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Prognostic Effect of Glycogen Synthase Kinase 3A (*GSK3A*) mRNA Expression in Breast Cancer Patients

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Abstract

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase and regulates glycogen synthase, cell-cycle progression and apoptosis. PI3K/PTEN/AKT/GSK3/mTORC1 pathway is often activated in multiple human cancers and activated AKT phosphorylates and inactivates GSK3. GSK3 exists as α and β isoforms in mammals; however which GSK3 isoform regulates cancer cell proliferation is still unclear. Notably, most studies have focused on GSK3 β and very few reports addressed the role of the alpha isoform in cancer. Current study explored the prognostic role of *GSK3A* in breast cancer patients using Kaplan-Meier plotter (KM plotter). *GSK3A* mRNA expression was significantly correlated with breast cancer patients survival. Lower *GSK3A* mRNA expression was significantly correlated to poorer relapse-free survival (RFS) in breast cancer patients (including luminal A and basal type). Luminal A breast cancer patients showed a better RFS with higher *GSK3A* mRNA expression in systemically untreated and chemotherapy only treated patients. Lower mRNA expression of *GSK3A* was significantly correlated to poorer RFS in luminal A breast cancer patients with grade 2 tumors and negative lymph node status. The distinct prognostic effect of *GSK3A* mRNA expression in breast cancer patients thus makes it a potential therapeutic target.

Keywords: Breast cancer, *GSK3A*, relapse-free survival, luminal A

1. Introduction

Breast cancer remains the primary cause of cancer-related deaths in women worldwide[1], regardless of headways in surgical and neoadjuvant/adjuvant therapy. About 30% of breast cancer patients develop metastatic disease[2] with resistance to conventional chemotherapeutic drugs. Based on distinctive gene expression, prognostic and therapy implications, breast cancer is of four intrinsic molecular subtypes: Luminal A and B, *HER2*-enriched (Human epidermal growth factor receptor 2) and basal-like[3]. Both Luminal A and B subtypes express estrogen receptor (ER), but

luminal B subtype has higher expression of proliferative genes and poorer prognosis than luminal A[4]. *HER2*-enriched and basal-like have worse survival and prognosis than the luminal subtypes. *HER2*-enriched subtype has augmented expression of *HER2* gene and includes *HER2*+ tumors independent of hormone receptors status[5]. The basal-like subtype is more aggressive than other subtypes, lacks ER, progesterone receptor (PR) and *HER2*, and has the shortest relapse-free and overall survival[3].

In addition to gene expression based heterogeneity, breast cancer has also shown some metabolic heterogeneity by using versatile energy sources and metabolic pathways for their energetic and anabolic requirements. Thus there is a critical need to find new prognostic markers as well as targeted therapeutic agents for breast cancer. Glycogen synthase kinase 3 (GSK3) is a serine/threonine protein kinase involved in glycogen metabolism[6,7] and is catalytically active in resting cells[8,9]. GSK3 regulates glycogen synthesis through insulin signaling, where insulin activates phosphatidyl-inositide 3-kinase (PI3K), leading to phosphorylation and activation of protein kinase B (AKT)[10]. AKT then phosphorylates and inhibits GSK3 and this results in dephosphorylation and activation of its substrates like glycogen synthase and eukaryotic initiation factor 2B (eIF2B) and subsequent synthesis of glycogen and protein[10]. GSK3 is inhibited by amino acids via mammalian target of rapamycin (mTOR) and the downstream S6K1 kinase[11], by epidermal growth factor (EGF) via MAPK and PI3K/AKT pathway and by tumor-promoting phorbol esters via MAPK cascade[8]. WNTs induce GSK3 inhibition by binding to their frizzled receptors[12] and absence of WNTs activates GSK3, which phosphorylates axin, β -catenin, and adenomatous polyposis coli (APC) and phosphorylated β -catenin undergoes ubiquitin-mediated proteolytic degradation[10]. GSK3 is also involved in Nuclear factor kappa B (NF- κ B)[13] and Hedgehog-Gli pathways[14] and these are aberrantly regulated during tumor progression. Thus the critical role of GSK3 in various oncogenic signaling pathways, makes it a prominent therapeutic target in various cancers[15-19].

GSK3 is expressed as two highly homologous forms in mammals namely GSK3 α and GSK3 β [10]. The isoforms are almost identical (98%) but differ in their N- and C-terminal domains[6]. AKT phosphorylates N-terminal serine 21 and serine 9 of GSK3 α and GSK3 β , respectively, leading to their inactivation[10].

Gene knockout studies show similar functions for GSK3 isoforms in various studies but these are not completely redundant. *GSK3A* null mice are viable but have metabolic defects like enhanced glucose, insulin sensitivity and reduced fat mass that cannot be prevented by the beta isoform[20]. *GSK3A* and GSK3B have distinct roles in developmental and differentiation processes[12] and in regulation of transcriptional activation[21]. However, the two isoforms have either redundant or distinct functions in cell survival, which varies with the cell type[22-24]. Mice with phosphorylation sites of endogenous alleles replaced by non-phosphorylable alanine (GSK3- α S21A, β S9A) show a high impairment (40%) in neurogenesis, increased susceptibility to hyperactivity and a heightened response to a novel environment[25]. These mice also have mild anxiety, increased immobility time and susceptibility to stress-induced depressive-like behavior[25].

Notably, most studies have focused on GSK3 β [26,27] and very few addressed the role of GSK3 α [27-29] in cancer. This study thus analyzed the previously unknown prognostic role of *GSK3A* mRNA expression in breast cancer patients.

