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Expression of reactive species related genes is associated with patient survival in luminal B breast cancer

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Abstract

Increased reactive species (RS; reactive oxygen and nitrogen species) are a byproduct of both enzymatic and non-enzymatic systems, and critical in cancer development, including breast tumorigenesis. To investigate the role of RS-related genes in breast cancer, expression levels of the most common annotated genes involved in regulating cellular RS levels and proteins that are substrates of RS in specific subtypes of breast cancer 9 were evaluated using public data bases. Based on the premise that increased RS promote tumor formation, and breast cancer subtypes vary in aggressiveness, we hypothesized that specific RS gene expression signatures are associated with breast cancer aggressiveness and patient survival. We identified a group of genes (*GSTK1*, *PRDX2*, *PRDX3* and *SLC36A1*) that differentiate Luminal B tumors in two clusters and predict survival of patients with Luminal B breast cancers. Furthermore, network analyses of these four genes revealed an overlap of known LumB related pathways with those of RS-related signaling, which included regulation of M-phase and mitochondrial functions.

Graphical Abstract

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Keywords

breast cancer; redox signaling; oncogenesis; patient survival; gene signature

1. Introduction

Reactive species (RS) are important in regulating normal cellular processes. Deregulation of RS contributes to the development of various human diseases, including cancers [1].

Environmental sources of RS such as ionizing radiation and redox-active compounds are carcinogens that damage cells and increase the rate of DNA mutation, as do RS generated during inflammation or as metabolic byproducts. It is widely accepted that cancer cells are under more RS stress compared to normal cells [2]. Cells rely on integrated antioxidant systems to limit RS generation, scavenge RS and repair RS-induced damage. Deterioration of these systems is likely to promote cancer. However, successful cancer cells require strong defenses to cope with a variety of oxidative insults, and selective pressure is placed on pre-neoplastic cells to ensure appropriate antioxidant systems. Therefore, while oxidative stress can promote cancer, it provides an ongoing challenge for tumor survival [2].

Breast cancer (BC) can be divided into four major molecular subtypes: luminal A, luminal B, basal-like/triple negative, and HER2 type. Luminal A (LumA) has high expression of estrogen receptor (ER) and progesterone receptor (PR), and low HER-2 expression. Luminal B (LumB) has relatively lower expression of ER and PR, variable expression of HER-2, and increased expression of proliferation-related genes. Basal-like subtypes are often referred to as triple negative because they are invariably estrogen receptor (ER) negative, progesterone receptor (PR) negative, and HER-2 negative. The intrinsic subtypes of breast cancer, the LumA, LumB, HER2 enriched, basal-like, and normal-like breast cancer subtypes were initially defined through unsupervised clustering analysis of global gene expression from RNA extracted from frozen tissue [3]. A 50-gene qPCR assay (Prediction Analysis of Microarray 50 =PAM50) was developed to identify the intrinsic biological subtypes using RNA isolated from more readily available formalin-fixed, paraffin-embedded (FFPE) tissue [4, 5].

Cancer cells are usually subjected to high levels of reactive oxygen species (ROS). Accordingly, it is assumed that cancer aggressiveness positively correlates with ROS levels. However, increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a readout for oxidized DNA, was observed in ER-positive malignant tissues to be 3.35-fold higher than in ER negative, which is the more aggressive phenotype in breast cancer. Also, increased levels of 8-OHdG in the DNA have been associated with early-stage cancer tissue as compared to that of later-stage cancer tissue, which suggests that ROS may play an important role in the early phases of carcinogenesis [6]. While this suggests that estrogen responsive breast cancers carry a higher ROS burden and modifications of DNA bases, basal like and Her2

breast cancers are mostly aneuploidy and highly genomic unstable compared to LumA or LumB breast cancers. These conflicting results prompted us to investigate if genes involved in ROS and reactive nitrogen species (RNS) metabolism and changes of RS substrates are expressed differently in BC subtypes.

As the work flow in Fig. 1 summarizes, we used different publicly available breast cancer gene expression data sets to examine if the expression of genes regulating ROS/RNS, or genes that are regulated by ROS/RNS correlate specifically with survival of a breast cancer subtype. Using data from The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) we identified a subset of genes that were differentially expressed among the subtypes [7, 8]. Using the BRB-Array tool, we specifically identified a subset of genes that were associated with patient survival in LumB cancers [9]. Significance of these genes in LumB patient survival was then further confirmed using different data bases from the Gene Expression-Based Outcome for breast Cancer Online (GOBO). The gene set containing not only the genes identified, but also their protein interaction partners were further examined for common pathways using IPA and DAVID, and commonalities in chromosomal localization or aberrations [10–12]. As it is commonly assumed that increase in RS and RS defenses correlate with tumor aggressiveness, we describe here for the first time that in breast cancer, RS genes don't associate with survival in the more aggressive subtypes such as Her2 positive or basal like breast cancers.

