



Investigating the Prognosis Gene Profile of Triple-Negative Breast Cancer

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Background: Current therapeutic strategies have poor effects in triple-negative breast cancer (TNBC) patients due to lack of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor-2 expression. Identification of novel genes of TNBC prognosis aids in the development of effective treatment strategies. **Aim:** We aim to explore key genes related to TNBC recurrence. **Methods:** RNAseq and clinical characteristics data were derived from The Cancer Genome Atlas Breast Invasive Carcinoma project. Ninety-seven TNBC patients were included. We used DESeq2 and Cox regression to identify significant genes to TNBC recurrence. Pathway enrichment analysis and protein–protein interaction plot were conducted to understand the functions of target genes. **Results:** We discovered top nine important genes for TNBC recurrence. Lower mRNA expression of potassium voltage-gated channel subfamily Q member 5, H3 clustered histone 10, and ADP-ribosylation factor-like protein 17 and higher mRNA expression of synuclein beta, interleukin 6 (IL-6), casein kappa, RHOC, phosphodiesterase 8B, and laminin subunit alpha 3 (LAMA3) were associated with higher risk of recurrence. IL-6, LAMA3, and Ras homolog family member V (RHOV) genes out of nine candidate genes can make the best prediction of TNBC recurrence (area under receiver operating characteristic curve: 0.95, sensitivity: 0.89 and specificity: 0.97). The top three significant Gene Ontology (GO) pathways are nucleosome, ion gated channel activity, and epidermis development. Significant GO pathways can be categorized into four functions: cell–cell adhesion, cell transportation, cell proliferation, ion channel and transporter, and immune. **Conclusion:** We discovered that the gene set of IL6, LAMA3, and RHOV can accurately predict TNBC recurrence. These genes warrant further study to confirm their causal association with TNBC prognosis and possible treatment targets.

Key words: Triple-negative breast cancer, relapse-free survival, genetic biomarkers

INTRODUCTION

Breast cancer (BC) is the most common cancer among women worldwide, with over 2 million new cases expected in 2020. Its incidence and mortality rates have risen in the previous three decades as a result of changes in risk factor profiles, improved cancer registry, and cancer detection. The number of risk factors for BC is substantial, and it includes both

modifiable and nonmodifiable factors.¹ TNBC is responsible for more than 15%–20% of all BCs and is of particular research interest as it is therapeutically challenging mainly because of its low response to therapeutics and highly invasive nature. The TNBC proportion in all age groups followed a similar

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trend.² TNBC is a subtype of BC that lacks the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2). Due to the lack of definite targets, novel therapeutic strategies have been restricted, and chemotherapy has remained the major treatment for decades with poor treatment effects.³ TNBC is still the most intractable subtype of BC to treat; however, immunotherapy can improve the prognosis of TNBC patients by modifying the tumor microenvironment and boosting antitumor immune response.³ Effective treatments for TNBC patients are still unmet medical needs.

A variety of new approaches, including gene profiling and sequencing, proteomics, and microRNA analysis, have been utilized to investigate human breast carcinogenesis and metastasis, including TNBC, potentially leading to new therapeutics. Chen *et al.* found novel genes *NCAPG* and *ABCA9* may be the key genes of TNBC using GSE76250, GSE31448, GSE43358, and METABRIC datasets.⁴ Liu *et al.* used the TCGA database to establish a ceRNA network and found that ERVMER61-1 in lncRNA was associated with poor prognosis in TNBC.⁵ Hsu *et al.* found the immunoglobulin genes (*IGHA1*, *IGHD*, *IGHG1*, *IGHG3*, *IGLC2*, and *IGLJ3*) associated with the prognosis of TNBC.⁶ Improved understanding of tumor-immune system co-evolution supports the need to adopt a more comprehensive view of TNBC as an ecosystem that encompasses the intrinsic and extrinsic features of cancer cells.⁷ The better we understand TNBC genetic features, the faster we can develop new therapeutic regimens. Therefore, we aim at exploring key genes related to TNBC prognosis using The Cancer Genome Atlas Program (TCGA) data set.

