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Research Article

Expression Patterns and Relevance of FN3K, Nrf2, and NQO1 in Breast Cancers

Narasimha Murthy Beeraka,[1,6,10,12] Jin Zhang,[5] Chinnappa Apattira Uthaiah,[9] Churganova A Anastasia,[6] Junqi Liu,[3,4] Hemanth Vikram Poola Ramachandrappa,[1,7] Priyanka MK,[2] Namitha Bannimath,[15] Rajashree Deka,[14] Prasath Manogaran,[11,13] Karen D Souza,[1] Namratha Mandya Parashivamurthy,[8] Vladimir Nikolaevich Nikolenko,[6] Kirill Vladimirovich Bulygin,[6] Prashant Vishwanath,[9] Ruitai Fan,[1,4] Gurupadayya Bannimath[1]

[1]Department of Pharmaceutical chemistry, JSS College of Pharmacy, JSS Academy of Higher Education & Research (JSS AHER), Mysuru, Karnataka, India
[2]Department of Pathology, JSS Medical College, JSS Academy of Higher Education and Research (JSSAHER), Mysore, India
[3]Cancer Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China
[4]Department of Radiation Oncology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China
[5]Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Mississippi
[6]I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, Russian Federation
[7]Xenone Healthcare Pvt. Ltd., New Delhi, India
[8]Department of Pharmacology, JSS College of Pharmacy, JSS Academy of Higher Education & Research (JSS AHER), Mysuru, Karnataka, India
[9]Center of Excellence in Molecular Biology and Regenerative Medicine, Department of Biochemistry, JSS Medical College, JSS Academy of Higher Education and Research, Mysore, India
[10]Raghavendra Institute of Pharmaceutical Education and Research (RIPER), Anantapuramu, Chiyyedu, Andhra Pradesh, India
[11]Department of Biotechnology, Bharathiar University, Coimbatore, Tamil Nadu
[12]Herman B. Wells Center for Pediatric Research, Department of Pediatrics, Indiana University School of Medicine, Indianapolis, USA
[13]Department of Clinical and Translational Sciences, Joan C. Edwards School of Medicine, Marshall University, Huntington, West Virginia, USA
[14]Animal Physiology and Biochemistry Laboratory, Department of Zoology, Gauhati University, Assam, India
[15]Department of Pharmacology, University of Galway, Galway, Ireland

Abstract

Objectives: Previous studies described the prognostic significance of nuclear factor erythroid 2-related factor 2 (Nrf2) in breast cancers. Nrf2 is significantly involved in inducing antioxidant responses in tumor cells to neutralize oxidative stress. A recent study by V Sanghvi et al 2019 demonstrated the role of fructosamine-3-kinase (FN3K) in Nrf2 deglycation in cancer but the prognostic significance based on pathological, clinical relevance has remained unknown in breast cancer patients.

In this study, we determined the relevance of FN3K based on Prediction Analysis of Microarray 50 (PAM-50)algorithm-based breast cancer classification & FN3K gene expression patterns on tumor-node-metastasis (TNM) wise using The

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Address for correspondence: Narasimha M Beeraka as 'MRes (UK), PhD; Dr. Gurupadayya BM, M.Pharm, PhD. Department of Pharmacology, Raghavendra Institute of Pharmaceutical Education and Research (RIPER), Anantapuramu, Chiyyedu, Andhra Pradesh 515721, India; Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS Academy of Higher Education & Research (JSS AHER), Mysuru, Karnataka, India
Phone: +918618382637 **E-mail:** bnmurthy24@gmail.com; bmgurupadayya@jssuni.edu.in

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Cancer Genome Atlas (TCGA) database subsequently determined its expression patterns in luminal A (HR+/HER2 – (human epidermal growth factor receptor 2), ER (Estrogen Receptor positive)+/PR+(Progesterone Receptor positive), ER+/PR–, and ER–/PR+), luminal B (HR+/HER2+, ER+/PR+, ER+/PR–, and ER–/PR+), HER 2+ (ER-, PR-, HER2+), and TNBCs (Triple-Negative Breast Cancers) (ER-, PR-, HER2-) along differential protein expression patterns of Nrf2, and NQO1(NAD(P)H quinone oxidoreductase) (markers of oxidative stress).

Methods: R-statistical computing, Kaplan Meier survival curves, immunohistochemistry, Western blotting was used to explore the expression of FN3K relative to the expression of Nrf2, and NQO1.

Results: We observed significantly a higher FN3K gene expression in luminal B when compared to its expression in other cancer types, Basal, Her2, Luminal A, and normal tissue as per TCGA database. FN3K gene expression is significantly higher ($P<0.05$) in stage IV, and T4 compared to the other stages or sizes of breast cancer. Correlated expression of both genes confers significant implications in the patient's overall survival. A control study pertinent to FN3K, Nrf2, and NQO1 immunoreactivity is performed and the expression was highly evident in luminal A, luminal B when compared to HER 2+, TNBCs and adjacent normal breast tissue. Breast cancer disease-specific survival was determined based on the FN3K and Nrf2 gene expression patterns.

Conclusion: Hence, this study described the relevance of FN3K-Nrf2 signaling in the breast cancers.

Keywords: FN3K, Nrf2, NQO1, PAM50 algorithms, breast cancer, TNM stages (tumor (T), nodes (N), and metastases (M))

Breast carcinoma (BC) continues to exhibit resistance to existing therapies due to dose-limiting toxicity and adverse effects to non-target tissues. Hence, identifying key protein(s) that play vital roles in drug resistance and metastasis in breast carcinoma conditions is highly significant for early prognosis and to choose early personalized oncomedicine.[1-3] Previous studies also delineated the significant association between the expressions of Estrogen receptor-alpha (ER-α) and activity of many kinases and phosphatases in clinical BC subtypes.[4-9] Several kinases are expressed in different subtypes of breast cancers. Giampaolo Bianchini et al. (2010) described the expression patterns of kinases in clinical subtypes of breast cancer and explored the prognostic relevance of the various kinase metagenes and correlated their functions through in vitro studies.[10] In this study, both immune kinase cluster and mitosis kinase cluster associated with prognostic relevance in the clinical BC subtypes and concluded that their expression varies according to the BC clinical subtype; for instance, the mitosis kinase score resulted in the worse prognosis but exhibited a higher pathological complete response (pCR) in breast cancers with ER+/HER- but this was not examined in ER-/HER2- or HER+ cancers. On the contrary, significantly a higher immune kinase score was reported a good overall survival in ER+/HER- and HER+ cancer subtypes.[10]

Previous reports described that the Nrf2 as a significant modifier in cancer development and its efficacy as a double-edged sword due to its role in tumor progression and tumor suppression.[11-13] For instance, the Nrf2 can activate antioxidant system through antioxidant response elements to mediate redox stress in cancer cells and on the contrary, it can also facilitate redox environment to neutralize the chemotherapy effects and enhance the chemoresistance of cancer cells.[13, 14] Hence, it has been concluded that the higher expression of Nrf2 in certain cancers is correlated to the poor prognosis.[15, 16] Nrf2 exhibits its significant implications in breast cancers, for instance, the nuclear Nrf2 levels could be considered as the prognostic factor in breast cancers.[17] Due to its double-edged role, it is insignificant to implicate its activity for efficient cancer prognosis. Hence, although there are several reports pertinent to the prognostic significance of the Nrf2 transcriptional signature as prognostic markers in breast cancers. Oncogenic activity of Nrf2 is regulated by deglycation by fructosamine 3-Kinase (FN3K), a protein kinase.

FN3K can induce deglycation through phosphorylation of basic amino acids include lysine, arginine in Nrf2 in hepatocellular carcinoma.[3, 18] Deglycated Nrf2 could cause tumor progression across liver, lung, brain, and pancreas.[19-22] Normally, the glycation of Nrf2 in cancer cells could maintain Nrf2 in inactivated state during post-translational sugar modifications.[3] FN3K sensitive glycation is substantially examined across various hepatic proteins include translation factors, DNA replication & repair proteins, splicing factors, and histone proteins in cancers.[3] Thus, FN3K has been reported to involve in protein glycation and induce accumulation of advanced glycation end products (AGEs).[3] Deglycation of Nrf2 is mediated through FN3K activity in hepatocellular carcinoma (HCCs) and concluding that Nrf2 activity requires FN3K. However, there are no reports pertinent to the expression patterns of FN3K in the breast cancers to denote the clinical molecular subtypes of BC in the patients based on its expression. In our study, we deciphered the expression patterns FN3K along with Nrf2, using breast cancer related data available through public databases including TCGA (The Cancer Genome Atlas). We performed a control study pertinent to the expression patterns of these proteins using both in vitro cell line studies and immunohistochemistry procedures on the breast cancer patient cohorts.