2. Material and methods

2.1. Generation of gene list

A list with ROS/RNS regulating genes (234 total) was generated using the gene ontology database (<http://amigo.geneontology.org>; 06/13/2016; AmiGO 2.3) selecting gene products directly regulating oxidative or nitrogen stress (Supplemental Excel file 1). In addition, gene products responsive to oxidative stress in breast cancer cells were selected from a previous study investigating gene expression in the ER-positive MCF-7 cell line treated with either diamide (a thiol oxidizing agent), H₂O₂, or menadione (a member of the vitamin K family known to produce peroxides and superoxide) [13]. Diamide regulated 232 genes, H₂O₂ 942 genes and menadione 922 genes. An overlap of genes was used in the analysis (Supplemental Excel file 2). Not all of these genes were probed for in the TCGA (Illumina HiSeq) and METABRIC (Illumina HT 12 arrays) data sets, resulting in a total of 128 ROS-target genes and 155 ROS and RNS regulating genes (Supplemental Table 1).

Genes on the 'intrinsic' list of 1906 genes, from Parker et al. were excluded. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2667820/bin/JCO.2008.18.1370_index.html - table 5)

2.2. Datasets used to evaluate expression levels of the selected genes

Data from two publicly available breast cancer datasets, TCGA BRCA (The Cancer Genome Atlas Breast Cancer) and METABRIC (Molecular Taxonomy of Breast Cancer International Consortium), were used to evaluate differential gene expression between breast cancer

subtypes [7, 8]. The RNASeq (Illumina HiSeq) gene expression data for TCGA Breast Cancer was downloaded from UCSC Cancer Browser on October 29, 2013. These data contain the gene-level transcription estimates, as in RSEM normalized count, which are $\log_2(x+1)$ normalized, for 991 primary tumor samples. The related clinical data was obtained at the same time. Gene expression data from 1981 breast tumors (Illumina HT 12 arrays) and outcome data for METABRIC was downloaded from Synapse on July 24, 2013 (www.synapse.org).

2.3. Consensus clustering

Consensus clustering using TCGA RNASeq expression data and METABRIC expression data for the selected genes was performed to identify robust clusters within breast tumors and to assess the stability of the identified clusters. Clustering was performed using the R package ConsensusClusterPlus (<https://www.bioconductor.org/packages/devel/bioc/vignettes/ConsensusClusterPlus/inst/doc/ConsensusClusterPlus.pdf>). Silhouette plots were plotted in R to assess the optimal number of clusters (k). The 'k' with maximum average silhouette width was chosen to be the optimal 'k'.

Heatmaps were generated in R using function heatmap.3. In the 'ordered' heatmaps, the samples were ordered by the 'cluster number' assigned by consensus clustering. In the 'clustered' heatmaps, the samples were allowed to cluster using hierarchical clustering. The 'cluster number' is added as a sample label to both heatmaps. Student's t-tests were performed using R to identify genes differentially expressed between the identified clusters.

2.4. Survival Analyses

As Figure 1 summarizes, to estimate and plot differences between the survival curves for the clusters only, METABRIC data were analyzed by consensus clustering. Kaplan-Meier survival analysis was performed in R using 'survival' package (<https://cran.r-project.org/web/views/Survival.html>). To identify genes that were significantly associated with overall survival for each breast cancer subtype, gene expression data from METABRIC were analyzed using the BRB-ArrayTools Version: 4.2.1, R version 2.13.2 (2011-09-30) and [9]. 19602 genes were analyzed using Cox proportional hazards model and Wald statistics. Genes called positive were significant at the nominal 0.01 level of the univariate test. As hazard ratio, the ratio of hazards for a two-fold change in the gene expression level was determined. It was called equal to $\exp(b)$ where b is the Cox regression coefficient. SD is the standard deviation of the $\log_2$ of the gene expression level. The analysis was done October 29th, 2014. Class comparisons were defined for two classes (class 1: good survivors and class 2: poor survivors) with the variable of OS less than 5 year versus greater than 10 years and determined by the univariate test two-sample T-test. The nominal significance level of each univariate test was 0.001.

The Gene Expression-Based Outcome for Breast Cancer Online (GOBO) tool was used to test each gene individually for survival-associated differences in LumB breast cancer cohorts [14]. Patient cohorts of Distant Metastasis Free Survival (DMFS) was investigated. Data sets for DMFS included Chin, GSE11121, GSE12093, GSE2034, GSE2603, GSE5327,

GSE6532 and GSE7390 (358 patients total); GOBO was also used to determine sample prediction. The analysis was done 12/12/2017.