MATERIALS AND METHODS

RNAseq analysis

RNAseq and clinical characteristics data were collected from the TCGA BRCA data set ($n = 1097$). We filtered out TNBC cases with the criteria of ER(-), PR(-), and HER2(-). We excluded metastatic cases ($n = 1$). There are 97 patients and 19,597 genes in total. First, we used DESeq2⁸ to filter out 284 significant genes with the criteria of $|\log_2 \text{fold change}| > 1$ and adjusted $P < 0.05$. Seventy-seven genes were upregulated and 207 genes were downregulated in TNBC recurrence. Second, we used Cox regression to filter out the significant genes ($P < 0.05$) for TNBC recurrence and 53 genes were left. Third, enter and stepwise multivariable Cox regression models were conducted to select the most significant genes to TNBC recurrence. There are nine candidate genes *casein kappa* (*CSN3*), *phosphodiesterase 8B* (*PDE8B*), *synuclein Beta* (*SNCB*), *Ras homolog family member V* (*RHOV*), *potassium voltage-gated channel subfamily Q member 5* (*KCNQ5*), *H3 clustered histone*

Table 1: Descriptive statistics of triple-negative breast cancer clinical characteristics

Item	Nonrecurrence (<i>n</i> =60), <i>n</i> (%)	Recurrence (<i>n</i> =37), <i>n</i> (%)	<i>P</i>
Ethnicity			
Asians	2 (3)	2 (5)	0.86
African-Americans	29 (50)	17 (46)	
White Americans	27 (47)	18 (49)	
Age (mean±SD)	55.9±9.9	53.7±11.9	0.33
Age groups (years)			
25-44	9 (15)	7 (19)	0.81
45-64	37 (62)	23 (62)	
65~	14 (23)	7 (19)	
Menopause (months)			
<6	11 (21)	8 (25)	0.72
6-12	3 (6)	3 (9)	
>12	38 (73)	21 (66)	
Primary site			
Left	32 (53)	22 (59)	0.55
Right	28 (47)	15 (41)	
Pathological stage			
I	18 (31)	4 (11)	2.7E-06*
II	38 (66)	15 (43)	
III	2 (3)	16 (46)	
Pathological T			
T1	22 (37)	8 (22)	0.36
T2	29 (48)	20 (54)	
T3	7 (12)	6 (16)	
T4	2 (3)	3 (8)	
Radiotherapy			
No	23 (44)	12 (34)	0.35
Yes	29 (56)	23 (66)	
Hormone therapy			
No	47 (98)	25 (83)	0.02*
Yes	1 (2)	5 (17)	

* $P < 0.05$. Statistical methods were Chi-square test and Fisher's exact test for two categorical features; Independent Student's t -test for the continuous feature by two groups. SD=Standard deviation

10 (H3C10), *ADP-ribosylation factor-like protein 17 (ARL17A)*, *interleukin-6 (IL-6)*, and *laminin subunit alpha 3 (LAMA3)* were selected for further analysis. This study used Rstudio software version 2022.2.3.492 (<http://www.r-project.org>).

Pathway enrichment analysis

Metascape⁹ is a web-based portal designed to provide a comprehensive gene list annotation and analysis resource for