Methods

Ethics Approval

A complete Institutional Ethics Committee approval (JSSMC/IEC/240921/09NCT/2021-22, dated 4 October 2021) was obtained from the JSS medical college and Hospitals to use paraffin-embedded patient's breast tumor blocks. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent: Written informed consent was obtained from all individual participants included in the study. Minors were not participated in this study.

TCGA Database Search & Patient Samples and R-statistical computing

RNA sequencing (RNAseq) expression data describing a total of 1,069 breast tumor samples and 96 matched adjacent normal and tumor samples were acquired from the UCSC xena (http://xena.ucsc.edu/) (Supplementary-2). The RNA-seq data (FPKM- Fragments Per Kilobase of transcript per Million mapped reads) of The Cancer Genome Atlas (TCGA)-BRCA (The Cancer Genome Atlas Breast Invasive Carcinoma) cohort were further transformed to log2 (TPM + 1) (Transcripts Per Million) and two-direction median centered. Breast cancer molecular subtypes based on the PAM50 classification was executed using R package 'genefu'. In addition to the Spearman rank correlation analysis, we have used online tools, such as GEPIA2 to validate the gene expression (Supplementary-2).

Statistical Computing

A paired t-test was performed to examine the differences in FN3K mRNA expression between 96 human breast tumors and matched adjacent normal tissue.

A Spearman rank correlation was conducted to determine the relationship between FN3K mRNA and Nrf2, Keap1 (Kelch-like ECH-associated protein 1), and Nrf2-target gene expression. To calculate the Nrf2-target gene signature for each sample, we calculated the mean expression of the 15 gene - gene expression signature as detailed by Hast et al. ("GCLC (Glutamate-Cysteine Ligase Catalytic Subunit)" "GCLM (Glutamate-Cysteine Ligase Modifier Subunit)" "G6PD (glucose-6-phosphate dehydrogenase)" "PRDX1 (Peroxiredoxin-1)" "GSTM4 (Glutathione S-Transferase Mu 4)" "MGST1 (microsomal glutathione S-transferase 1)" "NQO1 (NAD(P)H dehydrogenase quinone-1)" "HMOX1 (heme oxygenase 1)" "TXNRD1 (Thioredoxin Reductase 1)" "ABCC1 (ATP Binding Cassette Subfamily C Member 1)" "ABCC2 (ATP Binding Cassette Subfamily C Member 2)" "FASLG (Fas Ligand)" "GSR (Glutathione-Disulfide Reductase)" "SLC7A11 (Solute Carrier Family 7 Member 11)" "TXN (Thioredoxin)".[23]

Wilcoxon rank-sum tests were applied to compare the differences between different clinical features, clinical stage, tumor-wise (T) and node wise (N).

The survival curve was plotted by R package 'survminer', the function 'surv_cut point' were used to find a threshold of FN3K expression, showing variable FN3K gene expression. We used the two-stage test instead of log-rank test. Analytical methods can be referenced on this article.[24]

Chemicals and Cell Lines

Breast cancer cell lines such as MCF-7 (Michigan Cancer Foundation) (passage no. 45-55), T47D (Tumorigenicity 47D) (passage no. 60-72), BT-474 (Breast Tumour-474) (passage no. 48-58), and triple negative breast cancer (TNBCs) cell lines including MDA-MB-231 (MD Anderson-Metastatic Breast Cancer 231) (passage no. 52-62), MDA-MB-468 (MD Anderson-Metastatic Breast Cancer 231) (passage no. 58-63), and liver cancer cell line HepG2 (hepatoblastoma cell line) (passage no. 41-52) were procured from the National Center for Cell Science (NCCS), Pune, Maharashtra, India.

Primary antibody for FN3K was obtained from the Invitrogen, Thermofisher (Rabbit polyclonal, Catalog # PA5-28603) and secondary antibody (Rabbit cat#: SC2357 and Goat cat#:SC2020) were procured from Santa Cruz Biotech company, USA. DMEM (Dulbecco's Modified Eagle Medium) is procured from Thermofisher Scientific Ltd, USA. Other primary antibodies for Nrf2 (cat#: ab62352), NQO1 (cat#: 62262) were procured from Abcam, Cambridge, USA and Cell signaling Technologies.

Cell Cultures

Cell lines were cultured in 4.5% glucose containing DMEM medium having 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Invitrogen).

Tumor Sample Collection

A complete Institutional Ethics Committee approval (JSSMC/IEC/240921/09NCT/2021-22, dated 4 October 2021) was obtained from the JSS medical college and Hospitals for pathological assessment of luminal A (HR+/HER2–, ER+/PR+, ER+/PR–, and ER–/PR+), luminal B (HR+/HER2+, ER+/PR+, ER+/PR–, and ER–/PR+), HER 2+ (ER-, PR-, HER2+), and TNBCs (ER-, PR-, HER2-) for differential protein expression patterns of FN3K, Nrf2, and NQO1.

Tumor specimens pertinent to the above four categories of breast cancer subtypes of five each were obtained from

the breast cancer patients who were received surgical resection at Pathology department, JSS Medical College, JSS Academy of Higher Education & Research, Mysore, Karnataka, India during the period of 2017 to 2020. These tumor tissues and normal breast tissues were obtained in paraffin-embedded tissue blocks and subjected to immunohistochemistry procedures. Patient characteristics were given in Supplementary Tables. S1-S10.

Immunohistochemistry (IHC)

Paraffin-embedded breast tissues of luminal A, luminal B, HER 2+, and TNBCs were sectioned subsequently subjected to Hematoxylin-Eosin (H&E) staining. Hepatocellular carcinoma tissues, lung carcinoma of paraffin-embedded blocks was selected for FN3K, and Nrf2, NQO1 expression as a positive control. Tissue collection and punching was performed as the procedures described by Bovilla et al. 2021.[25] IHC procedures were executed to determine the differential expression patterns of FN3K, Nrf2 and NQO1 in the breast tissues.[17] Primarily the sections obtained after sectioning using microtome were mounted onto the slides subsequently subjected to deparaffinization using xylene, and antigen retrieval using antigen retrieval buffer at 100°C (10 mM sodium citrate, pH 6.0). 3% hydrogen peroxide was used to quench the endogenous peroxides within the tissues. Later, the sections were subjected to washing thrice with PBS for five minutes each. Subsequently, primary antibodies were used to probe FN3K (1:350), Nrf2 (1:200), and NQO1 (1:200) and incubated overnight at 4°C. Next day, Secondary antibody loading was performed with conjugated horseradish peroxidase (HRP) for 1 hour and incubated at room temperature. Subsequently, the TBS washings were given to the sections thrice for five minutes each. DAB (3,3'-diaminobenzidine: (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6)) chromogen loading for 20 minutes was used to probe bound antibodies. Later, the sections were subjected to counter staining using H&E (Hematoxylin and eosin) and the processed slides with sections were observed for tissue morphology and expression patterns of FN3K, Nrf2, and NQO1 markers using (BX 53, Olympus Corporation Shinjuku, Tokyo, Japan). Grading and IHC scoring was performed using two experienced expert pathologists for positively stained cells (% of stained cells in the field/slide) and determined intensity staining: 0: no staining), 1: weakly stained, 2: moderately stained, and 3: strongly stained.[26, 27]

Western Blotting

Total protein content of various breast adenocarcinoma (MDA-MB-231, MDA-MB-468, and MCF-7) and ductal invasive carcinoma (BT-474, T-47D) cancer cells was isolated with the aid of lysis buffer from several treated groups. Later, overall protein content was estimated using BCA (Bicinchoninic Acid Assay) method. Equal quantity of proteins (50 µg) was subjected to separation process using 12% gel in SDS-PAGE at 100V for two hours. Subsequently, the separated proteins were transferred onto PVDF (Polyvinylidene difluoride) membrane with the aid of semidry equipment. Membranes were subjected blocking using skimmed milk (5%) protein at least for one-hour TBST (Tris-Buffered Saline with Tween) buffer. Membranes were subjected to TBST washings 3 times and incubated with primary antibody to probe FN3K' at 4°C overnight. Next day, the TBST washings were given to the membranes and loaded with 'HRP-linked secondary antibody' for 2 hours. Afterward, the ECL (1:1 ratio of reagent 1 and reagent 2) was added to the above samples and observed for banding pattern in Chemi-UV tech in the dark till the bands appeared. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control.

Results

PAM-50 algorithm-based breast cancer classification & FN3K gene expression patterns on TNM wise: TCGA-BRCA cohort analysis through PAM-50 algorithm delineated the differential expression patterns in the different breast cancer types including Basal type, Her2, luminal B, luminal A, Normal-like subtypes. We observed significantly a higher FN3K expression in luminal B when compared to its expression in other cancer types, Basal, Her2, Lum A (Luminal A), and normal (Fig. 1A). Later, we executed the **Stage-wise** analysis of FN3K expression patterns from stage I to stage IV in this cohort (Fig. 1B) and results concluded the expression is significantly higher in stage IV compared to the other stages of breast cancer. Furthermore, the **tumor size-wise** expression of FN3K reported the expression of FN3K expression was higher in T4 than its expression in T1, T2, and T3 (Fig. 1C). But, the expression pattern of FN3K expression was non-significant in the **node wise** expression in N0, N1, N2, and N3 (Fig. 1D).