Kaplan-Meier Plotter was used to test each gene individually for survival-associated differences in LumB breast cancer cohorts [15]. The entire data collection for all breast cancers comprises 35 different data sets which includes the GOBO data sets listed above. The analysis was done 12/12/2017.

2.5. Gene co-expression and copy number variation (CNV) analyses

The co-expressed Genes module of GOBO allows a user to identify genes showing co-expression with a specified "prototype" gene across either a tumor data set. The Chin et al. data set was excluded from the correlation analysis across the tumor data set as this data set was generated on a different version of the U133A chip, which could potentially cause bias in unrestricted correlation analysis. The Pearson correlation was used. Correlation analysis is only performed if the prototype gene passes a log2 standard deviation cut-off threshold. The log2 standard deviation for the genes analyzed was 0.5. For CNV analyses, METABRIC data were analyzed by cBioPortal for gene amplification and gain [16, 17].

2.6. Pathway analyses

To identify enriched signaling pathways among associated proteins Ingenuity pathway analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA, and [18]) and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) was utilized [11].

3. Results

3.1. Differences in expression of RS-related genes correlate with breast cancer survival

To examine how differences in gene expression determine breast cancer sample grouping, the list of 283 genes were used to perform unsupervised Consensus Clustering [19]. This method that uses data resampling followed by iterative re-clustering to estimate cluster stability and to help determine the optimal number of clusters in the TCGA (The Cancer Genome Atlas). Data from the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) were also used to explore reproducibility of the results. While silhouette scores (coefficient value) for TCGA and METABRIC were higher for $k=2$ compared to $k=3$ (Supplemental Figs. 2A and 2D), the greatest increase in the area under the CDF curve (cumulative distribution function) was observed when 3 molecular subtypes are assumed (Figs. 2B and 2D and Supplemental Figs. 2B and 2D). In addition, highest consensus was achieved when $k=3$ (Figs. 2C and 2E).

Unsupervised and supervised clustering of TCGA and METABRIC samples was performed to visualize differences in gene expression associated with the clusters and analyze clusters for correlations with breast cancer subtypes (Fig 3A and 3B and Supplemental Figs. 3A and 3B). It was observed that clusters did not simply group the tumors based on the different molecular subtype as defined by PAM50. Importantly, the clusters obtained from METABRIC data, showed significant survival differences between the three clusters (log-rank test $p=0$ Fig. 3C): patients, whose tumors grouped in cluster 1 had extremely good

outcome, while patient survival in cluster 3 was shortened. TCGA survival data were not used for analysis as the patient follow up is too short to allow the determination of disease free or overall survival. To ensure that cluster-building and survival was not superseded by genes driving the PAM50 phenotype, we repeated the consensus clustering and survival analysis after removing PAM50 genes from the data set (Fig. 3D and Supplemental Fig. 3E). As Supplemental Figs. 3C and 3D show, similar consensus CDF, delta areas and silhouette width were obtained compared to Fig. 2D. In addition, a similar survival difference with a log-rank test p-value of 4.74×10^{-15} , (Fig. 3D) was determined. Again, cluster 3 showed distinct poorer survival, compared to cluster 1 and 2. In addition, it contained a significant portion of the LumB samples (21.6%) that clustered with the majority of HER2 (70.6%) and basal samples (89.02%), while in cluster 2 the majority of LumB (69.6%) grouped together with half of the LumA samples (53.4) and 25.6% of the Her2 samples. Cluster 1 was primarily dominated by LumA samples (41.2%) and 8.8% LumB, 3.8% Her2+ and 7.9% basal samples, respectively (Table 1).