2. Methods

Generation of survival Curves: The Kaplan-Meier (KM) plotter tool (http://kmplot.com/analysis/) was used to determine the prognostic value of *GSK3A* mRNA expression using microarray data in breast cancer patients[30]. In the KM plotter, *GSK3A* mRNA expression data was correlated with relapse-free (RFS), overall (OS), distant metastasis-free (DMFS) and post-progression (PPS) survival in breast cancer patients using all probe sets per gene for a follow-up threshold of 240 months. For mRNA expression analysis, samples were split into high and low expression groups based on the median expression of gene. The median expression was selected to split patients over other options of lower quartile, lower tertile, upper tertile and upper quartile

expression to give almost same sample numbers for both groups and hence less bias. The patients' samples were analyzed for subtypes[31] and different cohorts: systemically untreated patients, patients with systemic treatment (both endocrine and chemotherapy) and patient cohort similar to SEER prevalences. SEER refers to Surveillance Epidemiology and End Results - the population-based tumor registry program of the National Cancer Institute[32]. Patient cohort similar to SEER prevalences includes 500 patients of whom all clinical data are available and of whom the prevalences are similar to actual SEER based United States prevalence numbers[33]. Hazard ratio (HR), 95% confidence intervals and logrank P were analyzed and presented for all the survival curves and P value of < 0.05 was considered to be statistically significant.

3. Results

Effect of *GSK3A* mRNA expression on breast cancer patient's survival: KM plotter was used to analyze role of *GSK3A* mRNA expression in breast cancer patients for its effect on RFS, OS, DMFS and PPS. For *GSK3A* mRNA, the Affymetrix (Affy) IDs are 202210_x_at and 632_at.

GSK3A mRNA lower expression was significantly correlated to poorer RFS in all breast cancer patients till 150 months of follow-up threshold for Affy ID: 202210_x_at (Figure 1A) and till 100 months of follow-up threshold for Affy ID: 632_at (Figure 1B). This shows that a higher *GSK3A* mRNA expression favors RFS in breast cancer patients (Figure 1A and 1B). *GSK3A* mRNA expression was not significantly correlated to OS, DMFS and PPS in breast cancer patients (data not shown). To understand the overlap of survival curves in figures 1A and 1B post 150 and 200 months respectively, we analyzed three available patients' cohorts for effect of *GSK3A* mRNA expression on RFS in breast cancer patients. Figure 1C shows that systemically untreated breast cancer patients had a significantly poorer RFS with a lower *GSK3A* mRNA expression (only for Affy ID: 632_at) without any overlap. Results for patients with systemic treatment were same as Figure 1A (for Affy ID: 202210_x_at) and Figure 1B (for Affy ID: 632_at). There was no significant correlation of *GSK3A* mRNA expression to RFS in patient cohort similar to SEER prevalences (data not shown).

Effect of *GSK3A* mRNA expression on intrinsic breast cancer subtypes survival: The prognostic role of *GSK3A* was further evaluated within intrinsic molecular subtypes of breast cancer: luminal A, luminal B, basal and *Her2*+. For Luminal A breast cancer patients, lower *GSK3A* mRNA expression was significantly correlated with poorer RFS for both Affy IDs: 202210_x_at (Figure 2A) and 632_at (Figure 2B) till 150 months of follow-up threshold. *GSK3A* mRNA expression was not significantly correlated to OS and DMFS in Luminal A breast cancer patients (data not shown). PPS was also poorly correlated with lower *GSK3A* mRNA expression in Luminal A breast cancer patients till 200 months of follow-up threshold, for both Affy IDs (Figure 2C and 2D).

Luminal B and *Her2*+ breast cancer patients did not show significant correlation of RFS, OS, DMFS and PPS with *GSK3A* mRNA expression (data not shown). Luminal A breast cancer patients were further analyzed for three different cohorts: systemically untreated patients, patients with systemic treatment (both endocrine and chemotherapy) and patient cohort similar to SEER prevalences. Chemotherapy included both adjuvant and neoadjuvant treatment for breast cancer patients. Systemically untreated luminal A breast cancer patients showed significantly poorer RFS with lower *GSK3A* mRNA expression (Figure 3A) till 100 months of follow-up threshold for Affy ID: 632_at only. Similar results were seen for luminal A patients with systemic treatment, where lower *GSK3A* mRNA expression was significantly correlated with poorer RFS for both Affy IDs: 202210_x_at (Figure 2A) and 632_at (Figure 2B) and poorer PPS (Figure 2C and 2D). *GSK3A* mRNA expression was not significantly correlated to OS, DMFS and PPS for systemically untreated patients, OS and DMFS for systemically treated patients and RFS, OS, DMFS and PPS for patient cohort similar to SEER prevalences (data not shown) for Luminal A patients.