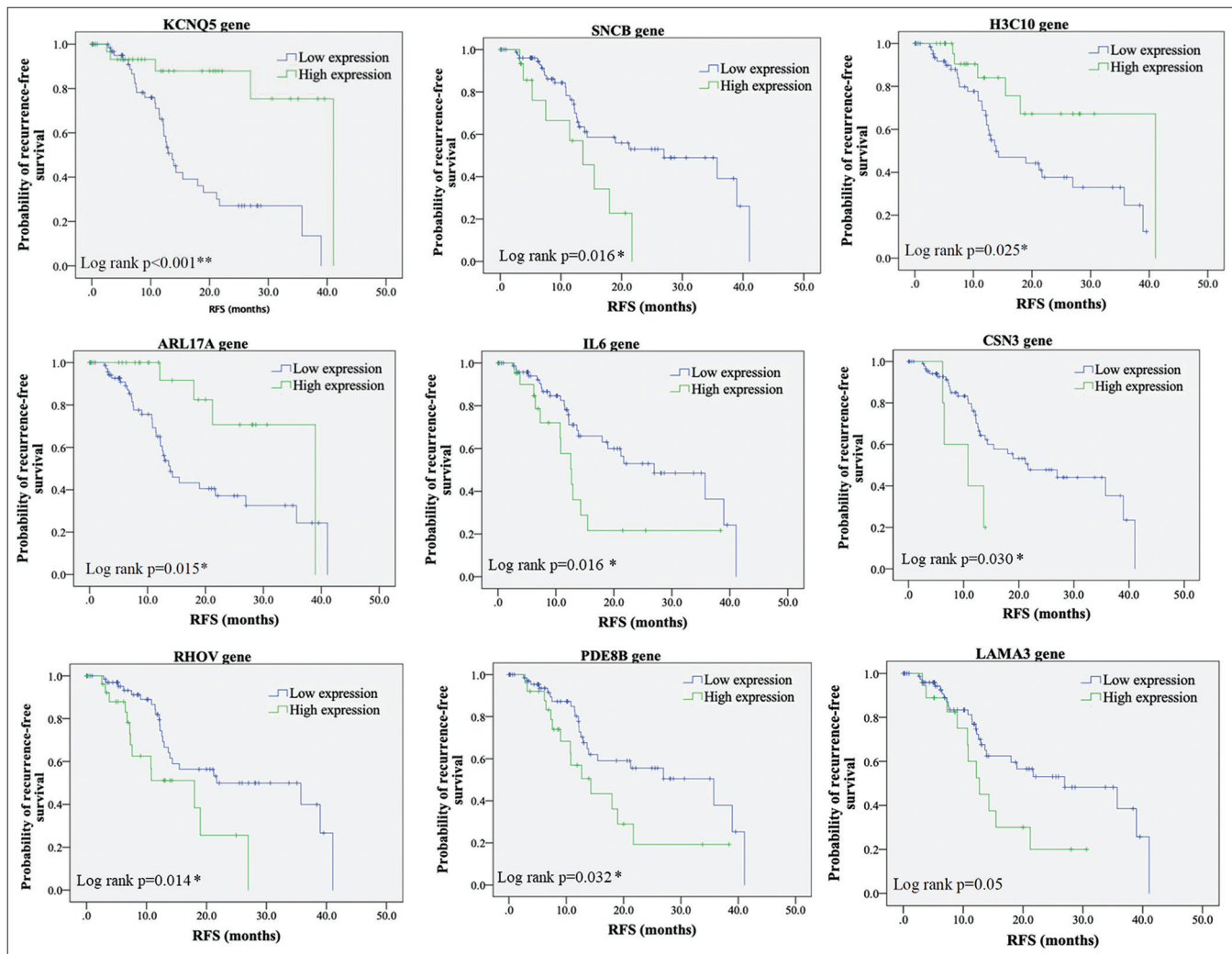


Figure 1: Kaplan–Meier plot of TNBC recurrence. Gene expression was categorized into high and low expression by the median value. P value was calculated using log rank and marked with an asterisk when $P < 0.05$. RFS = Relapse-free survival, *KCNQ5* = *Potassium voltage-gated channel subfamily Q member 5*; *SNCB* = *synuclein beta*; *H3C10* = *H3 clustered histone 10*; *ARL17A* = *ADP-ribosylation factor-like protein 17*; *IL-6* = *Interleukin-6*; *CSN3* = *Casein kappa*; *PDE8B* = *Phosphodiesterase 8B*; *RHOV* = *Ras homolog family member V*; *LAMA3* = *Laminin subunit alpha 3*; TNBC = Triple-negative breast cancer

experimental biologists. In terms of design features, Metascape combines functional enrichment, interactome analysis, gene annotation, and membership search to leverage over 40 independent knowledge bases within one integrated portal. Metascape was used to explore the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enriched pathways in TNBC recurrence.