FN3K expression in breast tumor tissue vs. adjacent tissue: FN3K gene expression is significantly higher in the breast cancer tissue than its expression observed in the adjacent tissues through paired t-test indicating its prognostic significance in breast cancer patients (Fig. 2).

Overall survival analysis: Breast cancer disease-specific survival was determined based on the FN3K and Nrf2 gene expression patterns. Results concluded the correlated expression of both genes has significant implications in the overall survival of the patients. The overexpression of Nrf2 caused low overall survival but statistically not significant (Fig. 3A). FN3K expression resulted in significant influence

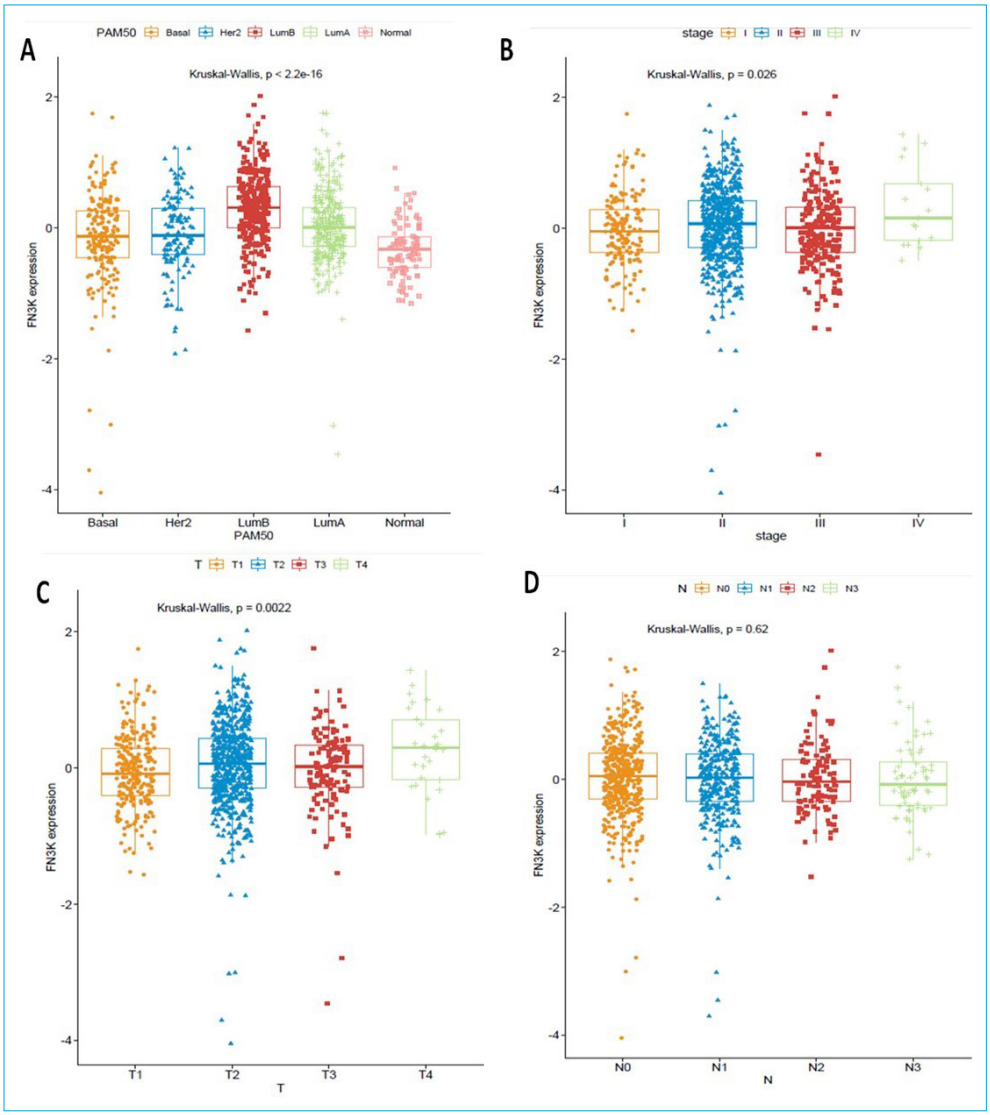

Figure 1. TCGA Database search and analysis: **(a)** FN3K is differentially expressed in breast cancers such as Basal type, Her2, luminal B, luminal A, Normal-like subtypes, which was analyzed by PAM50 algorithm, and the expression of FN3K in luminal B type breast cancer was significantly higher than the Basal, Her2, LumA, and normal, $p<0.05$. **(b)** Stage-wise (from stage I to stage IV) differential expression of FN3K in breast cancer patients. **(c)** FN3K expression based on T1, T2, T3, and T4 sizes of breast cancers or in different pathological grades, $p<0.05$; **(d)** There is no statistical significance in the node wise expression of FN3K in N0, N1, N2, and N3, $p>0.05$.

on patient's overall survival in the BC patients ($p<0.05$) (Fig. 3B). Correlated survival plots to denote the correlation between FN3K and Nrf2 expression: Low expression of both FN3K and Nrf2 resulted in higher disease specific survival whereas the 'low FN3K and high Nrf2' and 'high FN3K and Nrf2' expression patterns resulted in the poor disease specific survival (Fig. 3C).

Correlation of FN3K and Nrf2 transcription factor downstream-gene analysis: The relationship between FN3K mRNA and NRF2, KEAP1, and NRF2-target gene expression was executed and the Nrf2-target gene signature for each sample was determined by the mean expression of 15 gene - gene expression signature ("GCLC" "GCLM" "G6PD" "PRDX1" "GSTM4" "MGST1" "NQO1" "HMOX1" "TXNRD1" "ABCC1" "ABCC2" "FASLG" "GSR" "SLC7A11" "TXN" (PMID: 18829555). The mRNA expression of Keap1 and Nrf2 relative to FN3K mRNA levels was depicted and FN3K expression exhibited a positive correlation with 'Nrf2 target gene expressions' [r=0.7495], and negatively correlated to 'Keap1' [r=0.5240], and 'Nrf2 mRNA expression' [r=0.5214] (Fig. 4). The increased FN3K expression is correlated to the upregulation of Nrf2 downstream genes in human breast

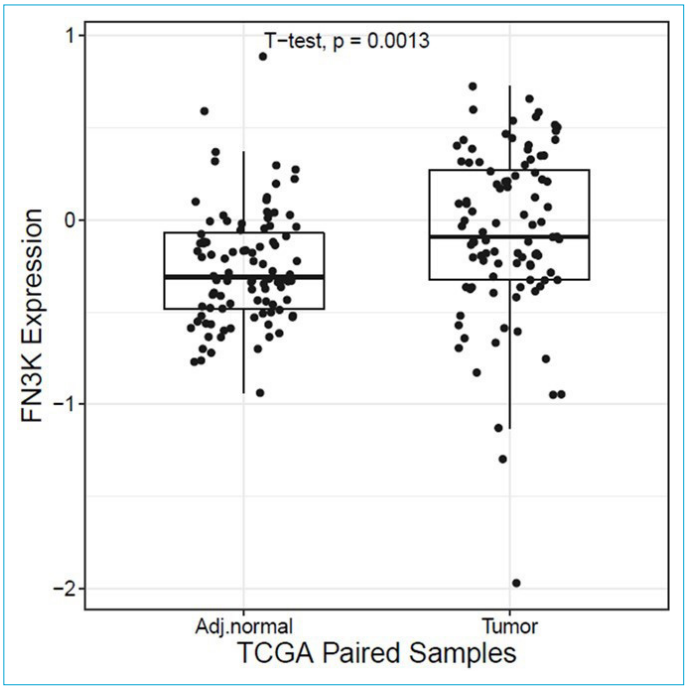

Figure 2. FN3K gene are differentially expressed in breast cancer (BC) and adjacent tissues, and the expression is significantly elevated (paired t-test) in breast cancer tumors than the adjacent normal tissue, p<0.05.