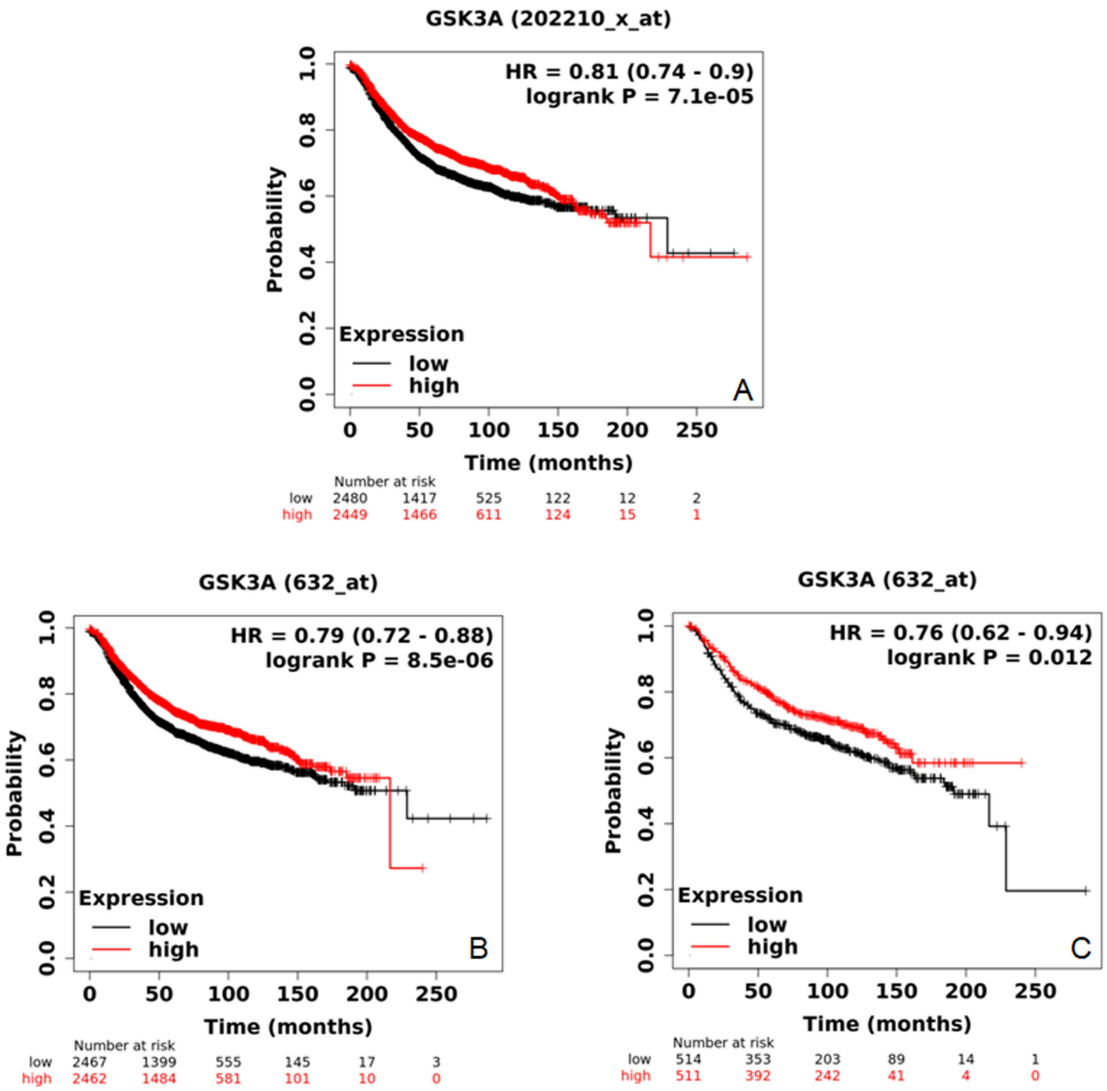

Figure 1: *GSK3A* mRNA has two Affy IDs: 202210_x_at and 632_at in KM Plotter. RFS curve for all breast cancer patients with Affy ID: 202210_x_at (A) and for Affy ID: 632_at (B). RFS curve for systemically untreated breast cancer patients (Affy ID: 632_at) (C).