Protein–protein interaction network

All the significant genes from DEeq2 were used to analyze the protein–protein interaction networks via the web-based tool STRING.¹⁰ STRING is a database of known and predicted protein–protein interactions. The interactions include both physical and functional correlations; they result from

computer prediction, information transmission across species, and interactions gathered from other database.

RESULTS

The study included 97 TNBC patients, with 60 developing recurrence. African-Americans and White Americans are the major population accounting for 96%. There was no statistical difference of age, menopause, primary site, pathological T, and radiotherapy between nonrecurrence and recurrence groups. All the patients had received chemotherapy. Pathological stage and hormone therapy were statistically associated with recurrence. However, only six patients had received hormone therapy [Table 1].

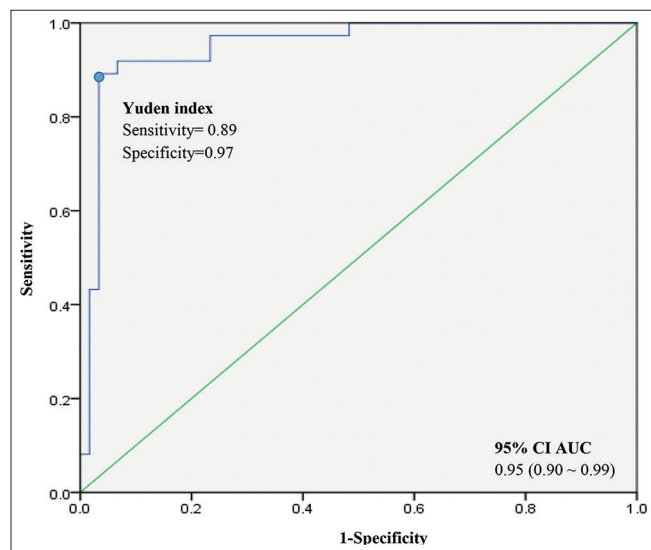


Figure 2: Receiver operating characteristic curve of Cox regression prediction models of TNBC recurrence using three candidate genes. TNBC = Triple-negative breast cancer; AUC = Area under the curve

Gene symbol	HR	HR 95% CI	P
<i>IL-6</i>	0.986	0.973-1.000	0.048*
<i>LAMA3</i>	1.001	1.000-1.003	0.016*
<i>RHOV</i>	1.001	1.000-1.002	0.003**

IL-6=Interleukin-6; *LAMA3*=Laminin subunit alpha 3; *RHOV*=Ras homolog family member V; HR=Hazard ratio; CI=Confidence interval; *P value < 0.05; **P value<0.01

We used DESeq2 and Cox regression to filter out final top nine important genes for TNBC recurrence. The survival plots of TNBC recurrence for low/high gene expression are shown in Figure 1. Lower mRNA expression of *KCNQ5*, *H3C10*, and *ARL17A* and higher mRNA expression of *SNCB*, *IL-6*, *CSN3*, *RHOV*, *PDE8B*, and *LAMA3* were associated with higher risk of recurrence. *IL-6*, *LAMA3*, and *RHOV* genes out of nine candidate genes can make the best prediction of TNBC recurrence. The area under of receiver operating characteristic (ROC) curve is 0.95 (95% confidence interval [CI]: 0.90–0.99) close to perfect prediction value 1 [Figure 2]. The sensitivity is 0.89 and specificity is 0.97 at the best cut point using Youden's index.