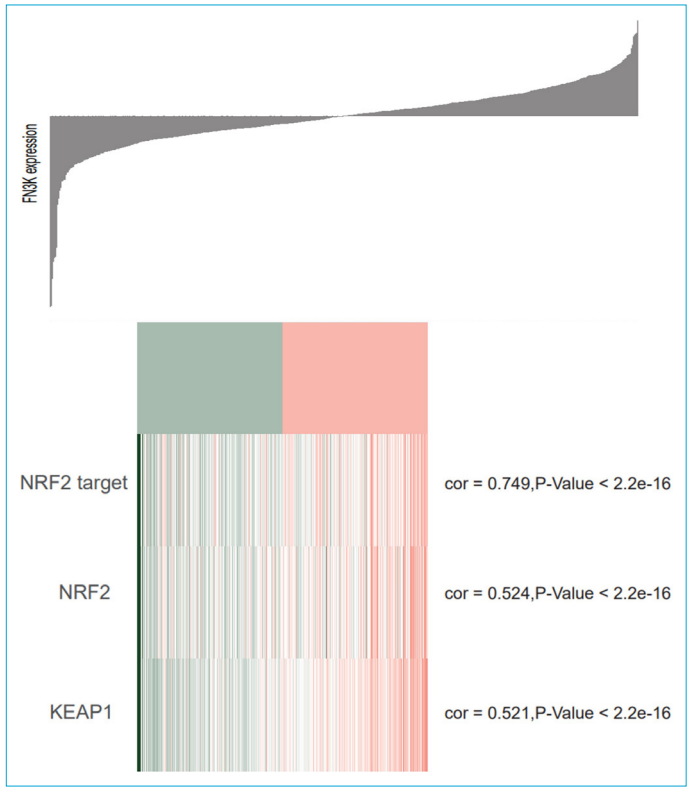

Figure 4. Spearman rank correlation analysis: The relationship between FN3K mRNA and NRF2, KEAP1, and NRF2-target gene expression was executed and the Nrf2-target gene signature for each sample was determined by the mean expression of 15 gene - gene expression signature ("GCLC" "GCLM" "G6PD" "PRDX1" "GSTM4" "MGST1" "NQO1" "HMOX1" "TXNRD1" "ABCC1" "ABCC2" "FASLG" "GSR" "SLC7A11" "TXN" (PMID:18829555). The mRNA expression of Keap1 and Nrf2 relative to FN3K mRNA levels was depicted and FN3K expression exhibited a positive correlation with 'Nrf2 target gene expressions' [r = 0.7495], and negatively correlated to 'Keap1' [r = 0.5240], and 'Nrf2 mRNA expression' [r = 0.5214]. The increased FN3K expression is correlated to the upregulation of Nrf2 downstream genes in human breast tumors concluding the FN3K-Mediated protection of Nrf2 activity.

tumors concluding the FN3K-mediated protection of Nrf2 activity.

Differential expression of FN3K, Nrf2, and NQO1 proteins in breast tumors than adjacent tissues: We have performed a simple control study for analyzing the protein expression patterns of FN3K, Nrf2, and NQO1 respectively. We have selected one category of each paraffin-embedded tumor block for screening the expression patterns of FN3K, Nrf2, NQO1 in different cancers. IHC studies revealed that the substantial protein expression of FN3K has been observed in luminal type in both luminal A (HR+/HER2−, ER+/PR+, ER+/PR−, and ER−/PR+), luminal B (HR+/HER2+, ER+/PR+, ER+/PR−, and ER−/PR+) compared to the expression observed in triple-negative breast cancer type (ER-, PR-, HER-), or basal, Her2, normal type.[28] The expression of tumor cells vividly observed with H&E staining for luminal A),

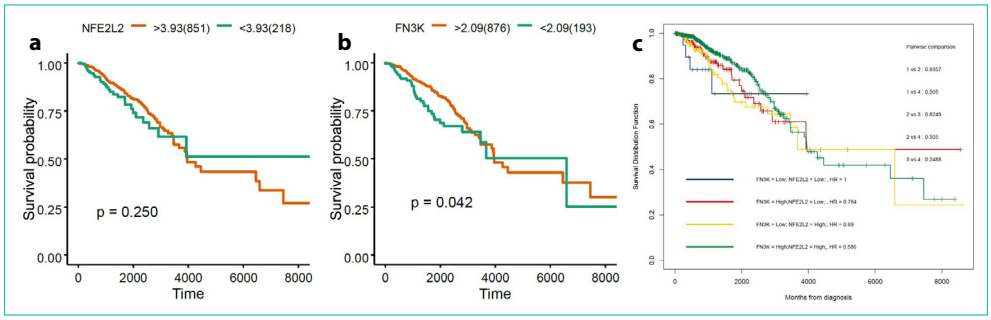

Figure 3. Kaplan Meier Survival curves: **(a)** Nrf2 survival plots, **(b)** FN3K survival plot, (p<0.05, HR: 0.68, 95% CI: 0.45-.02) **(c)** Both low expression of FN3K and Nrf2 together could produce a high disease-specific survival.

luminal B, HER 2+, and TNBCs. In addition, Ki67 tumor proliferation was observed in these four kinds of tumor types (Fig. 5A, 5B). Immunolocalization of FN3K substantially higher in luminal A and luminal B type tumors compared to other TNBCs and HER2+ tumors (Fig. 5C). Expression of the FN3K protein in HCC tissue was considered as positive control whereas lung carcinoma tumor tissue was considered as positive control for Nrf2, and NQO1 (Fig. 5D, 5E).

Expression of FN3K in ductal invasive breast carcinoma cells: Western blotting described that the FN3K expression is in the invasive ductal carcinoma cells such as T47-D, and BT-474. Its expression also observed in the adenocarcinoma cells, MCF-7. However, the expression of FN3K protein is significantly absent in other adenocarcinoma cells including MDA-MB-468, MDA-MB-231 (Fig. 6). FN3K expression in HepG2 liver cancer cells was used as an internal control from two random cell lysate samples.

Discussion

Breast cancer is a heterogenous and exhibits distinct morphological characteristics, and immunohistochemical profiles and unique histopathological subtypes. Several approaches such as MammaPrint[29], Oncotype DX[30], and PAM50[31], were developed in order to classify the breast tumors to inform prognosis subsequently to prescribe personalized treatment. For instance, PAM-50 is a 50-gene signature which typically can classify breast cancer into 5 molecular subtypes viz., 'Luminal A', 'Luminal B', 'HER2-