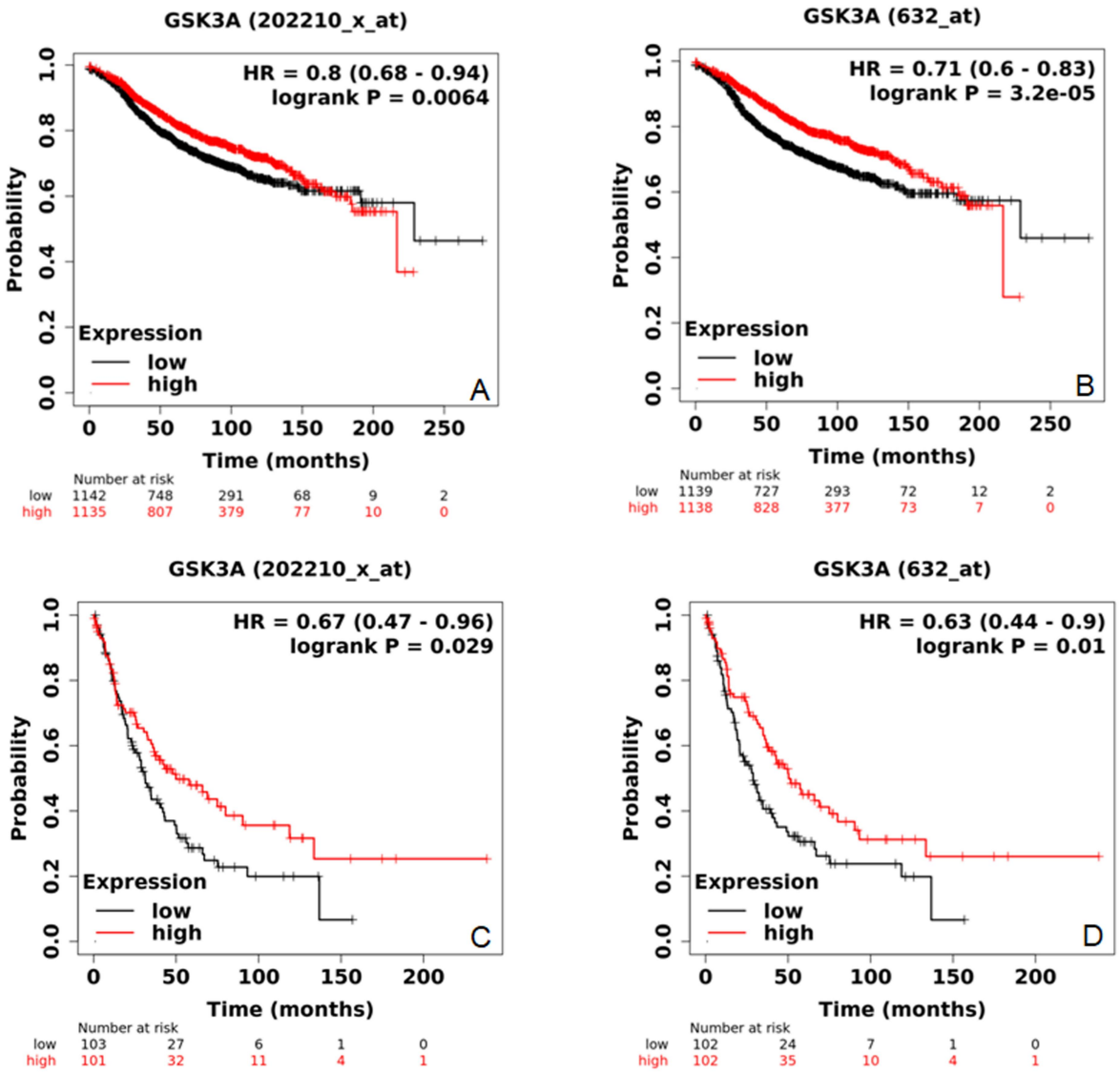

Figure 2: RFS curve for luminal A breast cancer patients with Affy ID: 202210_x_at (A) and Affy ID: 632_at (B). PPS curve for luminal A breast cancer patients with Affy ID: 202210_x_at (C) and Affy ID: 632_at (D).

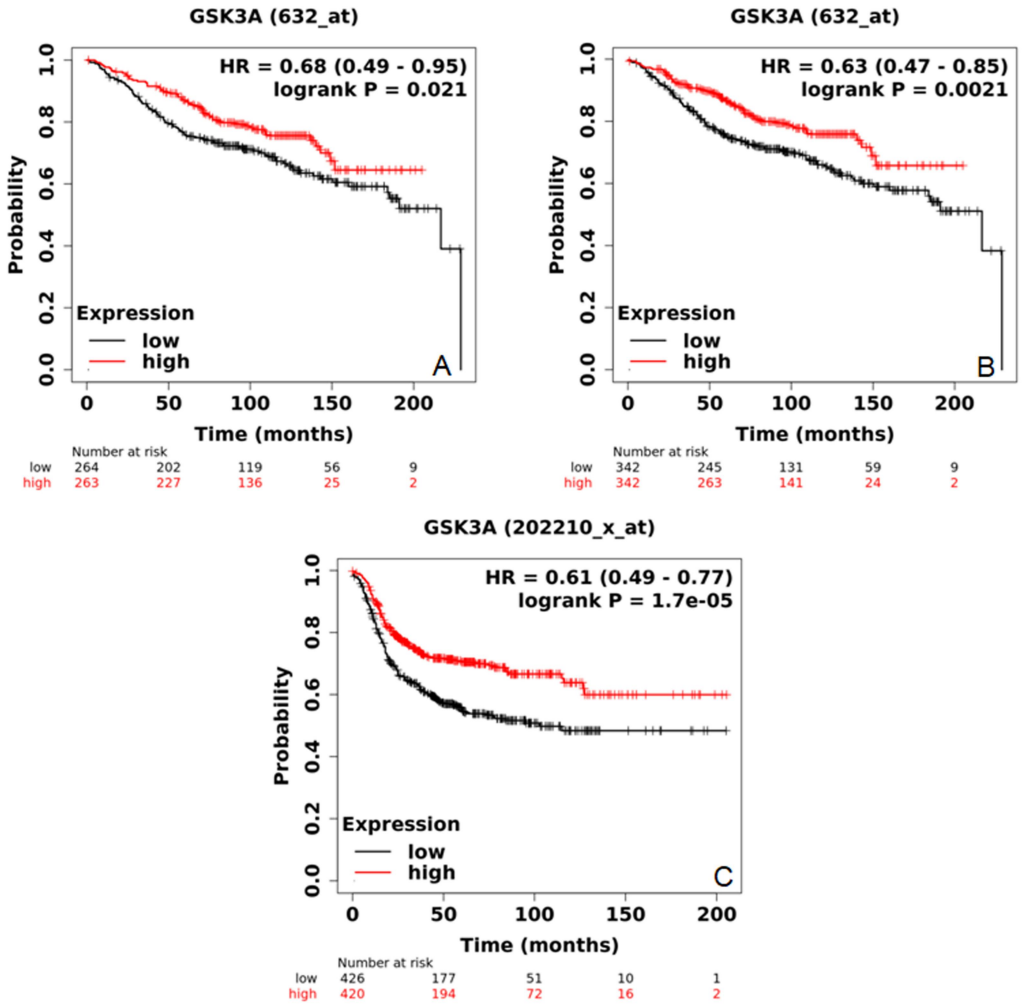

Figure 3: RFS curve for systemically untreated luminal A breast cancer patients with Affy ID: 632_at (A). RFS curve for chemotherapy only treated luminal A breast cancer patients with Affy ID: 632_at (B). RFS curve for basal breast cancer patients with Affy ID: 202210_x_at (C).

The systemically treated luminal A patients were further divided into two different groups for analysis: patients receiving only endocrine therapy and patients receiving only chemotherapy. Lower *GSK3A* mRNA expression was significantly correlated to poorer RFS for luminal A patients receiving only chemotherapy (Figure 3B) till 100 months of follow-up threshold and not endocrine only therapy (data not shown). Luminal A patients receiving endocrine only and chemotherapy only therapy did not show significant correlation of *GSK3A* mRNA expression to OS, DMFS and PPS (data not shown).

Basal breast cancer patients with lower *GSK3A* mRNA expression showed significantly poorer RFS for Affy ID: 202210_x_at only (Figure 3C) upto 200 months of follow-up threshold, but showed no correlation to OS, DMFS and PPS (data now shown).