A total of 284 significant genes of TNBC recurrence using DESeq2 analysis were included to conduct pathway enrichment analysis using GO and KEGG pathway database [Figure 3]. The most significant pathways that may be involved in TNBC recurrence were systemic lupus erythematosus (SLE) and nicotine addiction from KEGG. The top three significant GO pathways are nucleosome, ion gated channel activity, and epidermis development. Significant GO pathways can be categorized into four functions: cell–cell adhesion, cell transportation, cell proliferation, ion channel and transporter, and immune [Figure 3].

A total of 284 significant genes from DESeq2 were used to plot protein–protein interaction networks via STRING. No matched protein gene was displayed in the plot. There are five genes out of nine candidate genes shown in the plot. All the protein–protein interaction can be generally categorized into three clusters. *LAMA3* belonged to the blue cluster; *IL-6* and *CSN3* belonged to the green cluster, and *KCNQ5* and *SNCB* belonged to the red clusters [Figure 4].

DISCUSSION

We found nine important genes *CSN3*, *PDE8B*, *SNCB*, *RHOV*, *KCNQ5*, *H3C10*, *ARL17A*, *IL-6*, and *LAMA3* are related to TNBC recurrence. Among these genes, *IL-6*, *LAMA3*, and *RHOV* are great gene set of predicting TNBC recurrence, with perfect prediction performance with AUC 0.95. The major pathways that get involved in TNBC prognosis were nucleosome, ion gated channel activity, detection of chemical stimulus, and extracellular matrix organization.

IL-6 plays an important role in the immune response and participates in the host immune defense mechanism and the regulation of the growth and differentiation of various malignant tumors. After stimulating the combination of *IL-6* and *IL-6R*, it will trigger an inflammatory response and cause cancer cell proliferation. Blocking the combination of *IL-6* and *IL-6R* can inhibit TNBC tumor cells to grow and metastasize.¹¹ MCT-1/miR-34a/*IL-6*/*IL-6R* was a novel signaling axis identified in TNBC. MCT-1 inhibition combined with *IL-6*/*IL-6R* immunotherapy or with miR-34a expression would be a new stratagem for administration of TNBC.¹¹

LAMA3 is a widely studied methylated gene in multiple tumors. *LAMA3* hypermethylation is associated with chemotherapy resistance and poor prognosis in ovarian cancers. The expression level and base mutation of *LAMA3* gene can change the level of laminin, which have a certain influence on the onset and prognosis of ovarian cancer.¹² Laminin-332, an extracellular matrix (ECM) component composed of *LAMA3*, *LAMB3*, and *LAMC2* chains, is expressed by both tumor and stromal cells. The fibrosis caused by laminin-332 overexpressing myofibroblast formation (interface zone–fibroblast) via epithelial–mesenchymal transition may provide a specialized microenvironment for guiding breast tumor invasion.¹³ Marissa *et al.* compared the gene expression between primary TNBC and paired axillary and sentinel lymph node metastasis. They found that striking feature is the downregulation of genes known to regulate cell microenvironment interaction (*MMP2*, *MMP3*, *MMP7*, *MMP11*, *MMP14*, *COL1A1*, *COL1A2*,