3.2. Gene expression analysis identifies ROS related genes in the LumB subtype that correlate with survival

Patients in cluster 3 showed the poorest survival compared to cluster 2 and 1 (Fig 3E). 89% of all patients with basal-like breast cancer and 70.6% of all Her2+ patients dominate this cluster, taking up 61% of the total cluster. Since these two sub types present in general with shorter survival compared to LumA and LumB subtypes, we wondered if the remainder of the cluster 3 samples, which are mainly LumB patients (16.4%), exhibited significant differences in gene expression compared to other LumB patients found in clusters 1 and 2. To examine specifically the role of the 283 ROS/RNS-related genes in LumB survival, only genes that showed significant differential expression between LumB samples in cluster 3 vs LumB samples in cluster 2 and 3 were identified and analyzed by consensus clustering to identify the most informative number of clusters separating the patient groups. As shown in Fig. 4A and Table 1, 129 genes (46%) of the 280 ROS/RNS-related genes available in the LumB patient cohort, were significantly differently expressed in LumB cluster 3 samples compared with to LumB samples in clusters 1 and 2. Only genes with differential expression of a $p < 0.05$ were considered. As 70.6% of Her2+ patients are found in cluster 3 and 25.6 % are in cluster 2, that show better survival, we also assessed differential expression of Her2+ samples in cluster 3 compared to the remaining Her2+ samples. 53% of all 280 genes were significantly differently expressed in 238 Her2+ samples (Fig. 4A). Consensus clustering to all 490 LumB samples containing 47% differentially expressed ROS genes was applied and revealed a best average silhouette width of 0.71 with a k of 2 (Supplemental Figs. 4B–D). Log-rank test determined then if a significant difference in survival time exists between differential expressed genes of LumB patients in cluster 3 and LumB patients in cluster 1 and 2. Notably, survival was found to be significantly different between cluster 3 and cluster 1 and 2 LumB patients ($p = 0.045$) (Fig. 4B). As a control, Her2 patients in cluster 3 were compared to Her2 patients in cluster 1 and 2 by consensus clustering suggesting partitioning being best when $k = 2$ (Supplemental Figs. 4D and 4E). Log-rank testing showed no significant difference in patient survival ($p = 0.99$) although the majority of Her2 patients made up cluster 3. To evaluate which of the RS-related genes are significantly associated with patient survival in the LumB subtype, we used the BRB-ArrayTools [20]. As

summarized in Table 2 (Supplemental Fig. 3D), 5 genes were significantly associated with survival. Out of those, 4 were ROS regulating genes: PRDX2, PRDX3, KEAP1, GSTK1 and one gene SLC36A1 was identified to be responsive to ROS. BRB class comparison analysis demonstrated that PRDX2, PRDX3 and SLC36A1 were significantly differently expressed ($p=2.41e-05$, $9.56e-05$ and $2.21e-04$, respectively) in the population with shorter than 5 years and longer than 10 years survival and notably ranked high among all 19602 genes analyzed (Fig. 4D). To further confirm that the 5 genes were associated with survival in the LumB subtype in other data-bases, GOBO and Km plotter were investigated. As Table 2 shows, not all 5 genes were significantly associated with survival in other data bases. In fact, a significant association with survival could not be confirmed for KEAP1 by either GOBO (collection of 10 data sets) and Km plotter (collection of 44 data sets) [14]. Thus, both genes were excluded from further analysis.

3.3. GSTK1, PRDX3, PRDX2 and SLC36A1 predict survival in LumB breast cancers

GSTK1, PRDX2, PRDX3 and SLC36A1 show all individual survival differences in the LumB subgroup based on expression across three different data sets. We therefore asked whether these genes can predict survival in LumB cancers. Using Partitioning Around Methoids (PAM) analysis in GOBO building of three clusters across all data sets (8 data sets with 358 patients total, with a 10 yrs censored survival) showed that clinical outcomes for PAM_2 (115 patients) and PAM_3 (132 patients) cohorts were highly significant in univariate as well as multivariate analyses (Fig. 5A). As shown in Fig. 5B, all cohorts were examined in 8 different data sets and proportionally distributed. Intriguingly, as a comparison across all subgroups confirmed there was no significant difference in clinical outcome among LumA or other molecular subtypes, suggesting that expression of the 4 genes is only significantly associated with survival of LumB patients $p=4.53e-03$ (Fig. 5C and Table 3). Survival was also highly significantly associated with estrogen receptor (ER)-positivity ($p=5.11e-05$), negative lymph nodes (LNneg) ($p=9.62e-07$), ER-positivity plus LN-negativity ($p=5.96e-06$) and no treatment ($p=4e-06$). This suggests that higher expression these for genes may predict better outcome in LumB patients that ER-positive and Lymph node negative.

3.4. Pathway analyses of PRDX2, PRDX3, GSTK1 and SLC36A1 signaling networks

To explore functional roles that PRDX2, PRDX3, GSTK1 and SLC36A1 may play in LumB breast cancers, we explored if co-expressing genes of GSTK1, PRDX2, PRDX3 or SLC36A1 showed copy number variations that were specific to the LumB subtype. Co-expressed genes were determined in the GOBO database by screening all breast cancer subtypes using Pearson correlation with a 0.5 correlation cut off (Supplemental Table 2). GSTK1 correlated with 19 co-expressed genes, as PRDX2, PRDX3 and SLC36A1 did not. As analyzed by the cbiportal, although *PRDX2*, *NDFUA13*, *UBXN6* and *RPS9* are all localized on Chr19, as well as *FIS1*, *GSTK1*, *NDUFB2*, *ADCK2* and *LUC7L2* are localized on Chr7 and exhibited similar gain and amplification patterns among breast cancer samples, those patterns were not restricted to LumB alone and seen in other molecular subtypes as well (Supplemental Fig 6).