Correlation of *GSK3A* mRNA expression with lymph node status and tumor grade: All the breast cancer patients were further analyzed for correlation of *GSK3A* mRNA expression with lymph node status and tumor grade. Breast cancer patients with combination of negative lymph node status and grade 2 tumors had significantly poorer RFS (and not OS, DMFS and PPS, data not shown) with lower *GSK3A* mRNA expression till 200 months of follow-up threshold for Affy ID: 202210_x_at (Figure 4A) and till 100 months of follow-up threshold for Affy ID: 632_at (Figure 4B). All the other lymph node status and tumor grade combinations, negative or positive lymph node status alone and only tumor grades (1, 2 and 3) had

no significant correlation to *GSK3A* mRNA expression with RFS, OS, DMFS and PPS of breast cancer patients (data not shown).

Patients with combination of negative lymph node status and grade 2 tumors were further analyzed for correlation of *GSK3A* mRNA expression and survival within different patient cohorts. Systemically untreated breast cancer patients (with negative lymph node status and grade 2 tumors) had significantly poorer RFS with lower *GSK3A* mRNA expression for Affy ID: 632_at only (Figure 4C). Systemically treated breast cancer patients also had significantly poorer RFS with lower *GSK3A* mRNA expression for Affy ID: 202210_x_at (Figure 4A) and 632_at (Figure 4B). Systemically treated patients were divided into endocrine therapy and chemotherapy groups, and poorer RFS was significantly correlated to lower *GSK3A* mRNA expression for chemotherapy treated patients (with negative lymph node status and grade 2 tumors) for Affy ID: 202210_x_at (Figure 4D) and 632_at (Figure 4E) and not for endocrine treated patients (data not shown).

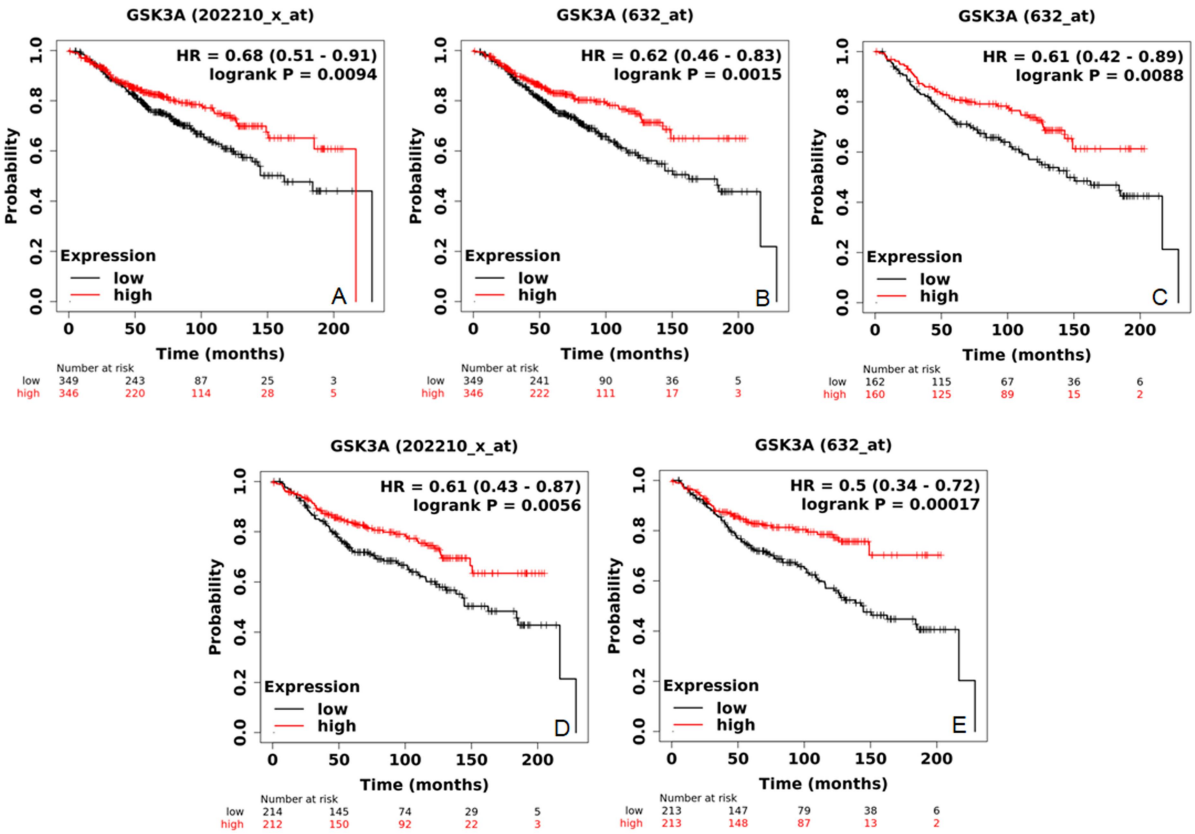

Figure 4: RFS curve for grade 2 tumors with negative lymph node status in all breast cancer patients for Affy ID: 202210_x_at (A) and 632_at (B). RFS curve for systemically untreated grade 2 tumors with negative lymph node status in all breast cancer patients for Affy ID: 632_at (C). RFS curve for chemotherapy only treated grade 2 tumors with negative lymph node status for Affy ID: 202210_x_at (D) and 632_at (E).

Both systemically untreated and treated patients (with negative lymph node status and grade 2 tumors) had no significant correlation of *GSK3A* mRNA expression to DMFS (data not shown). Patient cohort similar to SEER prevalences did not show significant correlation of above parameters to RFS, OS, DMFS and PPS (data not shown).