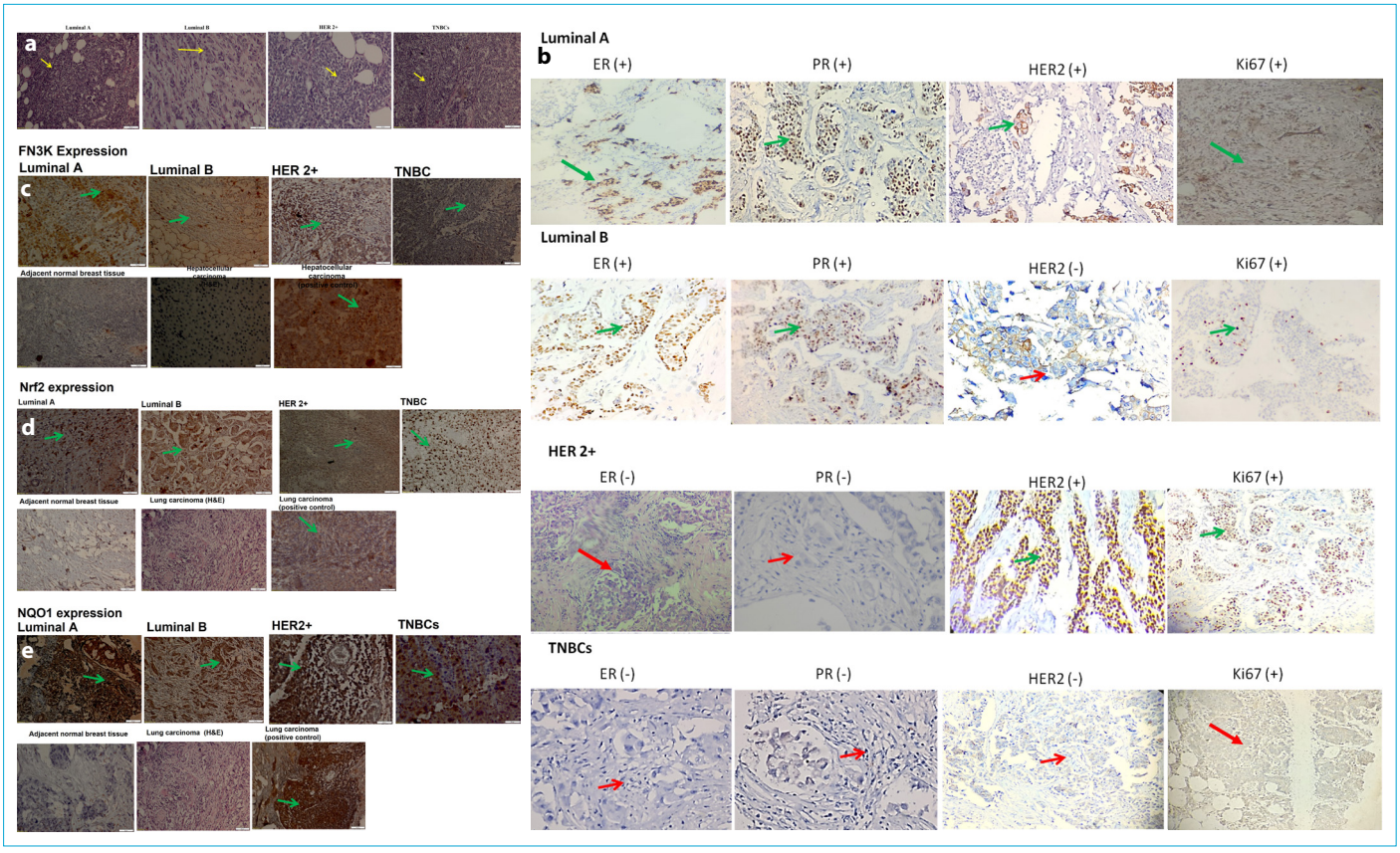

Figure 5. (a) H & E staining of tumor cells paraffin-embedded breast tumor blocks depicting luminal A, luminal B, HER2+, and TNBCs; **(b)** Receptor protein expression patterns of HR+/HER2–, ER+/PR+, ER+/PR–, and ER–/PR+ in luminal A tumor type, HR+/HER2+, ER+/PR+, ER+/PR–, and ER–/PR+ in luminal B tumor type, ER-, PR-, HER2+ in HER 2+ tumor type, and ER-, PR-, HER2 in TNBCs. Ki67 tumor proliferation was observed in these four kinds of tumor types. Green arrows: protein localization, yellow/red arrows: tumor cells, Magnification 40x. **(c)**. Expression patterns of FN3K in breast cancer tumors of luminal A, luminal B, HER 2+, TNBCs and hepatocellular carcinoma tumor sample was used as a positive control. Expression patterns of FN3K in luminal A, luminal B were higher compared to HER 2+ and TNBCs and adjacent normal breast tissue. Green arrows indicate the expression of Nrf2. **(d)** Expression patterns of Nrf2 in breast cancer tumors of luminal A, luminal B, HER 2+, TNBCs and lung carcinoma tumor sample was used as a positive control. Green arrows indicate the expression of Nrf2. **(e)** Expression patterns of NQO1 in breast cancer tumors of luminal A, luminal B, HER 2+, TNBCs and lung carcinoma tumor sample was used as a positive control. Green arrows indicate the expression of NQO1. Protein colocalization of Nrf2, and NQO1 across the luminal A, luminal B, HER 2+, and TNBCs were nonsignificant among them but higher than the expression patterns observed in adjacent normal breast tissue. The arrow points to the positive cells. Magnification: 10x, 20x.

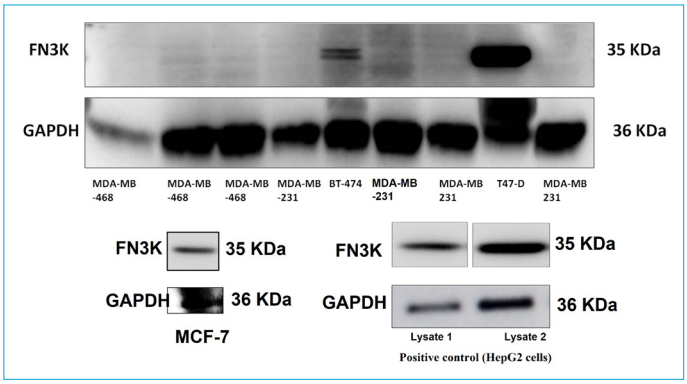

Figure 6. Western blot protein expression study delineated the expression of FN3K in ductal invasive carcinomas such as BT-474, T-47D (Luminal B subtype) compared to the other molecular subtypes as indicated the FN3K expression observed in TCGA samples analyzed through PAM50 algorithm. Expression of FN3K in duplicates of HepG2 samples was considered as a positive control.

enriched', 'Basal-like', and 'Normal-like'[32]. Every molecular subtype is variable according to its molecular properties and prognoses.[33] Disease-specific survival or prognosis with luminal A is significant but the HER2-enriched as well as basal-like exhibit poor disease-specific survival due to their aggressive pathology.[34]

Clinical subtypes of BC are predominantly exemplified by the altered gene expression and DNA copy number, and distinct mutational patterns.[35-37] The kinase expression patterns have been reported to be altered among different molecular subtypes of BC.[38] Furthermore, certain receptor tyrosine kinases (RTKs) reported to have prognostic relevance as they are actively involved in the cancer progression upon activation. For instance, the expression patterns of RTKs like HER2 and overall survival is reported in breast cancers.[39] A study by Finetti et al reported the involvement of 16 kinases pertinent to mitosis which can distinguishes luminal subtype from other molecular subtypes suggesting the prognostic significance of kinases within luminal A type cancers. Another study by Speers et al reported the novel kinase targets towards ER- molecular subtypes and concluded the kinases pertinent to the cancer cell proliferation could predict poor prognosis.[40, 41] We have chosen the receptor-based clinical categorization of the BC molecular subtypes to explore the patterns of FN3K expression.[10] For instance, the expression of ER is evident in two luminal subtypes; specifically luminal A molecular subtype is composed of 50 to 60% of breast cancers accompanied by the minimal levels of genes involved in cell proliferation[42, 43] whereas the luminal B is composed of 10–20% of tumors, accompanied by the substantial levels of proliferation-related genes and induce a poor prognosis.[44, 45] These two molecular subtypes can be differentiated according to the expression patterns of Ki67.[46] Total 15% to 25% of breast cancer tumors exhibit a higher HER2 expression and consequent HER2-related gene expression involved in proliferation process[47] suggesting a poor prognosis when compared to luminal molecular subtypes. Hence, the advent targeted therapy towards these molecular subtypes can enhance the patient's overall survival.[48-51] Basal-like BC tumors are featured by the substantial proliferation of myoepithelial cells and attain a poor prognosis.[52]

During metastatic breast cancer (ER/PR-positive, HER2-positive and TNBC) conditions, the clinical factors include long relapse-free intervals, and lack of brain/visceral metastases and the expression of estrogen receptors are the significant predictive factors.[53-58] De novo metastases breast cancers exhibits significant prognosis when compared to the recurrent breast cancer.[56, 59] MiRNAs, circulating tumor cells are other prognostic factors in the emerging metastatic breast cancers.[60-63] Expression of ER always varies in the metastatic breast cancers and the negative conversion of ER expression could be a better predictor of poor prognosis.[64, 65]

It has been found that the women with ER+ breast cancer continue to relapse 15 years after primary diagnosis.[66, 67] HER2+ breast cancer subgroups constitute <25% of breast cancers whereas the women with ER+/HER2+ breast tumors constitute significantly a higher proportion among all other breast tumor types.[68-72]. Hence, the ascertaining the long-term prognostic ability of PAM50 subtypes in the ER+/HER2- subgroup can clarify its clinical implications. Primarily, the implications of PAM50-prosigna signature was evaluated in postmenopausal breast cancer survivors but it may have significant prognostic implications in the premenopausal breast cancer.[73]

Normal-like BC tumors are composed of 5% to 10% categorized as fibroadenomas, and normal breast tissue cells.[42] Heba Alshaker et al 2020 described the higher expression of sphingosine kinase 1 in breast cancer cells could be considered as the negative prognostic marker in estrogen receptor BC molecular subtypes concluding as a target for chemosensitization therapy.[74] Another study by Zheng et al (2021) described the relation between the N6-methyladenosine (m6A) methylation and the breast cancer prognosis based on the clinicopathological features of several clinical BC molecular subtypes. m6A is significantly involved in the modulating the tumorigenesis and cancer progression in breast cancer.[75]