To understand better the signaling networks driven by PRDX2, PRDX3, GSTK1 and SLC36A1, we next identified known protein interactors utilizing the BioGRID^{3,4} database [21]. Interacting proteins were identified (Supplemental Excel file 3) and together with co-expressing proteins analyzed by pathway analyses by IPA (Table 4) and DAVID (Supplemental Excel file 4), respectively. Interestingly, both analyses overlapped in pathways relating to mitochondrial dysfunction, cell cycle/mitosis, neurological disorders, carbohydrate metabolism and DNA damage.

3.5. Signaling networks of PRDX2, PRDX3, GSTK1 and SLC36A1 can predict survival of LumB patients

Lastly, robustness of LumB associated pathways was tested for predicting LumB outcomes. Co-expressed genes and interacting proteins were combined and analyzed in LumB patient groups for expression-dependent survival. Using PAM analysis in GOBO, building of three clusters across all data sets with censored survival (10 years) showed that while clinical outcomes were significantly different, no significant Hazard Ratio was obtained for the individual groups (Fig 6A). As sample prediction for PRDX2, PRDX3, GSTK1 and SLC36A1 strongly associated with lymph node negative disease, suggesting a role for these genes in early disease, expression dependent-survival for 5 years was examined for interacting and co-expressing genes. This resulted in significant difference in survival overall among all LumB patients tested with a significant advantage of survival for the Pam_3 subgroup (HR=0.34, p=0.018) (Fig. 6B). Furthermore, as a comparison across all subgroups confirmed that there was no significant difference in clinical outcome among LumA or other molecular subtypes, it suggested that expression of the genes participating in PRDX2, PRDX3, GSTK1 and SLC36A1 networks are only significantly associated with survival of LumB patients (Fig. 6C). Lastly, significant pairwise gene correlation with eight different functional modules showed that M-phase genes correlated positively (ANOVA; p<0.00001). This finding reiterated the potential role for PRDX2, PRDX3, GSTK1 and SLC36A1 signaling networks in regulating mitosis.

4. Discussion

ROS are a group of highly reactive ions and molecules that are involved in a variety of biological processes including cancer. In cancer, elevated ROS contribute to its initiation, but as cancer progresses ROS are a product of oncogenic proliferation and mitochondrial dysfunction [22]. To shine more light on if expression of RS genes impact breast cancer survival, we analyzed a gene set of 155 ROS/RNS regulating genes and 128 ROS regulated genes (total of 283 genes), for significant association with breast cancer patient survival. Using TCGA and METABRIC we identified unique clustering for the 283 genes that led to discovery of survival associated genes in LumB breast cancer. Analyzing these 5 survival associated genes in other data sets confirmed 4 genes (GSTK1, PRDX2, PRDX3 and SLC36A1) to be significantly associated with LumB breast cancer survival. Intriguingly, extensive pathway analyses suggested that genes associated with GSTK1, PRDX2, PRDX3 and SLC36A1 are involved in LumB survival by regulating mitosis.

Clustering RS genes in the TCGA and METABRIC data sets revealed three main clusters. Of those, cluster 3 in the METABRIC data set showed a clear distinct shorter survival compared to cluster 1 and 2, which was independent from PAM50 genes within the ROS-genes cohort. Interestingly, 21% of all LumB samples co-clustered with the majority of Her2+ (70.6 %) and basal-like (89.0 %) tumors. This was surprising from the point of view that ROS also promote tumor progression, which has supported a dogma that tumor aggressiveness correlates positively with ROS levels because of the increase in mutagenesis [22, 23]. However, recent studies have shown that the correlation of ROS levels and tumor aggressiveness may not be linearly correlated as ROS-induced DNA damage such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) is significantly higher in ER-positive breast cancers when compared to ER negative breast cancers [24–27]. This suggests that ER signaling may promote oxidative stress. In fact, previous studies have confirmed that estrogen and estrogen metabolites increase not only ROS, but also the tolerance for oxidative damage by inducing antioxidant responses [28–31]. Moreover, growing evidence suggests that in contrast to earlier thoughts that hydroxylated estrogen metabolites are the main source of estrogen related ROS, ER increases ROS through the induction of pro-oxidant genes [28, 32]. As LumB cancers are positive for ER expression, the subset of LumB samples co-clustering in cluster 3 with the majority of Her2+ and basal-like cancers, may share other common genes that parallel the aggressiveness of ER negative tumors. PAM50 gene analysis shows that a subset of LumB cancers share proliferation-associated genes with the basal-like sub type such as MK167, BIRC5 and cyclin B1 [33]. Reverse phase protein assays of the breast cancer TCGA data sets have been investigated and compared with the transcriptomic patterns. Strikingly, protein expression of the G2/M-phase regulating cyclin B1, as well as the G1/S-phase specific cyclin E1, were also found highly distinct between LumA and LumB samples and similarly high in LumB, Her2 and basal-like breast cancers [34].