Patients with negative lymph node status and grade 2 tumors showed poorer OS with lower *GSK3A* mRNA expression for Affy ID: 202210_x_at only (Figure 5A). Systemically untreated breast cancer patients (with negative lymph node status and grade 2 tumors) did not show significant correlation of *GSK3A* mRNA expression to OS (data not shown). However, systemically

treated grade 2 tumors with negative lymph node status showed poorer OS with lower *GSK3A* mRNA expression for Affy ID: 202210_x_at only (Figure 5A). Chemotherapy only treated patients with grade 2 tumors and negative lymph node status showed poorer OS with lower *GSK3A* mRNA expression for Affy ID: 202210_x_at only (Figure 5B) and not endocrine treated patients (data now shown). Poorer PPS was also significantly correlated with lower *GSK3A* mRNA expression for grade 2 tumors with negative lymph node in breast cancer patients for Affy ID: 202210_x_at only (Figure 5C). Systemically treated patients with grade 2 tumors and negative lymph node status showed significantly poorer PPS with lower *GSK3A* mRNA expression for Affy ID: 202210_x_at only (Figure 5C) and not patients with no systemic treatment, endocrine treatment only and chemotherapy only (data now shown).

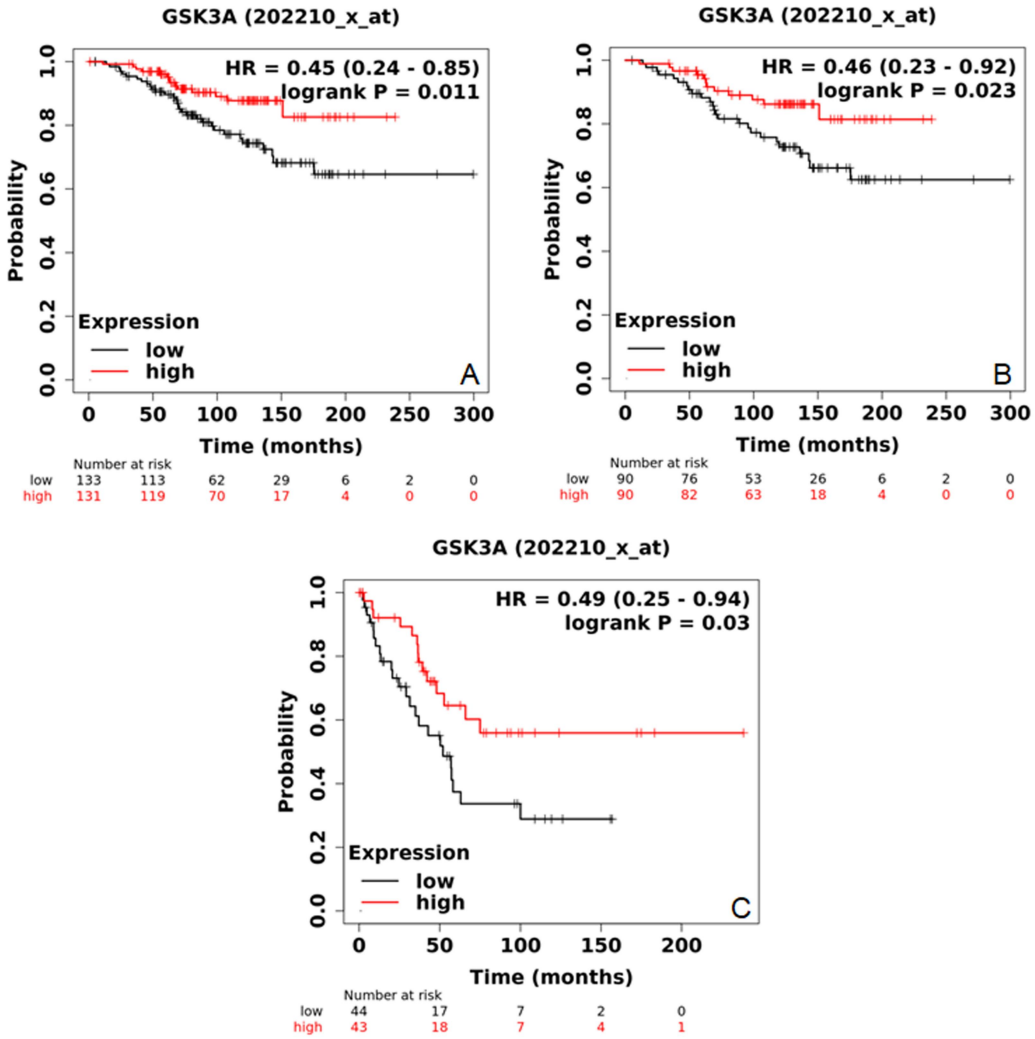

Figure 5: OS curve for grade 2 tumors with negative lymph node in all breast cancer patients for Affy ID: 202210_x_at (A). OS curve for chemotherapy only treated patients with grade 2 tumors and negative lymph node status for Affy ID: 202210_x_at (B). PPS curve for grade 2 tumors with negative lymph node in all breast cancer patients for Affy ID: 202210_x_at (C).

Correlation of *GSK3A* mRNA expression with lymph node status and tumor grade on patients' survival was further analyzed for intrinsic breast cancer types. Figure 6A shows that luminal A breast cancer patients with negative lymph node status has significantly poorer RFS with lower *GSK3A* mRNA expression for systemically untreated patients only (for Affy ID: 632_at), and not for patients with systemic treatment and patient cohort similar to SEER prevalences (data not shown). No significant

correlation of *GSK3A* mRNA expression was found for luminal A breast cancer patients with negative lymph node status to OS, DMFS and PPS and positive lymph node status to RFS, OS, DMFS and PPS (data no shown). Also, luminal A breast cancer patients showed no significant correlation of *GSK3A* mRNA expression to any tumor grade (1, 2 and 3) with RFS, OS, DMFS and PPS (data no shown). Also none of the above studied parameters were significantly correlated to RFS, OS, DMFS and PPS in luminal B, basal and *Her2*+ breast cancer patients and in any of the patients' cohorts (data not shown).