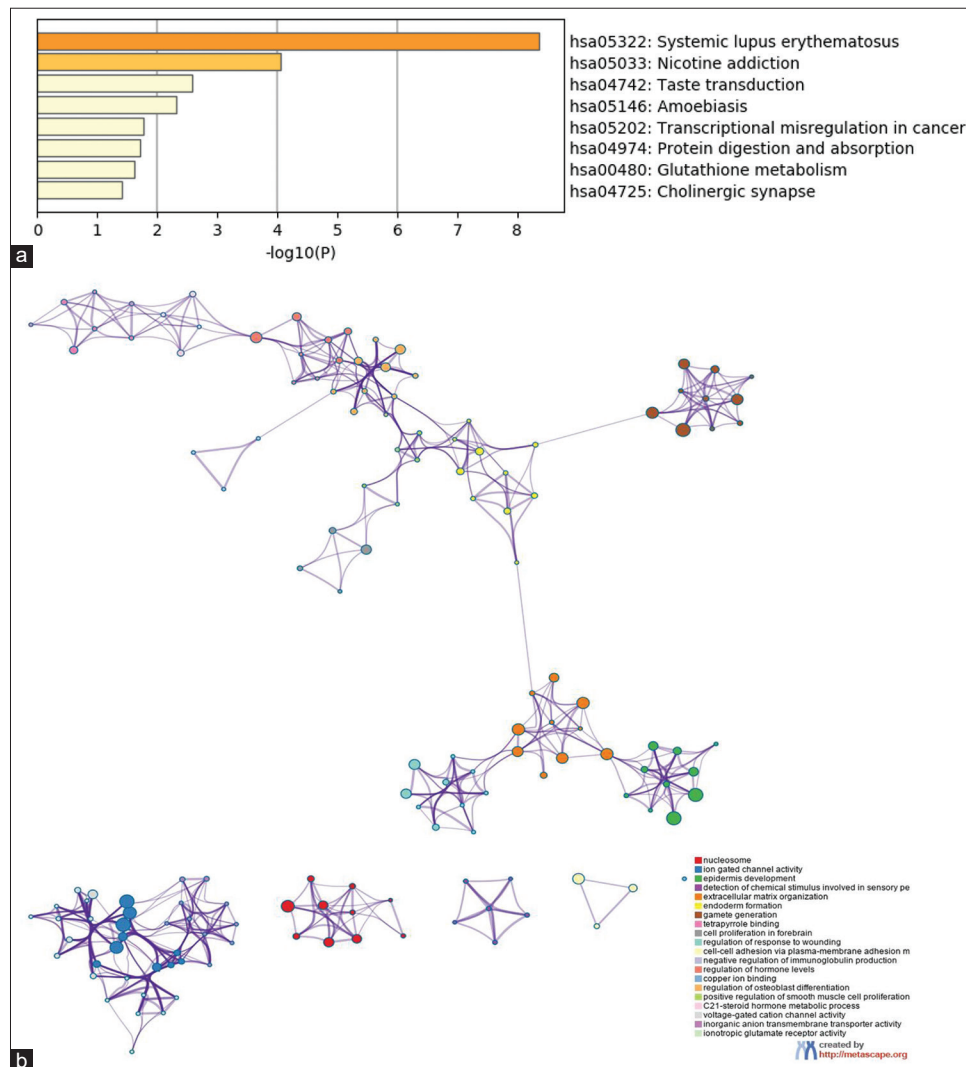


Figure 3: (a) Pathway enrichment analysis using Kyoto Encyclopedia of Genes and Genomes pathway database. (b) Top 20 enriched Gene Ontology pathway and genetic networks

COL3A1, COL5A1, COL5A2, COL6A6, COL11A1, and COL17A1).¹⁴ These features echoed the blue cluster of protein–protein interaction networks (including LAMA3).

RHOV has been widely studied to promote lung adenocarcinoma cell growth, metastasis, and therapeutic resistance.¹⁵ It has been revealed that *RHOV* serves as an EGFR-mediated metastasis mechanism activator and thus provides a mechanistic basis for TNBC metastasis. Knockdown of RHOV significantly suppressed cell invasion, migration, and metastasis both *in vitro* and *in vivo*.¹⁶ The increased regional and distant metastasis was observed in HER2+ tumors with high levels of ITGB6 (prognostic predictor).