In general it is well accepted that ROS promote cell proliferation in a context dependent manner via intermediate phosphorylation and ubiquitination of cell cycle dependent kinases and regulatory molecules [35]. For example, PRDX1, which shares over 90% similarity and can form heterodimers with PRDX2, has been shown to control pericentrosomal H₂O₂ levels. Whereas the centrosome is shielded from H₂O₂ through its association with the H₂O₂-eliminating enzyme PRDX1 during interphase, the centrosome-associated PRDX1 is selectively inactivated through phosphorylation by CDK1 during early mitosis, thereby exposing the centrosome to H₂O₂ and facilitating inactivation of centrosome-bound phosphatases [36]. The centrosome is an organelle that serves as a microtubule organizing center that includes proteins such as Microtubule-associated protein tau (MAPT) that ranked 2nd as upstream regulator in our performed IPA analysis. Notably, MAPT when overexpressed inhibits the function of taxanes and its expression was found increased in patients treated with tamoxifen, while fulvestrant decreased MAPT levels and sensitized patients to taxanes [37]. Further support of a possible role for GSTK1, PRDX2, PRDX3 and SLC36A1 networks to play a role in regulating LumB mitosis comes from the IPA analysis shown here, where “mitotic roles of Polo-Like kinases” were identified as third top canonical pathway. Thus, based on findings from the GOBO analyses that suggest expression of the GSTK1, PRDX2, PRDX3 and SLC36A1 networks to correlate positively with M-Phase gene expression and significantly with LumB survival, it is possible that

mitosis in LumB cancer is promoted by RS that are countered by GSTK1, PRDX2, PRDX3 signaling.

Sirtuin-3 (Sir3) is involved in mitochondrial metabolism and life span. Interestingly, Sirt-3 knock out mice exhibited dysregulated mitochondrial detoxification pathways, increased total and mitochondrial ROS levels and develop spontaneous breast cancers that resemble the pathology of LumB cancers as they were ER and PR positive, poorly differentiated and displayed high rates of proliferation (K67) [38]. The increased mitochondrial ROS in that model were attributed to dysfunctional manganese super oxide dismutase (SOD) activity. Notably, none of the three SODs were significantly associated with survival in LumB cancers in our analysis. Instead we found that mitochondrial localized PRDX3 correlated significantly with LumB patient survival [39, 40]. Interestingly, PRDX3 acetylation has been found decreased in Sirt3 depleted cells, however the consequences of this post-translational modification are currently unknown [41].

GSTK1 is a glutathione S-transferase, which is located in the peroxisomes and known to catalyze the conjugation of glutathione to a wide range of hydrophobic substances thus facilitating the removal of these compounds from the cell. GSTK1 has also been localized to the mitochondria where it is thought to participate in GSH metabolism. This suggests that together with PRDX3, GSTK1 could play a role in mitochondrial activities that affect specifically LumB breast cancer. As GSTK1 is involved in drug resistance mechanisms, further studies are needed to understand GSTK1's function in LumB breast cancers [42]. Similarly, the role of the proton-coupled amino acid transporter SLC36A1 in cancer is still unclear, however, it has been found to activate the rapamycin complex 1 (TORC1), which suggests a role for SLC36A1 in cancer metabolism [43].

5. Conclusions

LumB breast cancer is considered the more aggressive cancer type among luminal breast cancer with shortened survival and increased metastasis. In addition, its response to hormonal therapies is lessened compared to the LumA subtype [44]. Our findings described here support the notion that in contrast to the more aggressive TNBC and Her2 positive cancers, ER-positivity is associated with RS and oxidative stress as antioxidant genes GSTK1, PRDX2 and PRDX3 when higher expressed, associate with and predicting longer survival. As this also correlates with LN negativity, our data suggest that *GSTK1*, *KEAP1*, *PRDX2* and *SLC36A1* gene expression could be a predictor for early or less aggressive LumB breast cancers. As LumB breast cancer is considered the more aggressive cancer type among luminal breast cancer with shortened survival and increased metastasis, understanding the molecular pathways driving this subtype may support new treatment strategies in the future [44].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- ROS and RNS (reactive species = RS)-related genes have not been yet associated with breast cancer survival.
- RS-related genes correlate with breast cancer patient survival in a subtype specific manner.
- *GSTK1*, *PRDX2*, *PRDX3* and the RS-responsive target *SLC36A1* are significantly associated with patient survival in LumB breast cancer.
- *GSTK1*, *PRDX2*, *PRDX3* and *SLC36A1* can predict patient survival in LumB breast cancer.
- *GSTK1*, *PRDX2*, *PRDX3* and *SLC36A1* signaling networks can predict patient survival in LumB breast cancer and correlate with pathways regulating mitotic and mitochondrial functions.