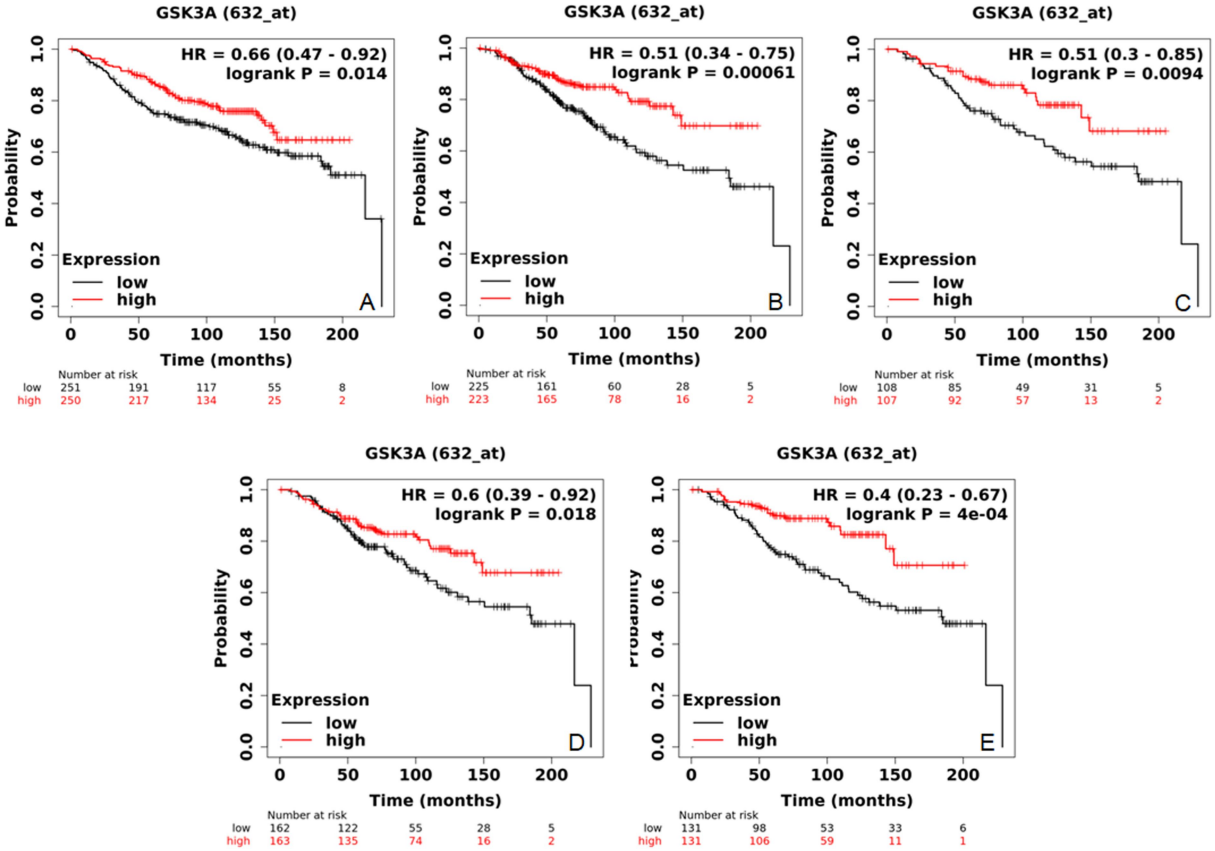

Figure 6: RFS curve for systemically untreated Luminal A breast cancer patients with negative lymph node status for Affy ID: 632_at (A). RFS curve for grade 2 Luminal A breast cancer patients with negative lymph node status for Affy ID: 632_at (B). RFS curve for systemically untreated grade 2 Luminal A breast cancer patients with negative lymph node status for Affy ID: 632_at (C). RFS curve for endocrine only treated grade 2 Luminal A breast cancer patients with negative lymph node status for Affy ID: 632_at (D). RFS curve for chemotherapy only treated grade 2 Luminal A breast cancer patients with negative lymph node status for Affy ID: 632_at (E).

Figure 6B shows that luminal A breast cancer patients with negative lymph node status and grade 2 tumors has significantly poorer RFS and not OS, DMFS and PPS (data not shown) for lower *GSK3A* mRNA expression (for Affy ID: 632_at). Luminal A breast cancer patients with negative lymph node status and grade 2 tumors were further analyzed in three patients' cohorts. Significantly poorer RFS and not OS, DMFS and PPS (data not shown) was seen with lower *GSK3A* mRNA expression in systemically untreated (Figure 6C) and treated patients (Figure 6B) and not in patient cohort similar to SEER prevalences (data not shown). Luminal A breast cancer patients with negative lymph node status and grade 2 tumors also showed significantly poorer RFS and not OS, DMFS and PPS (data not shown) with only endocrine (Figure 6D) and only chemotherapy (Figure 6E) treatment.

4. Discussion

Cancer cases continue to rise globally and breast cancer remains the most frequently diagnosed cancer in females and metastasis remains the leading cause of death by this cancer[1]. GSK3 regulates cell-cycle progression, differentiation and apoptosis[34,35] through phosphorylation of its targets[36]. Under basal conditions, GSK3 is active and its activity is regulated by selective phosphorylation by AKT. GSK3 α and β are activated upon phosphorylation of residues Tyrosine279 and Tyrosine216, respectively and inhibited upon phosphorylation of residues Serine21 and Serine9, respectively[10]. Which GSK3 isoform regulates cancer cell proliferation in a particular cell type is still not known[15,24]. Few reports have addressed the role of the alpha isoform (GSK3 α) in cancer cells[27-29]. Thus the present study assessed breast cancer patients' datasets to evaluate whether *GSK3A* mRNA expression is linked to different clinic-pathological features and prognostic signature of breast cancer and its subtypes.