It is notable that the GO pathway “negative regulation of immunoglobulin production” was associated with TNBC recurrence. There is growing evidence that tumor-infiltrating

B cells and plasma cells (together known as tumor-infiltrating B lymphocytes) play an important, synergistic role in tumor management. In the context of both standard therapies and immune checkpoint blockade, B cell, plasma cell, and IgG displayed high predictive and prognostic relevance in BC.¹⁷ Chang *et al.* identified IGLC2 as a novel prognostic biomarker for TNBC recurrence and may be a potential therapeutic target.¹⁸ Immunoglobulin-related gene set was found to play a crucial protective role in the prediction of TNBC prognosis.⁶ Intriguingly, the top two enriched KEGG pathways are SLE and nicotine addiction. BC cases occurring after SLE diagnosis was observed and a higher proportion of TNBC was reported in SLE patients.¹⁹ Nicotine promotes lung metastasis in TNBC through paracrine signaling in the tumor microenvironment.²⁰

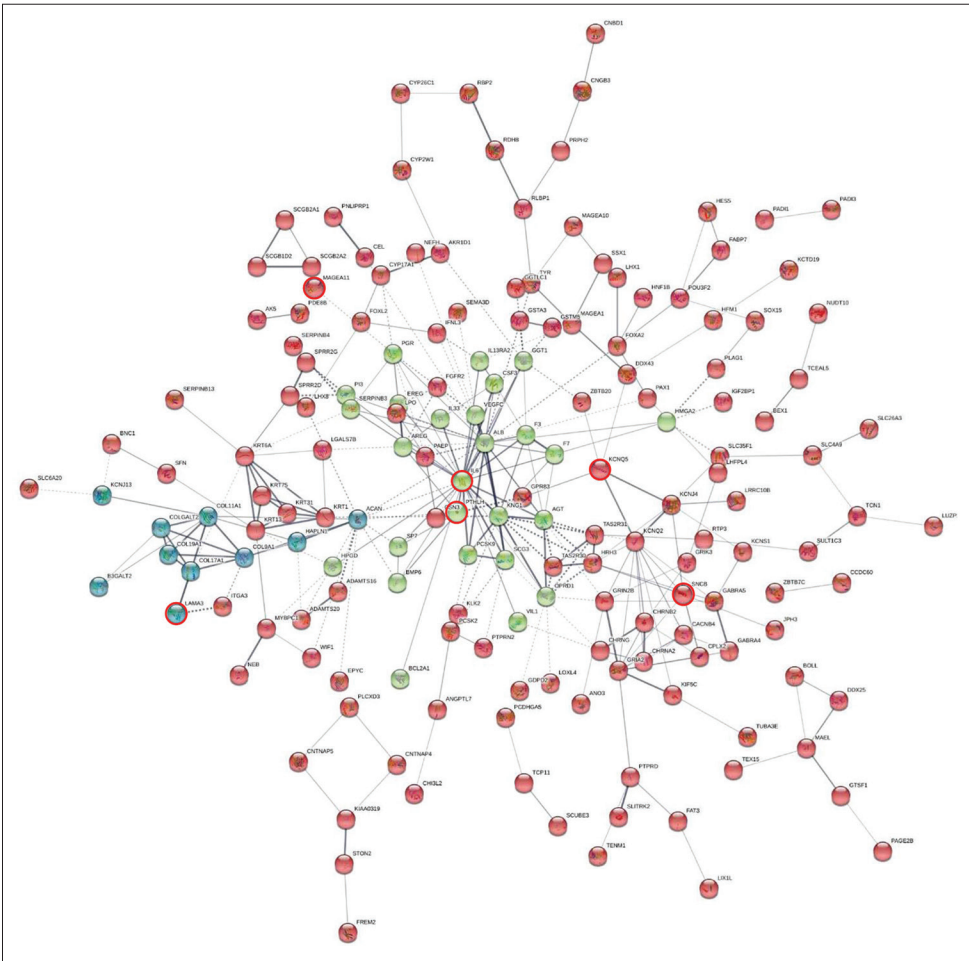


Figure 4: Protein–protein interaction networks of 284 significant genes using STRING. Genes without corresponding protein information in STRING were not plotted in the network. Different colors represent different protein clusters. Final target genes in the study are marked in red. The thickness of edge denotes the strength of interaction

