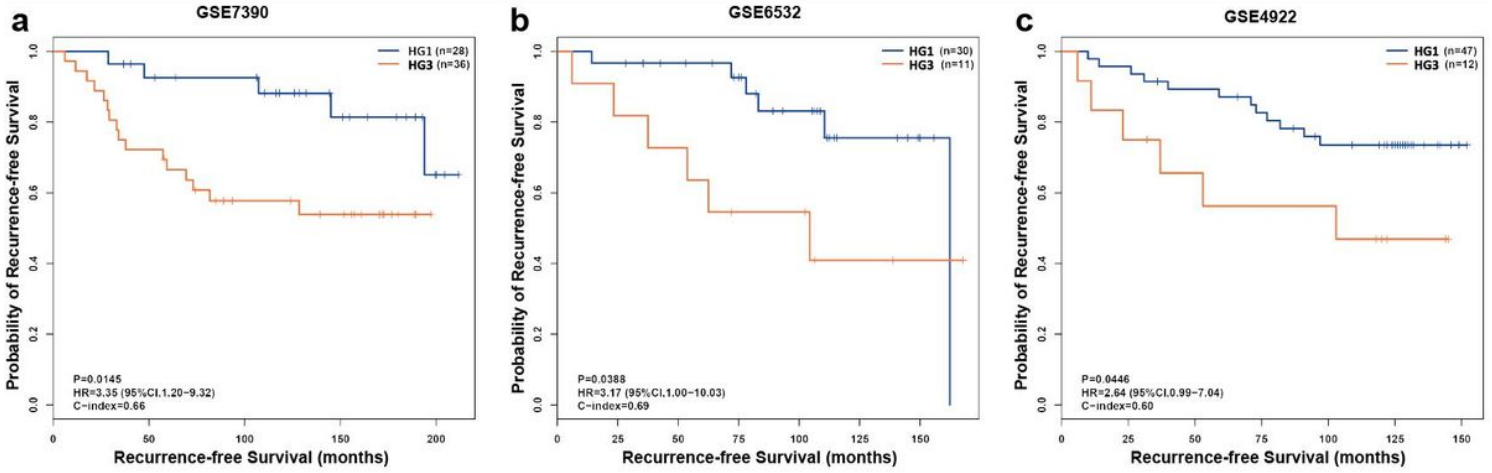

Figure 2

Kaplan–Meier estimates of relapse-free survival. (a) Relapse-free survival curves for HG1 and HG3 breast cancer patients in dataset GSE7390. (b) Relapse-free survival curves for HG1 and HG3 breast cancer patients in dataset GSE6532 . (c) Relapse-free survival curves for HG1 and HG3 breast cancer patients in dataset GSE4922.

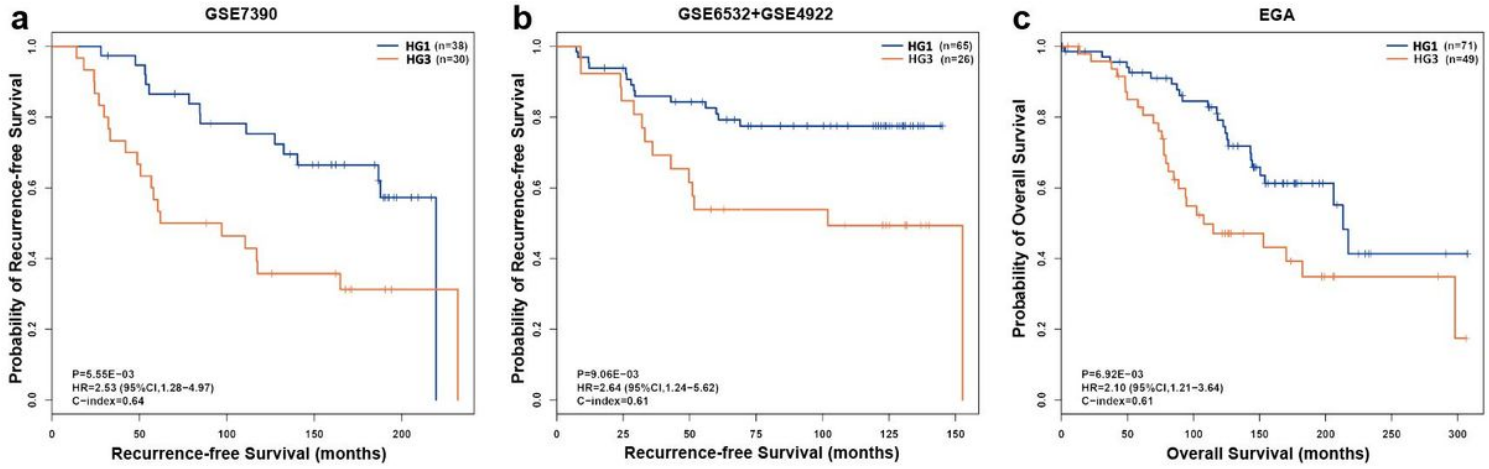

Figure 3

Kaplan–Meier estimates of survival. (a) Relapse-free survival curves for HG1 and HG3 patients reclassified from pHG2 breast cancer patients in dataset GSE7390. (b) Relapse-free survival curves for HG1 and HG3 patients reclassified from pHG2 breast cancer patients in dataset dataset merged from GSE6532 and GSE4922 . (c) Overall survival curves for HG1 and HG3 patients reclassified from pHG2 breast cancer patients in dataset EGA.