In addition to studies from our laboratory, several authors reported the role of Nrf2 in fostering breast cancer progression, chemoresistance, and metabolic reprogramming to attain tumor malignancy.[3, 13, 25] Nrf2 is actively involved in modulating the RhoA (Ras homolog family member A) ex-

pression subsequently promote Breast cancer progression. Dipeptidyl Peptidase 3 (DPP3) over-expression can induce modulatory role in the Nrf2/Keap1 pathway, and confer to the chemoresistance and breast cancer progression.[2] Oncoprotein Hepatitis B X-interacting protein (HBXIP) protein could modulate Nrf2/Keap1 signaling and foster cancer cell proliferation and metastasis in breast cancer subtypes.[76] Nrf2/keap1 signaling is involved in altering Notch1 (Neurogenic locus notch homolog protein 1) activity through G6PD (Glucose-6-phosphate dehydrogenase)/HIF-1α (Hypoxia-Inducible Factor) and EMT (Epithelial-Mesenchymal Transition) pathway consequently foster breast cancer progression.[77] It has recently reported that the Keap1 binding protein can promote Nrf2 accumulation through competitive binding and induce sequestration of Keap1. [78, 79] DPP3 expression is extensively higher in both ovarian and endometrial cancers.[79, 80] Kevin Lu et al described the overexpression of DPP3 in breast cancers and its enhanced expression resulted in the higher Nrf2 downstream gene expression and poor prognosis in the ER+ breast cancer subtype.[2] In our study, molecular subtyping can be executed with the aid of PAM50 gene signature obtained from microarrays, RNAseq, and qRT-PCR. The mRNA- RNA sequencing data obtained from TCGA database was executed to classify the molecular subtypes of breast tumors according to the expression levels of FN3K in the 5 subtypes. Four models of breast cancers with different immunohistochemical profiles were used to examine the expression patterns of FN3K, Nrf2, and NQO1. For instance, MCF-7 cell line indicates luminal A subtype and possess immunohistochemical profile of ER+, PR+, and HER2-[81] whereas BT-474 cell line is luminal B subtype and exhibits immunohistochemical profile of HR+/HER2+, ER+/PR+, ER+/PR−, and ER−/PR+. T47-D is a luminal A type and reported with HR+/HER2−, ER+/PR+, ER+/PR−, and ER−/PR+.[82] Luminal B sub type (BT-474) is associated with a substantial proliferation rate and poorer prognosis when compared to luminal A subtype.[28] MDA-MB-231, MDA-MB-468 cell lines indicates basal-like subtype and associated with triple-negative immunohistochemical profiles include ER-, PR-, HER2- respectively.[83, 84] FN3K has significant role in deglycating the Nrf2 in HCCs.[3, 85] Studies from our laboratory have demonstrated upregulation of Nrf2 in breast cancers.[3, 25] Our Western blot protein expression study delineated the vivid expression of FN3K in ductal invasive carcinomas such as BT-474, T-47D (Luminal B subtype) as indicated the FN3K expression observed in TCGA samples analyzed through PAM50 algorithm. In our study, the vivid TCGA analysis of breast cancer patients have resulted in the expression FN3K in Tumor size-wise (T1 to T4), stage-wise (stage I to stage IV). Comparatively the expression of FN3K is higher in patients with T4 and stage IV although there was no statistical significance in its expression in node-wise. Expression of FN3K was substantially higher in the breast tumors than the expression in adjacent normal tissues. Expression of FN3K in luminal A, luminal B, when compared to HER 2+, and TNBCs along differential protein expression patterns of Nrf2, and NQO1 (markers of oxidative stress). However, there are no substantial expression differences in Nrf2 or NQO1 among these 4 subtypes. But, the expression of FN3K, Nrf2, and NQO1 are higher than adjacent normal tissues.

Constitutive Nrf2 overexpression confers to the cancer progression and chemoresistance due to the enhanced expression of drug resistant proteins, and antioxidant proteins or detoxification genes.[15, 86-89] DPP3 overexpression promotes cancer progression through metastasis, and chemoresistance in breast cancer conditions by titrating Keap1, and by DPP3-mediated protection of Nrf2. The similar patterns of positive correlation between DPP3 mRNA level and Nrf2 target gene expression has been observed in squamous lung carcinoma,[78] ovarian and endometrial carcinomas.[2, 79, 80] A higher expression of DPPS is correlated to the poor prognosis mainly in the BC patients with ER (+) subtype.[2] Sanghvi et al (2019) reported that the knockdown of FN3K could invoke mitigation in Nrf2 target protein expressions in murine MYC/sgKeap1 HCC liver tumor isografts and similarly in three pairs of FN3K-proficient and FN3K-deficient human xenografts (Huh1, H460, and H3255) models. [3] Hence, the expression of FN3K gene may have significant modulatory role in the expression of Keap1, and Nrf2 target gene proteins (15 gene signature as mentioned in the results). The overexpression of DPP3 could promote the higher Keap1 levels subsequently makes keap1 levels stable.[2] In this study, authors concluded the DPP3 role as a prognostic gene as its expression is correlated with disease-specific survival of the breast cancer patients. Overexpression of DPP3 is positively correlated to the Nrf2-target gene expression and negatively correlated to the Nrf2 mRNA expression.[2] On the contrary, our study reported the positive correlation between the FN3K expression with the expression of 15 gene-gene Nrf2 target signatures indicating its role in breast cancers. Furthermore, FN3K expression is correlated to the disease-specific survival. Overall survival was comparatively higher among the patients with breast tumors with low FN3K and Nrf2 expression.

Conclusion

In breast cancer patients, FN3K expression is widely expressed in luminal molecular subtype compared to the other molecular subtypes as indicated by the in vitro studies, where BT-474 and T47D ductal invasive carcinoma cells expressed at higher level of FN3K compared to adenocar-

cinoma cells (Triple-negative breast cancer cells and MCF-7). Expression of FN3K is higher in luminal A and B tumor subtypes compared to HER2+, and TNBCs. However, the expression patterns of Nrf2, and NQO1 were not statistically significant among these subtypes. Furthermore, the FN3K gene expression positively invoked the expression of 'Nrf2 target gene expressions', and 'Nrf2 mRNA expression'. It has been explored previously that different molecular subtypes of breast cancer exhibit different kinase expression patterns due to their significant implications in the cell cycle, immune functions, and cancer progression. For example, the protein-kinase coding genes are reported to be differentially expressed in ER+ breast tumors. FN3K expression is reported in the hepatocellular carcinoma and its efficiency as a prognostic marker yet require extensive studies. Hence, the expression patterns of FN3K may be a possible prognostic marker which require future studies in elucidating the cancer progression.

Note: Patient characteristics pertinent to the selected breast cancer paraffin blocks of four categories of tumors were given in the attached as a supplementary Tables S1 to S9 and Master-chart (Supplementary Table S10).

Disclosures

Ethics Committee Approval: A complete Institutional Ethics Committee approval (JSSMC/IEC/240921/09NCT/2021-22, dated 4 October 2021) was obtained from the JSS medical college and Hospitals to use 20 paraffin-embedded patient's breast tumor blocks. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Conflict of Interest: None declared.

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Data Availability Statement: All data generated or analysed during this study are included in this published article [and its supplementary information files]. Furthermore, RNA sequencing (RNAseq) expression data describing a total of 1,069 breast tumor samples and 96 matched adjacent normal and tumor samples were acquired from the UCSC xena (http://xena.ucsc.edu/). The RNA-seq data (FPKM) was acquired from The Cancer Genome Atlas (TCGA)-BRCA database cohort which is a Universal database with complete permission of First affiliated Hospital of Zhengzhou University.

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Table S1. The clinical features of the beast tumors pertaining to Luminal A

Tumor type	Tumor tissue microarrays Serial number	Age (years)	Menarche	Menopause	Age at 1st childbirth	Side of the tumor	Location of the tumor	Desmoplasia	Necrosis
Patient tumor samples IV(600-17E)	4	64	12	1	1	1	5	1	1

Table S2. The clinical features of the beast tumors pertaining to Luminal A

Tumor type	Lymphovascular invasion	Perineuronal invasion	Histopathological grading(G)	Largest size of tumor (cm)	Pathological tumor size (pT)	Lymph node status (N)	Nottingham Prognostic Groups	Lymphocytic infiltration
Patient tumor samples IV (600-17E)	1	1	3	3	3	N3	5	1

Table S3. The clinical features of the beast tumors pertaining to Luminal B.

Tumor type	Tumor tissue microarrays Serial number	Age (years)	Menarche	Menopause	Age at 1st childbirth	Side of the tumor	Location of the tumor	Desmoplasia	Necrosis
Patient tumor samples III (1315-19)	6	38	11	0	2	1	4	1	1

Table S4. The clinical features of the beast tumors pertaining to Luminal B.

Tumor type	Lymphovascular invasion	Perineuronal invasion	Histopathological grading (G)	Largest size of tumor (cm)	Pathological tumor size (pT)	Lymph node status (N)	Lymphocytic infiltration	Nottingham Prognostic Groups
Patient tum1or samples III (1315-19)	0	2	2	2.8	1	1	0	1

Table S5. The clinical features of the beast tumors pertaining to HER2+.