The core center of protein–protein integration networks was green cluster in which major protein function was immune and angiogenesis. Recent studies of tumor lymphocytic immune infiltrates in BC have suggested an improved prognosis associated with increasing levels of tumor-infiltrating lymphocytes (TILs). TNBC has the greatest incidence of patients with a robust tumor immune infiltrate.²¹ Immunotherapy has prolonged survival in other solid tumors and represents a promising treatment strategy for TNBC. The most successful immunotherapeutic agents consist of immune checkpoint inhibitors (ICIs), which block immunosuppressive receptors, such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and PD-1, to improve the cytotoxicity and proliferative capacity of TILs. ICIs, including monoclonal antibodies against PD-1 (i.e., pembrolizumab, nivolumab), PD-L1 (i.e., atezolizumab, durvalumab, avelumab), and CTLA-4 (i.e., ipilimumab), have generated durable responses across many tumor types.²²

Potassium voltage-gated channel subfamily Q member 5

KCNQ5 has the best discrimination of recurrence or nonrecurrence TNBC patients in the Kaplan–Meier analysis. *KCNQ5* gene is a member of the KCNQ potassium channel gene family. A strong role for *KCNQ1*, *KCNQ3*, and *KCNQ5* in the progression of human gastrointestinal cancers was reported.²³ KCNQ potassium channel genes are located in the red cluster of protein–protein interaction networks. Ion gated channel activity was the top two enrich pathways of TNBC recurrence. It has been verified that altering the resting membrane potential of TNBC cells by potassium channel expression promotes *in vitro* invasion, *in vivo* tumor development, and metastasis, and is accompanied by alterations in cell adhesion-related genes. Bioelectric signaling mediated by potassium channels regulates metastasis in TNBC.²⁴

The description of other prognostic genes is listed as follows.

Casein kappa

CSN6 expression was dramatically increased in BC tissues compared with paired adjacent cancerous tissues. CSN6 promoted cell migration and wound-healing abilities in BC cell lines.²⁵ However, no study reports the association of CSN3 and TNBC up to date.

Phosphodiesterase 8B

Its protein is a cyclic nucleotide PDE that catalyzes the hydrolysis of the second messenger cAMP. PDE8B and DUOX2 were found significantly associated with the metastasis ability of thyroid carcinoma.²⁶ The mRNA levels of several PDEs, including PDE8B, PDE4B, and PDE5A, were higher in the BC metastatic bone in a mouse xenograft model.²⁷

Synuclein β

Synucleins are a tiny protein family with three known members: synuclein alpha (*SNCA*), synuclein beta (*SNCB*), and synuclein gamma (*SNCG*). *SNCG* expression predicts a poor prognosis in TNBC.²⁸ *SNCB* has not reported its association with BC.

H3 clustered histone 10

Histone family genes have demonstrated potential prognostic value in BC. H3C10 was previously reported upregulated in BC, and its high expression was associated with poor overall survival.²⁹

ADP-ribosylation factor-like protein 17

ARL17A was predicted to enable GTP binding activity, be involved in intracellular protein transport and vesicle-mediated transport. *ARL17A* gene encodes a protein of the ARF family that is involved in multiple regulatory pathways relevant to human carcinogenesis.³⁰

Limitation

Western patients account for the majority and Asian only accounts for 5%. The results may not represent the whole population. There are a lot of missing data in recurrence time. Small sample make it difficult to adjust other possible confounders. Large sample size is needed to further validate the findings in the future.

CONCLUSION

We discovered that the gene set of IL-6, LAMA3, and RHOV can accurately predict TNBC recurrence. These genes warrant further study to confirm their causal association with TNBC prognosis and possible treatment targets.

Ethical approval

This study proposal was approved by the Institutional Review Board of Tri-Service General Hospital. TSGHIRB:E202216030. 09/12/2022.

Data availability statement

The data that support the findings of this study are available from the corresponding author, YT Chang, upon reasonable request.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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