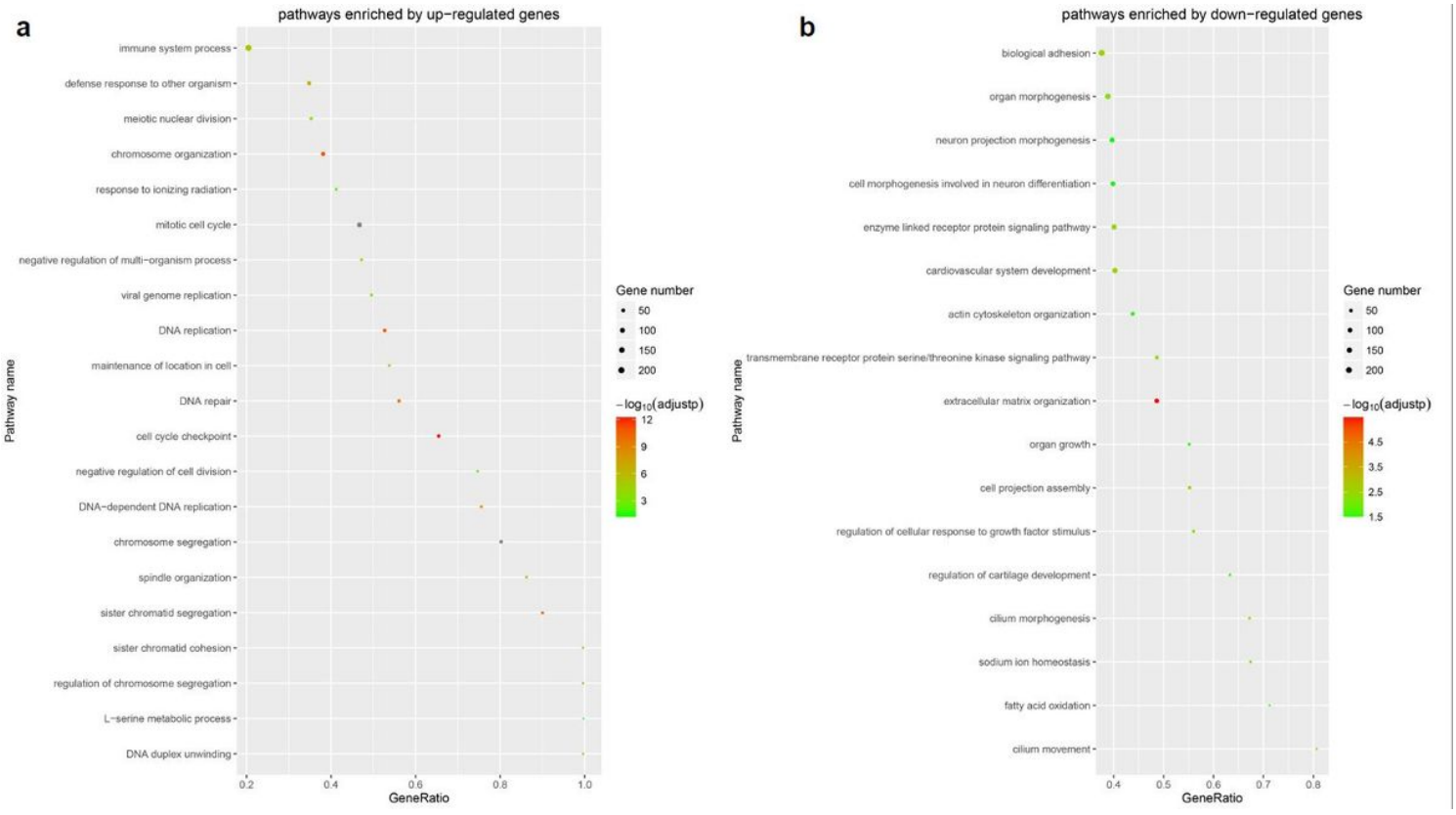

Figure 4

Functional pathways enriched with differential expressed genes between HG1 and HG3 groups. (a) Pathways enriched by up-regulated genes. (b) Pathways enriched by down-regulated genes.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.xlsx
- supplement2.pdf
- supplement3.pdf
- supplement4.pdf