Tumor type	Tumor tissue microarrays Serial number	Age (years)	Menarche	Menopause	Age at 1st childbirth	Side of the tumor	Location of the tumor	Desmoplasia	Necrosis
Patient tumor samples III (3553-20C)	11	53	13	1	1	2	4	1	1

Table S6. The clinical features of the beast tumors pertaining to HER2+

Tumor type	Lymphovascular invasion	Perineuronal invasion	Histopathological grading (G)	Largest size of tumor in centimeter	Pathological tumor size (pT)	Lymph node status (N)	Lymphocytic infiltration	Nottingham Prognostic Groups
Patient tumor samples III (3553-20C)	1	1	2	1.5	2	2	1	2

Table S7. The clinical features of the beast tumors pertaining to TNBCs

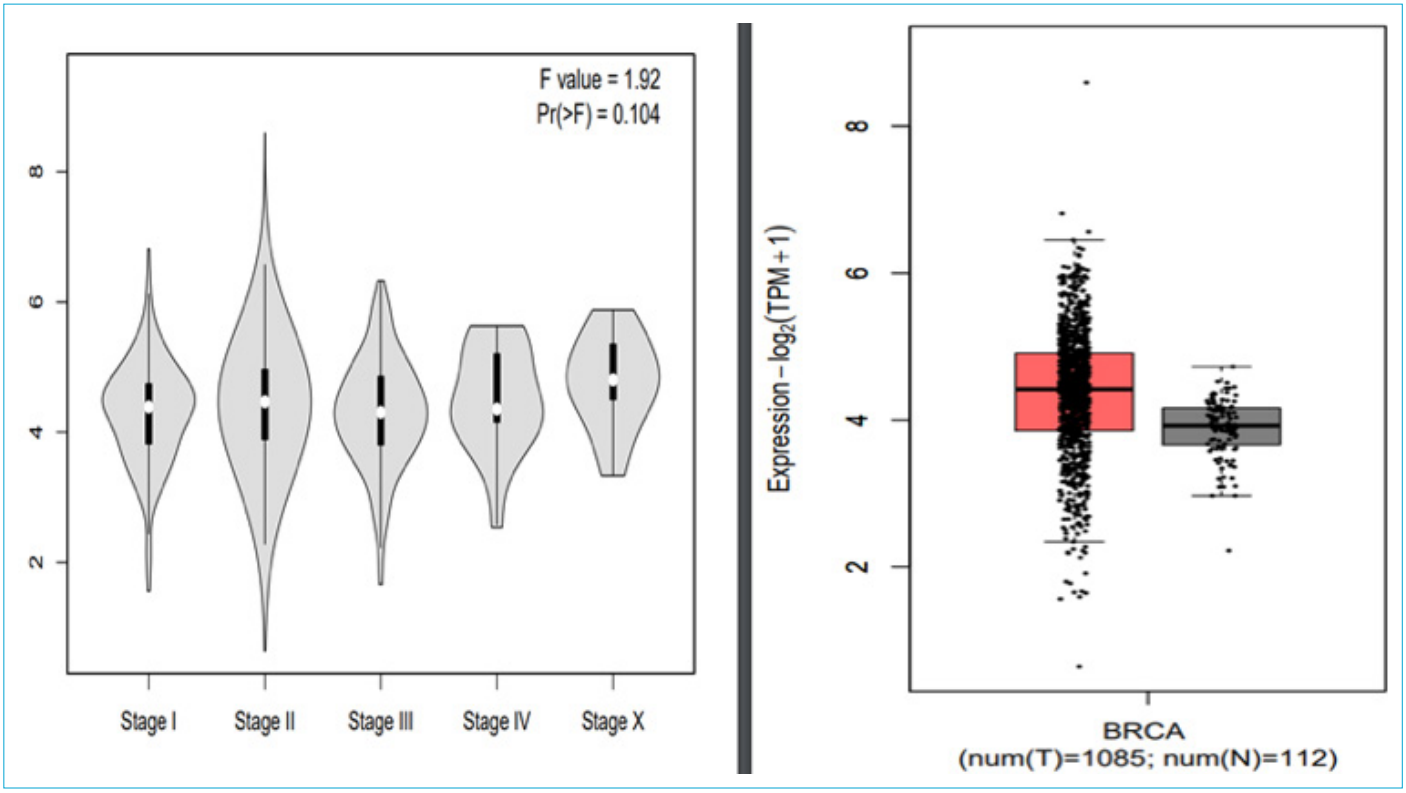
Tumor type	Tumor tissue microarrays Serial number	Age (years)	Menarche	Menopause	Age at 1st childbirth	Side of the tumor	Location of the tumor	Desmoplasia	Necrosis
Patient tumor samples III (2814-20C)	13	71	12	1	1	2	5	1	1

Table S8. The clinical features of the beast tumors pertaining to TNBCs

Tumor type:	Lymphovascular invasion	Perineuronal invasion	Histopathological grading (G)	Largest size of tumor (cm)	Pathological tumor size (pT)	Lymph node status (N)	Lymphocytic infiltration	Nottingham Prognostic Groups
Patient tumor sample I (2814-20C)	1	1	3	4.5	3	3	1	5

Table S9. The clinical features of the beast tumors pertaining to lung cancer & hepatocellular carcinoma

Tumor type	Age (years)	Gender	Description	Histopathological grading (G)	Lymph node status (N)	Pathological tumor size (pT)
Hepatocellular carcinoma	55	Male	Small size biopsy (less than 2 cm)	3	2	2
Lung carcinoma	64	Male	Small size biopsy (less than 2 cm)	2	3	2



Supplementary 2. Our dataset comes from the UCSC xena (http://xena.ucsc.edu/), and we downloaded 1,045 breast tumor samples and 96 matched adjacent normal to get our results. Based on your comments, we verified our results on the website. The following figures are the results from the website. The first figure is the difference in FN3K expression between stages, which corresponds to Figure 1B in the manuscript. The second figure is the difference between normal and tumor samples, which corresponds to Figure 2 in the manuscript. The above two figures are different from those in the manuscript. We think that this is because the sample size (Tumor = 1085, normal sample = 112 from the websites) was inconsistent with the data we downloaded from the UCSC xena. Even though they have inconsistent results, the trends of the results are very consistent, and the shape of the figures is very similar, which partly validates our results.

Table S10. The clinical features of the grade-II and grade-III tumors and keys to master chart

Keys to Master-chart	
	Serial number
	Menarche
	Menopause
0	Pre-menopause
1	Post-menopause
	Age at 1st childbirth
0	Nulliparous
1	15-19years
2	20-24years
3	25-29years
	Side of the tumor
1	Right
2	Left
	Location of the tumor
1	Upper outer quadrant
2	Upper inner quadrant
3	Lower outer quadrant
4	Lower inner quadrant
5	Nipple areola complex
	Lymphocytic infiltration
0	No
1	Yes
	Desmoplasia
0	No
1	Yes
	Necrosis
0	No
1	Yes
	Lymphovascular invasion
0	No
1	Yes
	Perineuronal invasion
0	No
1	Yes
	Histopathological grading (G)
1	Grade 1
2	Grade 2
3	Grade 3
	Pathologic tumor size (pT)
1	T1
2	T2
3	T3
4	T4
3	**Lymphnode status (N)**
1	N0
2	N1
3	N2
4	N3
	Nottingham Prognostic Groups
1	Good Prognostic Group
2	Moderate Prognostic Group I
3	Moderate Prognostic Group II
4	Poor Prognostic Group
5	Very poor Prognostic Group

Supplementary 2. RNA sequencing (RNAseq) expression data describing breast tumor samples and matched adjacent normal and tumor samples were acquired from the UCSC xena (http://xena.ucsc.edu/).