Figure 1. Workflow of the study

RS-related genes were examined in the TCGA and METABRIC data bases for differential expression by consensus clustering. A k of 3 was deemed best and examined for differences in survival. Clusters were then examined for PAM50 molecular subtype distribution that revealed a subgroup of LumB patients in cluster 3 that showed distinct differences in survival compared to patient groups in clusters 1 and 2. BRB analysis determined significant associated of candidate genes with LumB patient survival that was confirmed in GOBO and km plotter data bases. Selected candidate genes were further evaluated for survival prediction, interacting proteins and pathways analysis and copy number variation (CNV).

Consensus Clustering of ROS genes plus targets in TCGA and Metabric data set

Figure 2. Differential expression of RS-related genes divides patient cohorts in three groups in TCGA and METABRIC datasets

A. Generation of RS related gene list: ROS/RNS regulating genes were added to overlap of the genes regulated by either Diamide, H₂O₂ and Menadione (K3) (Venn diagram). **B and D.** Cumulative distribution functions (CDF) of the consensus matrix for each number k of clusters ($k=2, 3, \dots, 6$, top) and delta area plot showing the relative change in area under the CDF curves (bottom) of 961 breast cancer patient samples from the TCGA (C) and 1981 breast cancer patient samples from the METABRIC data set. The shape and area under CDF curves allow one to select the appropriate number of clusters [45]. Here partitions into 3

clusters seem appropriate. **C and E.** Consensus matrices. The consensus matrices genes (rows) and tumor samples (columns). Consensus values range from 0 (white, never clustered together) to 1 (dark blue, always clustered together) marked by white to dark blue. The consensus matrices are then ordered by the consensus clustering which is depicted as a dendrogram atop the heatmap.

Figure 3. RS-related genes determine differences in patient survival that is independent of Perou genes

A and B. Heatmaps showing RS-related genes differentially expressed between three clusters identified in Figure 2 by consensus clustering from the TCGA and METABRIC data set, respectively. Each gene is normalized to standard normal distribution with green indicating lower expression and red indicating higher expression. **C.** Kaplan-Meier curve analyzing overall patient survival of METABRIC data from 3B with patient samples in cluster 3 showing the shortest survival. **D.** Kaplan-Meier curve analyzing overall patient survival of METABRIC data from 3C with Perou genes removed and patient samples in cluster 3 showing the shortest survival.

Figure 4. Differential expression of RS-related genes shows differences in survival of LumB patients

A. Numbers of RS-related genes that are significantly differential expressed (cutoff $p < 0.05$) between cluster 3 and cluster cluster 1 and 2 in patients of LumB and Her2 subtypes, respectively. B. Kaplan-Meier survival curve comparing survival of LumB patients from cluster 3 with patients from cluster 1 and 2. C. Kaplan-Meier survival curve comparing survival of Her2 patients from cluster 3 with patients from cluster 1 and 2. D. Gene expression of RS-related genes is ranked in the LumB patient group for longer than 10yr and shorter than 5yr survival using METABRIC data in the BRB-array tool.

Figure 5. *GSTK1*, *PRDX2*, *PRDX3* and *SLC36A1* expression correlates with survival of LumB patients

A. Kaplan-Meier analysis, using DMFS as endpoint, for LumB tumors (n = 449) stratified into the five groups based on *GSTK1*, *PRDX2*, *PRDX3* and *SLC36A1* gene expression level. On the right, HR and p-values for univariate and multivariate analyses for three patient groups differing significantly in survival based on gene expression. Confidence interval (CI) was set to 95%. **B.** Gene expression distribution of groups across data sets used in analysis. **C.** Association with outcome for *GSTK1*, *PRDX2*, *PRDX3* and *SLC36A1* expression in subgroups of breast cancer using DMFS as endpoint and 10-year censoring. Samples in the 358-sample set were stratified into five groups based on correlation to the gene expression of all three genes, followed by Kaplan-Meier survival analysis in 17 subgroups using 358 cases with DMFS follow-up. Logrank p-values are shown as $-\log_{10}(\text{P-value})$.