Patient survival analysis show that lower *GSK3A* mRNA expression is significantly correlated with worse RFS and not OS, DMFS and PPS in breast cancer patients (Figure 1A and 1B), suggesting that *GSK3A* may serve as a useful prognostic marker for predicting RFS and can be a therapeutic target for breast cancer. Lack of overlap of survival curves in systemically untreated patients (Figure 1C) compared to systemically treated patients (Figure 1A and 1B) suggest that *GSK3A* mRNA levels may be modulated by systemic treatment over time and thus *GSK3A* is a potential target in drug- and chemotherapy-resistant breast cancer patients. AKT-directed phosphorylation inhibits GSK3[37,38], which results in β -catenin aggregation and CD1 activation[10,39]. Brazilein (isolated from Caesalpinia sappan plant) treatment induced cell death and arrested proliferation in MCF-7 cell line by decreasing GSK3 phosphorylation by AKT and ensuing degradation of phosphorylated β -Catenin[40]. Further, over-expression of GSK3 and GSK3-inactive mutant induced and prevented apoptosis in Rat-1 fibroblasts and PC12 cells, respectively[41]. Thus higher *GSK3A* mRNA expression may promote better RFS in breast cancer patients through its role as a tumor suppressor.

The relationship between *GSK3A* mRNA expression and intrinsic breast cancer subtypes were also analyzed. Figure 2 shows that lower *GSK3A* mRNA expression is significantly correlated with worse RFS (Figure 2A and 2B) and PPS (Figure 2C and 2D) in Luminal A breast cancer patients and not in Luminal B and *Her2*+ patients. Analysis of RFS for Luminal A type patients within available cohorts show that patients who are systemically untreated (Figure 3A) and received only chemotherapy (Figure 3B) has better RFS with higher *GSK3A* mRNA expression and not the patients receiving endocrine only treatment.

Correlation of *GSK3A* mRNA with Luminal A subtype predominantly might be due to the fact that about 70% of breast cancers express estrogen receptor (ER)[42] and GSK3 regulates ER α activity by Ser118 phosphorylation[43]. Tamoxifen and aromatase inhibitors (AI) are the most frequently used treatment for hormone-dependent breast cancer, but are not without adverse effects due to significant estrogen depletion. Thus the results (Figure 2, 3A and 3B) suggest that *GSK3A* has the potential to be used along with hormone-dependent treatment in breast cancer patients to overcome endocrine treatment resistance.

Basal breast cancer patients showed worse RFS with lower *GSK3A* mRNA expression (Figure 3C). Basal breast cancer spreads faster with worse prognosis than luminal subtypes and has limited treatment options. The results thus suggest *GSK3A* both as a novel prognostic marker and a potential therapeutic target for basal breast cancer patients.

Correlation of *GSK3A* mRNA expression was also assessed for lymph node status and tumor grade in breast cancer patients. The results show that only grade 2 tumors (well/moderate differentiation) with negative lymph node status had significant correlation of lower *GSK3A* mRNA expression with poorer RFS (Figure 4A and 4B), poorer OS (Figure 5A) and poorer PPS (Figure 5C). These tumors were further analyzed within different cohorts and systemically untreated patients had significantly poorer RFS with lower *GSK3A* mRNA expression (Figure 4C). Systemically treated patients showed significant correlation of poorer RFS (Figure 4A and 4B), poorer OS (Figure 5A) and poorer PPS

(Figure 5C) to lower *GSK3A* mRNA expression. Among the systemically treated patients, only chemotherapy treated patients showed significantly poorer RFS (Figure 4D and 4E) and poorer OS (Figure 5B) with lower *GSK3A* mRNA expression.

These breast cancer patients were then analyzed within intrinsic subtypes and only luminal A subtype with no systemic treatment correlated lower *GSK3A* mRNA expression to poorer RFS for negative lymph node status (Figure 6A). Luminal A breast cancer patients with grade 2 tumors and negative lymph node status (Figure 6B) also showed poorer RFS with lower *GSK3A* mRNA expression. This set of patients (i.e. luminal A, grade 2 tumor and negative lymph node) was further analyzed for different cohorts. Significantly poorer RFS was correlated to lower *GSK3A* mRNA for systemically untreated (Figure 6C), endocrine only treated (Figure 6D) and chemotherapy only treated (Figure 6E) patients till 100 months of follow-up threshold. These results might explain the intersection of RFS survival curves in luminal A type patients (Figures 2A and 2B) post 150 months of follow-up threshold, suggesting that higher *GSK3A* mRNA expression may promote survival in early stage breast cancer patients than advanced stage. *GSK3A* mRNA expression correlation to RFS, OS and PPS in systemically treated patients with well/moderate differentiated tumors and no cancer in lymph node may help predict the outcome of hormone-dependent treatments in breast cancer patients. Thus correlation of *GSK3A* mRNA expression with above clinic-pathological features of breast cancer patients suggests that *GSK3A* is a potential prognostic marker for early stages of breast cancer.

Sample sizes for *GSK3A* mRNA expression for some clinic-pathological features were too low to reach a significant correlation and thus further studies are needed. Current results showed that lower *GSK3A* mRNA expression is closely associated with poorer survival and thus is a promising therapeutic target in breast cancer patients, especially in luminal A subtype. These results are useful to further understand the heterogeneity and complexity of breast cancer, and identified *GSK3A* as a novel target to predict prognosis of breast cancer patients.

Conflict of Interest

The author does not have any conflict of interest in the manuscript.

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