SamID	Tissue	SamID	Tissue
TCGA-BH-A0DG-01A	paired.tumor	TCGA-BH-A18Q-01A	paired.tumor
TCGA-BH-A18L-01A	paired.tumor	TCGA-E2-A153-01A	paired.tumor
TCGA-E2-A1IG-01A	paired.tumor	TCGA-BH-A0BT-01A	paired.tumor
TCGA-E9-A1N6-01A	paired.tumor	TCGA-A7-A0DB-01A	paired.tumor
TCGA-BH-A0DP-01A	paired.tumor	TCGA-BH-A18J-01A	paired.tumor
TCGA-BH-A18P-01A	paired.tumor	TCGA-A7-A13E-01A	paired.tumor
TCGA-A7-A0DC-01A	paired.tumor	TCGA-E9-A1RB-01A	paired.tumor
TCGA-BH-A0DZ-01A	paired.tumor	TCGA-E9-A1RC-01A	paired.tumor
TCGA-E9-A1ND-01A	paired.tumor	TCGA-BH-A1EO-01A	paired.tumor
TCGA-E2-A1LB-01A	paired.tumor	TCGA-BH-A0H5-01A	paired.tumor
TCGA-E9-A1RD-01A	paired.tumor	TCGA-BH-A0E1-01A	paired.tumor
TCGA-BH-A0DV-01A	paired.tumor	TCGA-E9-A1RF-01A	paired.tumor
TCGA-BH-A1FN-01A	paired.tumor	TCGA-E2-A1LS-01A	paired.tumor
TCGA-BH-A18V-01A	paired.tumor	TCGA-BH-A0B7-01A	paired.tumor
TCGA-BH-A0HA-01A	paired.tumor	TCGA-AC-A2FF-01A	paired.tumor
TCGA-BH-A0BV-01A	paired.tumor	TCGA-BH-A0BA-01A	paired.tumor
TCGA-E9-A1RH-01A	paired.tumor	TCGA-BH-A0B5-01A	paired.tumor
TCGA-BH-A208-01A	paired.tumor	TCGA-BH-A0AZ-01A	paired.tumor
TCGA-BH-A0C0-01A	paired.tumor	TCGA-E2-A158-01A	paired.tumor
TCGA-AC-A2FB-01A	paired.tumor	TCGA-BH-A204-01A	paired.tumor
TCGA-BH-A0DD-01A	paired.tumor	TCGA-BH-A0B8-01A	paired.tumor
TCGA-BH-A0DK-01A	paired.tumor	TCGA-E2-A15M-01A	paired.tumor
TCGA-E2-A1BC-01A	paired.tumor	TCGA-A7-A0CH-01A	paired.tumor
TCGA-A7-A0CE-01A	paired.tumor	TCGA-E2-A15I-01A	paired.tumor
TCGA-BH-A0HK-01A	paired.tumor	TCGA-BH-A0H7-01A	paired.tumor
TCGA-BH-A1EU-01A	paired.tumor	TCGA-BH-A0BM-01A	paired.tumor
TCGA-AC-A23H-01A	paired.tumor	TCGA-BH-A0BZ-01A	paired.tumor
TCGA-BH-A0BW-01A	paired.tumor	TCGA-GI-A2C8-01A	paired.tumor
TCGA-BH-A0E0-01A	paired.tumor	TCGA-E2-A1LH-01A	paired.tumor
TCGA-BH-A1FU-01A	paired.tumor	TCGA-BH-A18R-01A	paired.tumor
TCGA-BH-A0AY-01A	paired.tumor	TCGA-BH-A18U-01A	paired.tumor
TCGA-BH-A0H9-01A	paired.tumor	TCGA-BH-A1F2-01A	paired.tumor
TCGA-BH-A18N-01A	paired.tumor	TCGA-BH-A18M-01A	paired.tumor
TCGA-GI-A2C9-01A	paired.tumor	TCGA-E9-A1R7-01A	paired.tumor
TCGA-E9-A1NG-01A	paired.tumor	TCGA-E2-A1L7-01A	paired.tumor
TCGA-BH-A0DQ-01A	paired.tumor	TCGA-BH-A18S-01A	paired.tumor
TCGA-E9-A1N9-01A	paired.tumor	TCGA-E9-A1NF-01A	paired.tumor
TCGA-BH-A1EN-01A	paired.tumor	TCGA-BH-A209-01A	paired.tumor
TCGA-E9-A1RI-01A	paired.tumor	TCGA-BH-A0BQ-01A	paired.tumor
TCGA-BH-A0BJ-01A	paired.tumor	TCGA-E9-A1N4-01A	paired.tumor
TCGA-BH-A1FB-01A	paired.tumor	TCGA-A7-A13F-01A	paired.tumor
TCGA-E9-A1N5-01A	paired.tumor	TCGA-A7-A0D9-01A	paired.tumor
TCGA-BH-A18K-01A	paired.tumor	TCGA-BH-A0C3-01A	paired.tumor
TCGA-BH-A0DT-01A	paired.tumor	TCGA-BH-A1EV-01A	paired.tumor
TCGA-E2-A15K-01A	paired.tumor	TCGA-E9-A1NA-01A	paired.tumor
TCGA-BH-A0DL-01A	paired.tumor	TCGA-BH-A0AU-01A	paired.tumor
TCGA-BH-A1FC-01A	paired.tumor	TCGA-BH-A0DH-01A	paired.tumor
TCGA-BH-A0BC-01A	paired.tumor	TCGA-BH-A203-01A	paired.tumor

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TCGA-BH-A18L-11A	paired.normal	TCGA-E2-A153-11A	paired.normal
TCGA-E2-A1IG-11A	paired.normal	TCGA-BH-A0BT-11A	paired.normal
TCGA-E9-A1N6-11A	paired.normal	TCGA-A7-A0DB-11A	paired.normal
TCGA-BH-A0DP-11A	paired.normal	TCGA-BH-A18J-11A	paired.normal
TCGA-BH-A18P-11A	paired.normal	TCGA-A7-A13E-11A	paired.normal
TCGA-A7-A0DC-11A	paired.normal	TCGA-E9-A1RB-11A	paired.normal
TCGA-BH-A0DZ-11A	paired.normal	TCGA-E9-A1RC-11A	paired.normal
TCGA-E9-A1ND-11A	paired.normal	TCGA-BH-A1EO-11A	paired.normal
TCGA-E2-A1LB-11A	paired.normal	TCGA-BH-A0H5-11A	paired.normal
TCGA-E9-A1RD-11A	paired.normal	TCGA-BH-A0E1-11A	paired.normal
TCGA-BH-A0DV-11A	paired.normal	TCGA-E9-A1RF-11A	paired.normal
TCGA-BH-A1FN-11A	paired.normal	TCGA-E2-A1LS-11A	paired.normal
TCGA-BH-A18V-11A	paired.normal	TCGA-BH-A0B7-11A	paired.normal
TCGA-BH-A0HA-11A	paired.normal	TCGA-AC-A2FF-11A	paired.normal
TCGA-BH-A0BV-11A	paired.normal	TCGA-BH-A0BA-11A	paired.normal
TCGA-E9-A1RH-11A	paired.normal	TCGA-BH-A0B5-11A	paired.normal
TCGA-BH-A208-11A	paired.normal	TCGA-BH-A0AZ-11A	paired.normal
TCGA-BH-A0C0-11A	paired.normal	TCGA-E2-A158-11A	paired.normal
TCGA-AC-A2FB-11A	paired.normal	TCGA-BH-A204-11A	paired.normal
TCGA-BH-A0DD-11A	paired.normal	TCGA-BH-A0B8-11A	paired.normal
TCGA-BH-A0DK-11A	paired.normal	TCGA-E2-A15M-11A	paired.normal
TCGA-E2-A1BC-11A	paired.normal	TCGA-A7-A0CH-11A	paired.normal
TCGA-A7-A0CE-11A	paired.normal	TCGA-E2-A15I-11A	paired.normal
TCGA-BH-A0HK-11A	paired.normal	TCGA-BH-A0H7-11A	paired.normal
TCGA-BH-A1EU-11A	paired.normal	TCGA-BH-A0BM-11A	paired.normal
TCGA-AC-A23H-11A	paired.normal	TCGA-BH-A0BZ-11A	paired.normal
TCGA-BH-A0BW-11A	paired.normal	TCGA-GI-A2C8-11A	paired.normal
TCGA-BH-A0E0-11A	paired.normal	TCGA-E2-A1LH-11A	paired.normal
TCGA-BH-A1FU-11A	paired.normal	TCGA-BH-A18R-11A	paired.normal
TCGA-BH-A0AY-11A	paired.normal	TCGA-BH-A18U-11A	paired.normal
TCGA-BH-A0H9-11A	paired.normal	TCGA-BH-A1F2-11A	paired.normal
TCGA-BH-A18N-11A	paired.normal	TCGA-BH-A18M-11A	paired.normal
TCGA-GI-A2C9-11A	paired.normal	TCGA-E9-A1R7-11A	paired.normal
TCGA-E9-A1NG-11A	paired.normal	TCGA-E2-A1L7-11A	paired.normal
TCGA-BH-A0DQ-11A	paired.normal	TCGA-BH-A18S-11A	paired.normal
TCGA-E9-A1N9-11A	paired.normal	TCGA-E9-A1NF-11A	paired.normal
TCGA-BH-A1EN-11A	paired.normal	TCGA-BH-A209-11A	paired.normal
TCGA-E9-A1RI-11A	paired.normal	TCGA-BH-A0BQ-11A	paired.normal
TCGA-BH-A0BJ-11A	paired.normal	TCGA-E9-A1N4-11A	paired.normal
TCGA-BH-A1FB-11A	paired.normal	TCGA-A7-A13F-11A	paired.normal
TCGA-E9-A1N5-11A	paired.normal	TCGA-A7-A0D9-11A	paired.normal
TCGA-BH-A18K-11A	paired.normal	TCGA-BH-A0C3-11A	paired.normal
TCGA-BH-A0DT-11A	paired.normal	TCGA-BH-A1EV-11A	paired.normal
TCGA-E2-A15K-11A	paired.normal	TCGA-E9-A1NA-11A	paired.normal
TCGA-BH-A0DL-11A	paired.normal	TCGA-BH-A0AU-11A	paired.normal
TCGA-BH-A1FC-11A	paired.normal	TCGA-BH-A0DH-11A	paired.normal
TCGA-BH-A0BC-11A	paired.normal	TCGA-BH-A203-11A	paired.normal