Figure 6. Expression of interacting proteins and co-expressed genes *GSTK1*, *PRDX2* and *SLC36A1* correlates with survival of LumB patients

A. Kaplan-Meier analysis, using DMFS as endpoint, for LumB tumors ($n = 358$) stratified into the three groups based on gene expression levels. **B.** As in (A) for 5yr censored survival. **C.** Association with outcome for *GSTK1*, *PRDX2*, *PRDX3* and *SLC36A1* expression in subgroups of breast cancer using DMFS as endpoint and 5-year censoring. Samples in the 358-sample set were stratified into five groups based on correlation to the gene expression of all three genes, followed by Kaplan-Meier survival analysis in 17 subgroups using 358 cases with DMFS follow-up. Logrank P-values are shown as $-\log_{10}(P\text{-value})$. **D.** Correlation of

matched predictor genes show significant correlation. For each gene module and gene in the centroid of the 157 genes, a Spearman correlation value is computed by comparing the expression pattern across all samples for a specific gene to the corresponding rank sum for each sample in the specific module. Red dots indicate actual correlation values.

Table 1
Distribution of Pam50 molecular subtypes among clusters formed in 1981 METABRIC samples.

Cluster	NC	Basal	Her2+	LumA	LumB	Normal-like
1 numbers (%)	4	26 (7.9)	9 (3.8)	296 (41.2)	43 (8.8)	131 (65.5)
2 numbers (%)	2	10 (3.0)	61 (25.6)	383 (53.3)	341 (69.6)	27 (13.5)
3 numbers (%)	0	292 (89.0)	168 (70.6)	40 (5.6)	106 (21.6)	42 (21.0)
Total samples: 1981	6	328	238	719	490	200

Table 2

Determination of Kaplan-Meier survival.

Web tool	BRB		GOBO		Km Plotter
Gene	Parametric p-value	Hazard Ratio	Kaplan Meier p-value	Kaplan Meier p-value	Kaplan Meier p-value
PRDX2	0	15	0.7	6.5e-05	7.9e-05
SLC36A1	0	25	2.33	0.027	NS
PRDX3	0	46	0.73	7.6e-04	NS
KEAP1	0	88	0.53	0.031	NS
GSTK1	0	99	0.65	0.005	4.5e-04

P-values for PRDX2, SLC36A1, PRDX3, KEAP1 and GSTK1 of LumB patients were generated using METABRIC (491 patients), GOBO (449 patients) and KmPlotter (1149 patients) data sets.

Table 3

Log Rank p-values for all 17 subsets.

Subset of data set	logrank p
<i>All</i>	8.76E-06
<i>ER-positive</i>	5.11E-05
<i>ER-negative</i>	0.63
<i>PAM50_Basal</i>	0.82
<i>PAM50_HER2enriched</i>	0.34
<i>PAM50_Luminal A</i>	0.18
<i>PAM50_Luminal B</i>	4.52E-03
<i>PAM50_Normal-like</i>	0.12
<i>LNneg</i>	9.62E-07
<i>LNpos</i>	0.87
<i>ERposLNneg</i>	5.96E-06
<i>ERnegLNneg</i>	1
<i>Grade1</i>	0.13
<i>Grade2</i>	0.15
<i>Grade3</i>	0.15
<i>Untreated</i>	4.00E-06
<i>TAM</i>	0.10

Table 4

Top candidates in IPA analysis

IPA	p-value
<i>Top Canonical Pathways</i>	
PI3K/AKT Signaling	1.3e-07
Mitochondrial dysfunction	5.0e-07
Mitotic Roles of Polo-Like Kinases	1.2e-06
Telomerase Signaling	4.0e-06
Xenobiotic Metabolism Signaling	6.4e-06
	p-value
<i>Top Upstream Regulators</i>	
p53	2.3e-13
Microtubule associated protein tau (MAPT)	1.2e-12
MYC	2.9e-10
Amyloid β precursor protein (AAA)	1.6e-09
Nitrofurantoin	1.9e-09
	Molecules
<i>Top Diseases and Biofunction</i>	
Cancer	179
Organismal Injury and Abnormalities	183
Cellular Assembly and Organization	65
Cellular Function and Maintenance	59
Cell cycle	55
	Score
<i>Top Networks (Score >30)</i>	
Carbohydrate Metabolism, Small Molecule Biochemistry, Post-translational Modification	61
Free Radical Scavenging, Small Molecule Biochemistry, Amino Acid Metabolism	55
Developmental Disorder, Hereditary Disorder, Neurological Disease	42
Nucleic Metabolism, Small Molecule Biochemistry, Cell Signaling	40